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Behavioral and physiological responses to central administration of corticotropin-releasing factor in the bluebanded goby (*Lythrypnus dalli*)

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ABSTRACT

Central manipulation of neuromodulators is critical to establishing causal links between brain function and behavioral output. The absence of a rigorous method of evaluating intracerebroventricular (icv) injection efficacy in small model organisms is one reason why peripheral administration of neuroactive substances is more common. We use the bluebanded goby (*Lythrypnus dalli*), a small, highly social fish, to 1) validate our method of icv injection by testing the hypothesis that corticotropin-releasing factor (CRF) elevates ventilation rate (VR) and 2) propose a novel bioassay using basal physiology and behavior during recovery from anesthesia/icv administration to assess injection efficacy, neuromodulator activity, and procedural confounds. Central CRF administration significantly increased ventilation rate, demonstrating successful delivery of CRF to the brain. There were no significant differences in cortisol among treatments. The injection procedure did, however, decouple the temporal relationship between the initiation of ventilation and time to regain equilibrium present in control fish. Importantly, neither icv vehicle nor CRF injection affected the initiation of ventilation, disrupted the stereotyped recovery pattern following anesthesia, or initiated an endocrine stress response. Taken together, we suggest that 1) icv injection can be effectively used to manipulate central levels of CRF in *L. dalli* and 2) physiological and behavioral recovery from anesthesia may be used to evaluate injection/technique efficacy. We will use these data in future studies as a measure of effective CRF delivery, to allow for appropriate recovery from icv injection, and to better evaluate independent effects of CRF on social and/or sexual behavior.

1. Introduction

Rapid and context-specific behavioral responses can involve local and transient changes in brain chemistry that precede circulating or systemic changes [1–3]. Brain-level manipulation of neuropeptides, neurotransmitters, hormones, or enzymatic activity is critical to establishing causal relationships between brain function and subsequent downstream behavioral processes. A variety of small model organisms are used to address scientific questions that are central to unraveling the brain–behavior relationship [1,4–8]; however, the absence of an equivalent to mammalian stereotaxic injection limits the scope of questions that are addressed with these organisms. Size itself is not a technological hindrance, as small-scale recording and manipulative techniques are commonly used [9,10], and a number of laboratories have developed intracerebroventricular (icv) injection procedures [4,8,11]. Still, many studies that exogenously administer neuroactive substances opt to use intraperitoneal injection or implant [5,7]. The interpretation of these data may be limited, however, given that the synthesis and action of neuromodulators occur locally in the brain and many substances may not cross the blood brain barrier. Among many potential reasons for peripheral rather than central manipulation, the desire to minimize invasiveness and recovery time (especially for social behavior studies), and the absence of a standard assessment of injection consequences (i.e., stress or injury related effects) or efficacy may have limited the use of icv techniques. In this paper, we 1) validate our minimally-invasive method of icv injection in the bluebanded goby (*Lythrypnus dalli*) by testing the hypothesis that icv corticotropin-releasing factor (CRF) increases ventilation rate (VR) and 2) propose that a physiological and behavioral analysis of the recovery from injection/anesthesia provides real-time validation of neuromodulator delivery and quantifies the potential negative consequences of icv administration that might compromise other behaviors of interest (e.g., social behaviors).

The bluebanded goby is a small (adult standard length (SL): 20–45 mm, mass: 0.2–1.5 g), highly-social, marine fish that undergoes socially-regulated, bidirectional sex change [12,13]. The high degree of social and reproductive plasticity that the bluebanded goby maintains throughout all life history stages makes it a powerful model for understanding how environmental cues, and their internal representation,

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produce rapid and dramatic neural, behavioral, and, ultimately, morphological changes [1,12–14]. Corticotropin-releasing factor is a highly-conserved vertebrate neuropeptide that is released in response to environmental stressors and modifies biological function at multiple levels of organization. Corticotropin-releasing factor may be best known for its role as the initiator of the classical neuroendocrine stress axis, wherein it induces adrenocorticotropic hormone release from the pituitary, which subsequently induces the release of glucocorticoids from the interrenal organ (in fishes). The rapid alteration of physiology and behavior by glucocorticoids is just one mechanism by which CRF mediates homeostatic processes. In addition to this classical function, CRF receptors 1 and 2 are widely distributed throughout the vertebrate brain [15–17], and direct, central action of CRF has been shown to mediate a variety of neural [18–20], physiological [21], and behavioral processes [4,18,22–25]. One of the most well established roles for CRF in vertebrates is as a modulator of VR [21,26,27].

2. Materials and methods

2.1. Study organism

We collected *L. dalli* offshore of Santa Catalina Island, California (California Fish and Game permit no. SC-10676) and maintained them at our fish facility at Georgia State University (Atlanta, GA, USA). Fish acclimated for 3 months before the initiation of this study. Fish were housed in 38 L aquaria on a 12:12 light/dark cycle at a temperature of 18–20 °C and were fed brine shrimp once daily. Each aquarium contained a social group of one large male and 3–6 females of varying sizes. We only used females (20.3–33.6 mm SL (tip of the lower jaw to the caudal peduncle); average 27.5 ± 0.5 s.e.m.) for this study. We randomly assigned fish to one of the following groups: control (n = 11), vehicle (phosphate buffer solution) injection (n = 13), or CRF injection (n = 13). All experiments were carried out during the afternoon to reduce diel variation in cortisol levels [28].

