Physiology & Behavior 106 (2012) 619–625

Contents lists available at SciVerse ScienceDirect

Physiology & Behavior

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

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

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ARTICLE INFO

Article history:
Received 16 October 2011
Received in revised form 16 April 2012
Accepted 17 April 2012

Keywords:
Corticotropin-releasing factor
Intracerebroventricular injection
Ventilation
MS-222
Anesthesia

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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doi:10.1016/j.physbeh.2012.04.017

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

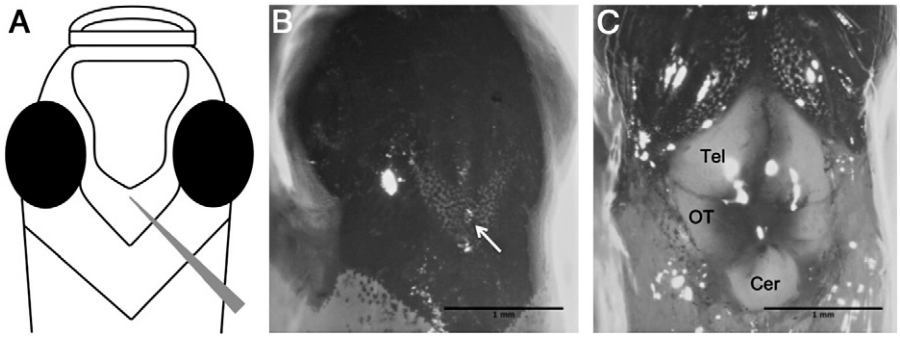

Fig. 1. Dorsal views of the *L. dalli* head: a schematic drawing with injection site indicated (A), and the injection site (arrow, B) and brain following the intracerebroventricular injection of methylene blue (C). Thirteen pilot injections were scored to establish experimenter accuracy in dispensing the solution into the third ventricle. The injection was 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. Optic tectum (OT); telencephalon (Tel); cerebellum (Cer).

on the supplied EIA plate. The two samples omitted had average values for SL, IoV, and RE.

2.6. Statistics

Statistics were performed using JMP 7.0. Results were considered significant at the $p<0.05$ level, and the data presented in the text are means ± standard error of the mean (s.e.m.). We conducted one-way analysis of variance (ANOVA) to assess differences in SL, time to catch, CoV, IoV, RE, and cortisol among control, vehicle, and CRF-injected groups. The data for RE were not normally distributed, so we performed a square root transformation prior to analysis. The Tukey–Kramer HSD test was used for post hoc analysis of all significant ANOVA results.

We used linear regressions to test for the following relationships between variables. We examined whether conditions immediately prior to placement in the anesthesia (time to catch) influenced CoV. We tested whether body size (SL) influenced IoV, RE, cortisol production, or VR. Finally, we analyzed whether RE or cortisol was associated with each other, IoV, and/or VR.

Ventilation rate data were analyzed using a two-way Mixed Factorial ANOVA with time interval (following IoV) as the within subjects factor and treatment group as the between subjects factor. The simple effects of this analysis were evaluated using one-way ANOVAs and the Tukey–Kramer HSD test for pairwise comparisons of significant one-way ANOVA results. We also compared control, vehicle, and CRF-injected group VR to baseline rates in the first and last 30 s intervals of the 300 s recording period. Data from the first 30 s were analyzed using a one-way ANOVA. Data from the last 30 s were not normally distributed, and transformations failed to create a normal distribution. As a result, data from the last 30 s were analyzed using a Kruskal–Wallis one-way ANOVA.

3. Results

3.1. Allometry, cessation of ventilation, initiation of ventilation, and regaining equilibrium

Control, vehicle, and CRF-injected groups did not differ in SL ($F(2,34)=0.89$, $p=0.42$), the time required to net and transfer fish into the MS-222 (time to catch) ($F(2,34)=0.60$, $p=0.56$), or CoV (Fig. 2A) ($F(2,34)=1.15$, $p=0.33$), and time to catch was not associated with CoV (Fig. 2A, inset) ($p=0.83$). There were also no differences in IoV among control, vehicle, and CRF-injected fish (Fig. 2B) ($F(2,33)=0.15$, $p=0.87$), and IoV was not associated with SL (Fig. 2B, inset) ($p=0.07$). We analyzed treatment effects on RE from two different temporal starting points: from time 0 (when the fish was first put into the beaker of salt water to recover) and from IoV, which may be more sensitive to individual variation in IoV. In both analyses, CRF-injected fish required significantly more time to RE than control fish but did not differ significantly from vehicle-only fish. The data presented are from time 0 ($F(2,31)=5.12$, $p=0.012$) (Fig. 2C). There was also a significant, positive association between IoV and RE, beginning from time 0, ($R^2=0.28$, $p=0.001$) in all fish (Fig. 3A). Latency to IoV explained almost all of the variation in RE for control group fish ($R^2=0.90$, $p<0.0001$), but this relationship was not maintained in the vehicle ($p=0.10$) or CRF-injected groups ($p=0.06$) (Fig. 3B).

