Effect of neurotransmitter antagonists on the sea urchin righting response and tube foot motility

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ABSTRACT

The purple sea urchin, *Strongylocentrotus purpuratus*, has been extensively studied for its developmental and reproductive characteristics, but knowledge regarding the neurobiology of the adult sea urchin is lacking. Specifically, the understanding of the different neurotransmitters involved in the systems controlling sea urchin behavior are widely under-researched. To further investigate the neurotransmitter systems and their implications in sea urchin behavior and locomotion, we have conducted behavioral and motility assays. The behavioral assay employs a righting response, in which a sea urchin is inverted and the amount of time for the urchin to right itself with the use of its spines and tube feet is recorded. Righting assays were performed following drug exposure via immersion in varying concentrations of propranolol, isoprenaline, or bicuculline. In vivo administration of propranolol, a beta-adrenergic receptor antagonist slowed the righting response of the sea urchins in a dose-dependent manner (IC50 = 39.8 μM). When the beta-adrenergic receptor agonist isoprenaline, and propranolol were administered together, isoprenaline was not able to reverse the effects of propranolol, and in fact isoprenaline actually slowed the righting response at high concentrations (IC50 = 400 μM). Bicuculline, a $GABA_A$ antagonist, had no significant effect on the righting response up to 100 μM, but did visibly increase tube feet motility. The effect of bicuculline on tube feet motility led to the development of a motility assay. The motility assay utilizes a small tank in which sea urchins are isolated following drug immersion, and Behavioral Observation Research Interactive Software (BORIS) will be used to analyze the video footage of tube feet movement. The motility assay will quantitatively measure tube feet motility in response to drug exposure.

INTRODUCTION

The purple sea urchin, *Strongylocentrotus purpuratus,* is classified as an Echinoderm, which is a diverse group of marine invertebrates that includes starfish, sea cucumbers, and close relatives. The development of the purple sea urchin, *Strongylocentrotus purpuratus,* has been widely studied and extensive knowledge exists regarding their reproductive and developmental biology. However, studies regarding the neurobiology of the adult purple sea urchin lack far behind that of the purple sea urchin larvae (Shah *et al.,* 2018). The whole genome of *Strongylocentrotus purpuratus* has been sequenced and reveals homologies between Echinoderms and Chordates, and more specifically, many similarities to the human genome (Sodergren *et al.,* 2006; Burke *et al.,* 2006). Further analysis of the genes involved in the nervous system of sea urchins revealed that they share a broad range of neurotransmitters with humans, but despite being an invertebrate that is phylogenetically similar to humans, sea urchins lack sensory and motor input from a central nervous system. Therefore, the neurotransmitters involved in mediating behavior and locomotion in the sea urchin's non-centralized nervous system are not well studied (Cobb, 1985).

The adult purple sea urchin is equipped with a protective calcium carbonate test, from which spines and tube feet, used for both protection and locomotion, protrude. The tube feet are contractile appendages that are important in anchoring, locomotion, and sensory perception. Sea urchins utilize a water vascular system to control tube feet relaxation and extension, and movement of these highly mobile tube feet allows sea urchins to translocate (Nichols, 1966; Billack *et al.*, 1998). The adult sea urchin, like all adult echinoderms, have a non-centralized nervous system, which allows them to sense their environment from all sides. The echinoderm nervous system, which consists of the ectoneural and the hyponeural, is made up of a peripharyngeal nerve ring encircling the esophagus close to the oral cavity within the Aristotle's lantern (Cobb, 1985). Nerve cords radiate from this nerve ring, which connects to the subepithelial nerve net that innervates the tube feet and spines (Florey *et al.,* 1975; Smith *et al.,* 1985; Burke *et al.*, 2006).

Previous studies have shown that sea urchin motility and locomotion can be influenced by the use of different chemicals and neuromodulators (Shah *et al.,* 2018). In this study, we used a neuropharmacological approach via the in vivo administration of a GABA antagonist, bicuculline, a beta adrenergic receptor antagonist, propranolol, and a beta adrenergic receptor agonist, isoprenaline, to further investigate the role of neurotransmitter systems in controlling behavior and locomotion of the purple sea urchin.

METHODS

Purple Sea Urchins / Identification:

Strongylocentrotus purpuratus, from the Pacific Ocean were obtained from Marinus Scientific (Long Beach, CA) and were maintained in an aerated multi-tank setting at 13°C containing artificial seawater (ASW) (Instant Ocean; That Pet Place, Lancaster, PA) prepared according to the manufacturer's protocol. All sea urchins used in this experiment were tagged with colored plastic beads for identification purposes. Each sea urchin was removed from ASW, and three to five colored beads were attached to individual spines using superglue. The sea urchins were returned to ASW tanks once the glue had dried fully.