2.2. Anesthesia and recovery

We netted the fish from the aquaria and transferred them to the anesthetic tricaine methanesulfonate (MS-222; 500 mg/L salt water) in less than 60 s (time to catch; average 31.3 ± 1.47 s s.e.m.). We chose to use MS-222 because it is a common and safe fish anesthetic, and previous studies have used MS-222 at this dosage to perform similar icv injections [18]. Thirty seconds after the opercula stopped moving (cessation of ventilation; CoV), the fish were removed from the MS-222 and kept out of water for the following 120 s, during which the CRF and vehicle groups were injected icv. Control fish were similarly handled but received no injection. The fish recovered unaided (e.g., no artificial ventilation) in a 200 mL plastic beaker of fresh salt water. We recorded the time until initiation of ventilation (IoV), signaled by the first movement of the opercula, and time until positional equilibrium was regained (RE), when the dorsal fin of the fish first reoriented to a vertical position. Ventilation rate was recorded for the 300 s following IoV, in 30 s increments, by counting opercular beats. Observers were blind to the treatment of the recovering fish.

We determined baseline VR by observing random, undisturbed fish housed in social groups in the fish facility. These fish were not involved in the manipulative study. Opercular beats were counted in 30 s intervals in fish (n = 11) not currently swimming or engaging in social interactions. Baseline VR recording was replicated in each fish between one and five times, then averaged for each individual. Recording bouts were excluded if the fish began to swim or interact before a 20 s minimum to ensure that our measurements were representative of baseline VR.

2.3. Chemicals

We purchased ovine CRF from Sigma-Aldrich (St. Louis, MO, USA) and dissolved it in 0.1 M sterile phosphate buffer solution for icv administration. Corticotropin-releasing factor-injected fish received 500 ng CRF/50.6 nL PB, a dose we chose based on previous icv CRF experiments in fishes [18,23,24]. Vehicle-injected fish received the same volume injection of phosphate buffer only. We prepared fresh phosphate buffer at the start of the experiment and sterilized it immediately prior to use with a sterile 0.2 μm Nalgene syringe filter (Rochester, NY, USA). The solution was stored at 4 °C in between uses.

2.4. Intracerebroventricular injections

We performed icv injections under a dissecting microscope using the Nanoject II Auto-Nanoliter Injector (Drummond Scientific Company, Broomall, PA, USA). The solution was injected into the third ventricle by penetrating the skull with a pulled capillary tube needle at the intersection of the midline and the posterior edge of the eyes. We assessed injection accuracy by scoring 13 pilot injections of methylene blue. The goal of the icv injection was to bathe the brain and third ventricle with solution (as opposed to delivering solution into specific brain cells) while avoiding tissue damage. Thus, injections were scored as successful if methylene blue was visible along the midline, between the right and left telencephalon and optic tectum, and/or bilaterally between the telencephalon and optic tectum (Fig. 1). Anatomical analysis verified that 85% of injections were accurate. For both pilot and experimental injections, detailed notes were recorded about the penetration of the skull, ease of injection, and injection location based on the visible needle entry point (Fig. 1B). Fish were excluded from analysis if the needle failed to penetrate the skull.

In this experiment, the needle was changed as soon as there was any detected resistance in penetrating the skull (indicates needle dulling), and between CRF and PB injections. Between injections, we wiped the needle with ethanol and allowed it to dry. Following each injection, the needle was kept in the skull for an additional 5 s to reduce leakage (as in [23]). To verify proper Nanoject function, we followed every injection with a test injection under the dissecting scope to confirm that the needle was unobstructed. Fish were excluded from analysis if the test injection showed that the needle was obstructed.

2.5. Cortisol

We placed fish in 200 mL beakers containing 100 mL of fresh salt water for 1 h immediately following VR recording or RE, whichever occurred later. All beakers were washed with soap and water, sterilized with ethanol, and rinsed with fresh and salt water before use. After 1 h, we removed the fish, and the water-borne steroids were extracted and measured as in [28]. Briefly, steroid was extracted from the water sample using 3 cm^3 Sep-Pak Vac C18 columns (Water Associates, Milford, MA, USA) and then eluted from the columns into 13× 100 mm vials with two consecutive washes of 2 mL HPLC-grade methanol. The samples were maintained at 40 °C (water bath) while the eluted solvent was evaporated by a constant, gentle stream of nitrogen directed to the samples through an evaporating manifold. We then resuspended the hormone pellet in 600 μL of 5% EtOH, 95% enzyme immunoassay (EIA) buffer from the Cayman Chemical Cortisol EIA kit (Ann Arbor, MI, USA). We completed the assay according to the supplied instructions, and all samples were assayed in duplicate. We read the plate 90, 105, 120, and 135 min following the addition of the developing reagent (Ellman's reagent). We chose to analyze data based on the accuracy of the standard curve (135 min, $R^2 = 0.99$). The data are presented as pg/sample (pg/mL multiplied by 0.6 mL, the volume of EIA buffer used to resuspend the sample). Water samples from one vehicle and one CRF-injected fish were excluded due to space constraints