3.2. Ventilation rate

A two-way mixed factorial ANOVA with time interval (following IoV) as the within subjects factor and treatment group as the between subjects factor revealed a significant interaction between treatment (control, vehicle, or CRF-injected) and time ventilating for VR ($F(2,368)=7.69$, $p=0.0005$). Post hoc analysis revealed a significant simple effect of treatment ($F(2,35)=8.24$, $p=0.0012$). Pairwise analysis of the simple effect test using the Tukey–Kramer HSD test demonstrated that icv CRF significantly increased VR in comparison to control and vehicle-only fish (control vs. vehicle, $p>0.05$; control vs. CRF, $p<0.05$; vehicle vs. CRF $p<0.05$). There was no independent simple effect of time interval on VR over the 300 s recording period ($F(9,367)=1.78$, $p=0.07$). In the first 30 s, a one-way ANOVA revealed a significant effect of treatment group (including baseline) on VR ($F(3,44)=13.58$, $p<0.0001$). Post hoc analysis demonstrated that control, vehicle, and CRF-injected fish VR was significantly higher than baseline rates. Control and CRF-injected fish VR did not differ ($p>0.05$) and was statistically higher than vehicle-only fish ($p<0.05$). In the last 30 s of the recording period, a Kruskal–Wallis analysis showed a significant effect of treatment group (including baseline) on VR ($H=18.51$, $df=3$, $p=0.0003$). Post hoc analysis demonstrated that the VR of CRF-injected fish was significantly higher than control, vehicle, and baseline rates ($p<0.05$), which were not statistically different ($p>0.05$).

Central CRF administration significantly elevated VR and, because of the injection, increased time to RE. To determine whether VR affected RE, we used a linear regression to determine whether RE (time 0) was associated with VR in the first 30 s of ventilation. Ventilation rate was not associated with RE when analyzed in all fish ($p=0.38$) or separately for control ($p=0.59$), vehicle ($p=0.60$), or CRF-injected fish ($p=0.75$).

3.3. Cortisol

Levels of water-borne cortisol did not differ among control, vehicle, or CRF-injected fish (Fig. 5) ($F(2,32)=0.32$, $p=0.41$). Cortisol levels in CRF-injected fish were significantly and positively associated with RE. Regain equilibrium measured from IoV explained more of the variation in cortisol ($R^2=0.52$, $p=0.012$) than beginning at