Pharmacological Solutions / Drug Administration:

Artificial seawater (ASW) was composed of (in mmol l^{-1}) Na^+ (462), K^+(9.4), Mg^{2+} (52), Ca^{2+} (9.4), Cl^- (550), SO_4^{2-} (28), and Li^+ (20) (Atkinson and Bingman, 1997). ASW solutions were used for all control conditions. For experimental conditions, bicuculline, propranolol, and isoprenaline were purchased from Sigma-Aldrich (St. Louis, MO) and made into 100 mM drug stocks. Drug stocks were then diluted in ASW to make up the desired final concentrations. For drug administration, live sea urchins were immersed in the drug solution that was kept at approximately 12-14 °C for 1 hour before performing behavioral assays.

Righting Assay:

A righting assay was used to test whether drug exposure affected the sea urchin's ability to move. Control experiments were performed in ASW prior to drug exposure. The sea urchins were placed upside down on their aboral side in the tank and the time it took for urchins to fully right was recorded. Urchins that did not right after the cutoff time of 900 sec were excluded from the study. Drug righting experiments were performed following immersion in the drug solution for 1 hour, following the same exclusions as in the control righting assay. Each urchin was allowed to recover for at least 48 hours between drug exposures.

Motility Assay:

A motility assay was used to test whether bicuculline affected the movement of the sea urchin's tube feet. Following immersion in drug solution or control ASW, urchins were individually isolated in a small tank to limit mobility and ensure the urchin fit into the field of view of the video camera. Urchins were recorded at 4K resolution, 30 frames per second, using the Olympus TG-5 camera for 60 sec in order to provide ample observation intervals and to avoid whole-urchin movement. Behavioral Observation Research Interactive Software (BORIS) (Friard, O. and Gamba, M. (2016), BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. Methods Ecol Evol, 7: 1325–1330) was used for analyzing video footage and coding movement. A 6.25" segmented circle template was used as an underlay under the small tank, and tube foot movement over each of the 16 segment lines was tracked. Each time a tube foot crossed the line of interest, it was coded as an event. 60 sec of coding was performed for each of the 16 segment lines for each sea urchin. Total number of events were recorded for each urchin in control and drug conditions.

Data Analysis:

GraphPad Prism was used to further analyze the data of righting times and create dose response curves to determine the IC50 for both propranolol and isoprenaline. Righting time data were fitted with the the Hill equation of the form of y=min+(xn(max-min)xn+IC50n). All error bars in the figures are standard errors of the mean (SEM). The motility assay data obtained from BORIS analysis was analyzed to determine any significant differences in tube foot motility between urchins in bicuculline solution and control solution. T-Test: Paired Two Samples for Means was used to determine statistical significance between control and experimental drug conditions. A p value of less than 0.05 was determined to be significant.

RIGHTING AND MOTILITY ASSAY METHODOLOGY

Fig.1. Righting Assay. Frame by frame of an urchin 'righting'

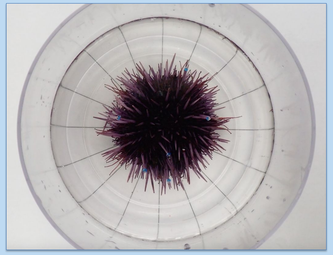

Fig.2. Screenshot from video analysis of urchin isolated in motility assay tank with circle template underlay.

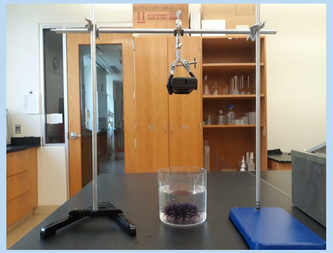

Fig.3. Photograph of the motility assay recording apparatus.

RIGHTING ASSAY RESULTS

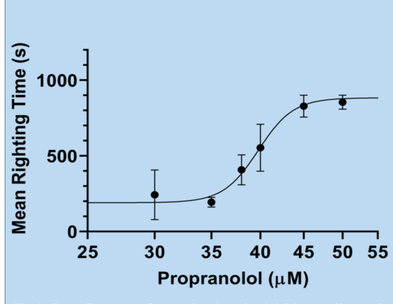

Fig.4. Dose Response Curve showing the inhibitory effects of propranolol on the mean righting time. Dose response curves were generated to determine the IC50 of propranolol using a range of concentrations from 1 to 100 μM (minimum righting time = 190 s, maximum righting time = 883 s). (b) The best fit value of the IC50 of propranolol is 39.8 μM with the 95% confidence interval ranging from 37.9 to 42.3 μM (slope = 21.0).

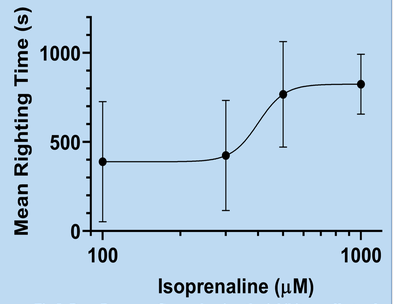

Fig.5. Dose Response Curve showing the inhibitory effects of isoprenaline on the mean righting time. The IC50 of isoprenaline was estimated to be 400 μM by using a range of concentrations from 100 to 1000 μM. (slope = 8.5, minimum righting time = 389 s, maximum righting time = 825 s). Not enough data was obtained to determine the 95% confidence interval.