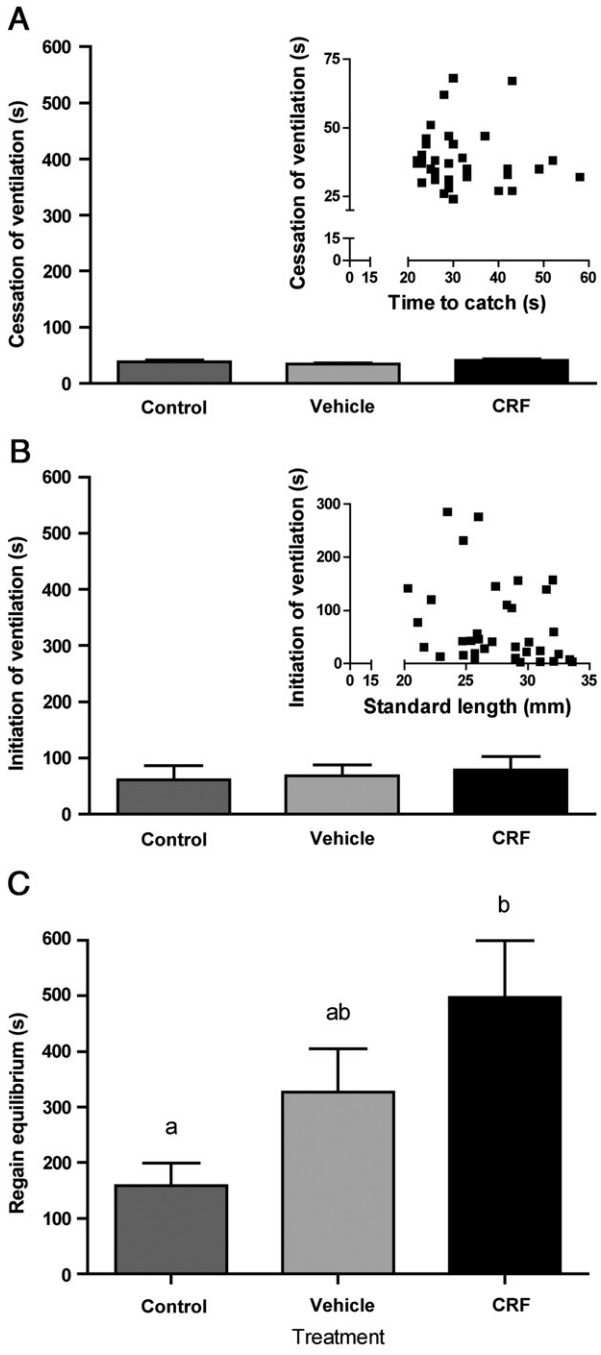

Fig. 2. Effect of the anesthetic MS-222 on *L. dalli* cessation of ventilation (A), and the initiation of ventilation (B) and regaining of equilibrium (C) following no injection (control; n = 11) or the intracerebroventricular injection of vehicle (n = 13) or corticotropin-releasing factor (CRF; n = 13). Regain equilibrium data refer to the time period starting when the fish first begins recovery (time 0). Values are represented as means (± s.e.m). Treatment groups differed significantly in time to regain equilibrium ($F(2,31) = 5.12$, $p = 0.012$). Different letters indicate significant differences (control vs. vehicle, $p > 0.05$; control vs. CRF, $p < 0.01$; vehicle vs. CRF $p > 0.05$). Inset to (A) shows the relationship between the time required to catch and place the fish in the anesthetic and the time until the cessation of ventilation. Inset to (B) shows the relationship between standard length and time to initiate ventilation.

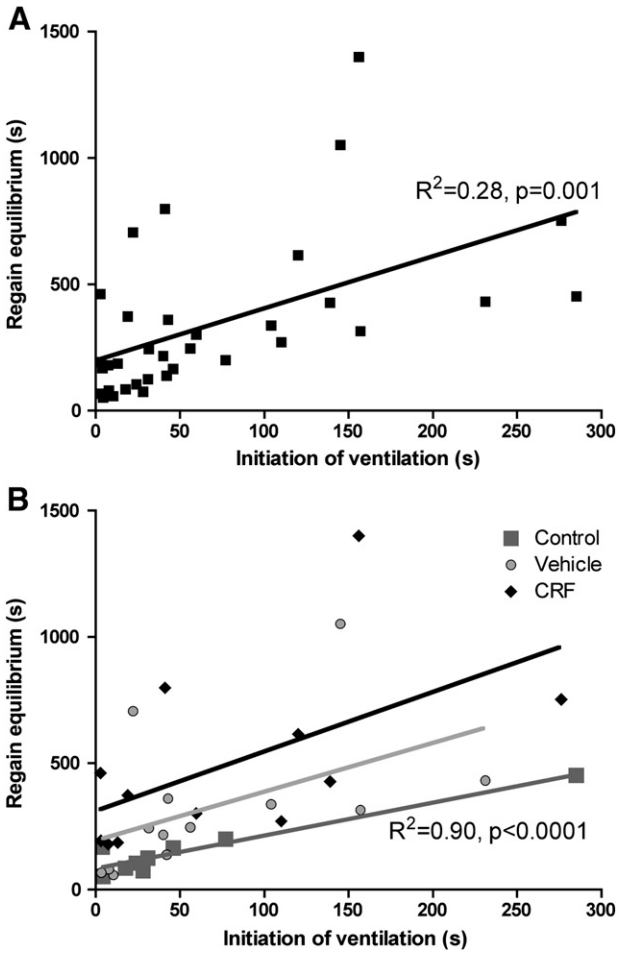

Fig. 3. Effect of initiation of ventilation on regaining equilibrium in fish from all treatment groups (A) and separated by fish that received no injection (control; n = 11) and intracerebroventricular injection of vehicle (n = 13) or corticotropin-releasing factor (CRF; n = 13) (B). Regain equilibrium data refer to the time period starting when the fish first begins recovery (time 0). R^2 values are indicated for statistically significant relationships.

time 0 ($R^2 = 0.43$, $p = 0.029$). Regain equilibrium was not associated with cortisol in the control ($p = 0.57$) or vehicle groups ($p = 0.71$).

4. Discussion

Increased VR in response to icv CRF injection indicates the successful elevation of brain-level CRF in the bluebanded goby. Our data also reveal that icv injection in the bluebanded goby has effects independent of the pharmacological action of CRF, for example, altering RE. The injection procedure does not, however, disrupt the highly stereotyped pattern of recovery from anesthesia [29], alter IoV—the stage of recovery that is a necessary prerequisite for RE—or initiate a stress response as measured by changes in glucocorticoid levels. Given these robust similarities among treatment groups, our research supports the use of icv injection as an effective means of manipulating central levels of neuromodulators in *L. dalli*, without significant negative consequences of the injection procedure. In our laboratory, we have injected fish as small as 25 mm SL. As a result, we anticipate being able to use icv injection with the entire adult population of *L. dalli* (18–45 mm SL), as well as other similarly small fish. Finally, we propose that quantitative analysis of physiology and behavior during recovery from an invasive procedure can provide real-time information about the action of pharmacological agents and consequences of injection, a novel application for these data.

4.1. Corticotropin-releasing factor elevates ventilation rate through central mechanisms

The excitatory effect of CRF on VR is the strongest indication that icv injection delivers physiologically active levels of CRF to the brain. One of the most robustly replicated roles for CRF is as a modulator of both respiratory [21] and cardiovascular processes in a fish

[30] and other vertebrates [26,27]. Our results are consistent with these studies and show that CRF significantly increases VR compared to the control and vehicle-only groups (Fig. 4). This persistent elevation of VR by CRF may be best explained by direct modulation of hindbrain central pattern generators [21]. Ventilation in fish is driven by motor nuclei in the medulla that make up portions of the trigeminal (V^{th}), facial (VII^{th}), glossopharyngeal (IX^{th}), and vagal (X^{th}) cranial nerves, along with the reticular formation. Ventilatory rhythms are primarily dictated by a pattern generator in the reticular formation [31], although the midbrain exerts some influence over ventilatory movements [31,32] and chemoreceptors in the gills can initiate ventilatory reflexes [31,33]. Multiple analyses confirm the presence of endogenous CRF [34] and CRF receptor mRNA [35] in the teleost hindbrain, and exogenous CRF injected into the third ventricle could access ventilation control centers located around the fourth ventricle [32] via the ventricular system.

It is our goal in future studies to use icv injection to elevate CRF centrally; therefore, it is necessary to consider the likelihood that the ventilatory effects of CRF in our study originate in the periphery. First, the stress response in fishes involves both a sympathetic (e.g., catecholamines) and neuroendocrine (e.g., cortisol) response and increases the capacity for oxygen uptake, in part, by elevating VR [36]. Our data show no differences in cortisol levels across treatment groups (Fig. 5), demonstrating that general stress axis activity was not affected by icv injection or CRF administration any more than by the handling and anesthetization experienced by the control group. Furthermore, there is no evidence from other cortisol analyses in *L. dalli* (R. Earley and M. Grober, unpublished data) that a ceiling effect is responsible for the similarity in cortisol levels among treatment groups. Second, it is possible that CRF cleared from the ventricles in the circulation of cerebral spinal fluid could act at the level of the gills. Assuming that peripheral concentrations of CRF are lower than central concentrations due to the location of administration, and that CRF acting at the gills would be more rapidly washed out during ventilation, we predict that peripheral CRF would only cause transient changes. In contrast, our data support lasting effects of CRF: VR is elevated for at least 300 s, and the effects on RE persist even longer (~420 s). Taken together, our data, and a similar study in which CRF was injected icv in a teleost [21], suggest that the observed ventilatory effects result from central actions of CRF.