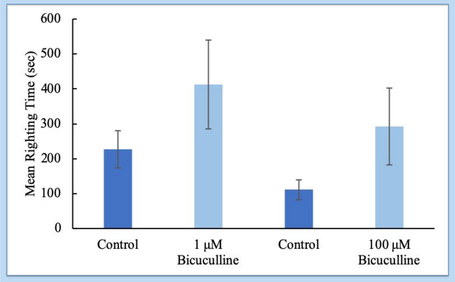

Fig.6. Bicuculline has no significant effect on the righting response. Control mean righting time (n=8, mean = 226.8 sec, SD=149.9 sec) compared to 1 μM Bicuculline mean righting time (n=8, mean=412.9 sec, SD= 359.7 sec) (t-Test p=0.20). Control mean righting time (n=8, mean=111.3 sec, SD=81.6 sec) compared to 100 μM Bicuculline mean righting time (n=8, mean = 292.5 sec, SD=311.1 sec) (t-Test p=0.15).

MOTILITY ASSAY RESULTS

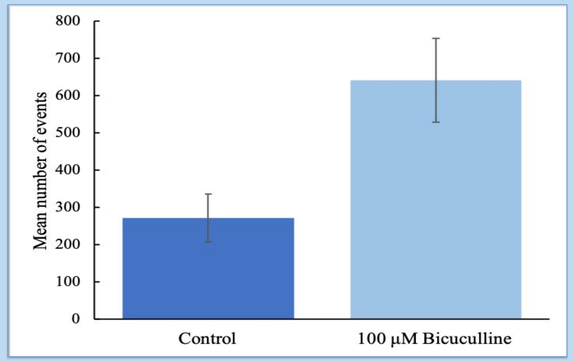

Fig.7. Bicuculline has an excitatory effect on tube feet motility. In vivo administration of bicuculline increased the mean number of events. A tube foot crossing a segment line of interest during video analysis was coded as an event. Control mean number of events (n=4, mean = 272, SD= 128.0) compared to 100 μM bicuculline mean number of events (n=4, mean = 642.3, SD= 225.1) (t-Test p= 0.006).

DISCUSSION

The primary purpose of this study was to further investigate the sea urchin nervous system and its implications in controlling behavior and locomotion. Propranolol, known for its implications in treating patients with acute chest pain related to cardiac issues, acts by blocking epinephrine from binding to beta adrenergic receptors (Gillam & Prichard, 1966; Epstein & Braunwald, 1966). According to our righting assay results, propranolol and isoprenaline both slowed the sea urchin righting response in a dose dependent manner; whereas, bicuculline showed no significant effect on the righting response at the concentrations used. It is possible that using a higher concentration of bicuculline may change the results found here.

Since propranolol is a beta adrenergic receptor antagonist and isoprenaline is a beta adrenergic receptor agonist, we expected that isoprenaline would be able to reverse propranolol's effects on the righting time when administered together. Surprisingly, the righting time was still slowed compared to the control when they were administered together, suggesting that either propranolol or isoprenaline act differently on beta adrenergic receptors of the purple sea urchin than it does in other organisms. When isoprenaline was administered alone at varying concentrations, the sea urchin righting response was slowed, but at much higher concentrations than propranolol. Because it takes a higher concentration of isoprenaline to slow the righting response by 50%, propranolol is a more potent drug. Further investigations using epinephrine would reveal additional characteristics about propranolol and isoprenaline and their mechanism of action as antagonists or agonists of beta adrenergic receptors in the purple sea urchin. If the administration of epinephrine, an endogenous ligand of beta adrenergic receptors, caused the righting response to be slowed, we would be able to conclude that the propranolol acts as a beta adrenergic receptor agonist in sea urchins, rather than an antagonist as it has previously shown to be in other organisms.

Although bicuculline did not significantly affect the righting response, the in vivo administration of bicuculline did have an observable effect on tube feet motility. This observation led to the design of a motility assay, which quantifies sea urchin locomotion by tracking changes in tube movement in response to drug exposure. From the motility assay results using the in vivo administration of bicuculline, we determined the motility assay to be an effective measure of sea urchin locomotion. This new locomotion assay will allow future studies to research a different element of sea urchin behavior by quantitatively assessing how sea urchin motility and locomotion can be influenced by the use of different chemicals and neuromodulators. This motility assay will provide further insight into the field of echinoderm neurobiology by allowing researchers to explore the different neurotransmitters systems involved in controlling sea urchin behavior and locomotion.

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