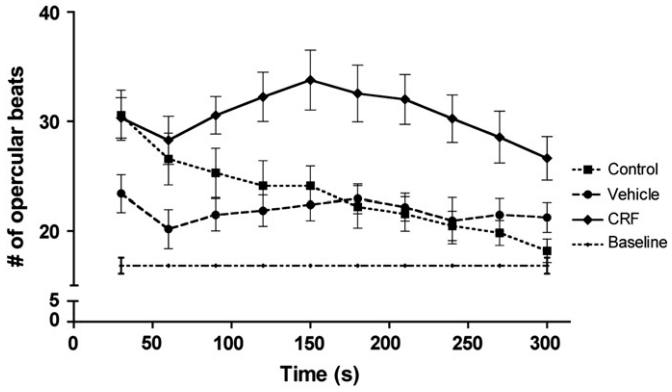

Fig. 4. Mean (± s.e.m.) number of opercular beats recorded in 30 s intervals during the first 300 s following initiation of ventilation. A two-way mixed factorial ANOVA revealed a significant interaction between treatment and time ventilating for ventilation rate ($F(2,368) = 7.69$, $p = 0.0005$). Post hoc analysis revealed that icv CRF significantly increased ventilation rate compared to control and vehicle-injected fish ($F(2,35) = 8.24$, $p = 0.0012$). Comparisons between experimental groups and baseline were conducted for the first and last 30 s intervals. In the first 30 s, CRF-injected and control group ventilation rates were significantly higher than vehicle group ventilation rates, which were significantly higher than baseline ($F(3,44) = 13.58$, $p < 0.0001$). In the last 30 s, ventilation rates of CRF-injected fish were significantly higher than all other groups, including baseline ($H = 18.5$, $df = 3$, $p = 0.0003$).

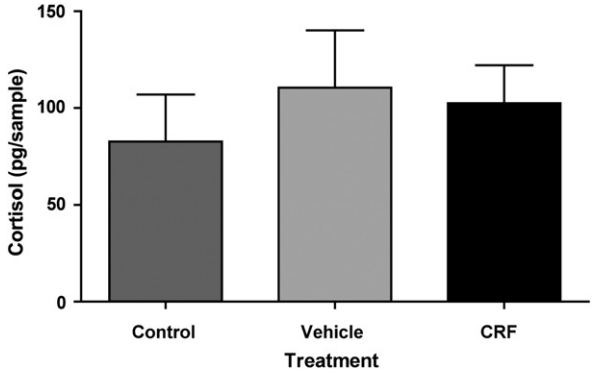

Fig. 5. Effect of no injection (control; n = 11) and intracerebroventricular injection of vehicle (n = 13) and corticotropin-releasing factor (CRF; n = 13) on water-borne cortisol levels. Data are represented as means (± s.e.m.). There were no significant differences among treatment groups ($p = 0.41$).

4.2. Corticotropin-releasing factor effects on ventilation rate are independent of MS-222

Ventilation rate was elevated by MS-222 in this and previous studies [37,38]. The VR of control group fish was initially elevated to levels equivalent with CRF-injected fish and significantly higher than both vehicle-only fish and baseline rates. By the end of our 300 s recording period, the VR of control fish returned to baseline levels. The persistent elevation of VR in CRF-injected fish, therefore, indicates a stimulatory role for CRF on VR and not a prolonged effect of MS-222 (Fig. 4). These data confirm that observing VR over the first 300 s of recovery is sufficient to understand how potential VR effects of handling, injection, and CRF change over time as the influence of MS-222 is eliminated. Our study is consistent with a previous study on anesthetization in a teleost fish demonstrating that MS-222 is cleared from circulation in less than 300 s [38].

4.3. Neither icv injection nor CRF administration affects cortisol levels

Although there are differences in VR and RE, our results show no treatment effects on water-borne cortisol. This indicates that despite the physical insult of icv injection, injected fish are not more classically stressed than control fish. It also strongly suggests that the effects of CRF are central and that 'leakage' to the pituitary or peripheral receptors is not significant, as this should drive an increase in circulating cortisol levels. A number of previous studies have administered icv CRF in a variety of fishes using administration procedures similar to the one described in the current study [18,23,24,39], via cannula [21], or via open-skull injection [40]. These studies have yielded mixed results on the ability of icv CRF to activate the neuroendocrine stress pathway in fishes. Our study is consistent with some [18,21,23,24,39], but inconsistent with others (e.g., [22]). Interestingly, in the study showing icv CRF elevated cortisol, the effect was not dose dependent [40]. Additionally, a study using a similar dosage did not result in activation of the neuroendocrine stress axis [24], suggesting that the effect of icv CRF on cortisol is not dependent on dosage. Because elevated cortisol reduces central CRF via negative feedback [41], the fact that icv CRF does not drive cortisol production in the current study validates our ability to exclusively manipulate brain-level CRF.

4.4. Behavioral and physiological correlates of icv injection

4.4.1. Intracerebroventricular injection influences ventilation patterns

The consistency in IoV across all treatment groups and the initial elevation of VR compared to baseline rates indicate that ventilation processes following anesthesia are under strict physiological control.

After the initial phase of elevation, additional mechanisms, such as vagal inhibition [42], can establish control of VR, possibly to resynchronize ventilation with heart rate [43], which may be slowed, but is not stopped, by anesthetic doses of MS-222.

The VR of vehicle-only fish also starts significantly lower than either control or CRF-injected fish. In a similar study, CRF and vehicle (Ringer's solution) were delivered icv via cannula to unanesthetized trout; vehicle administration had no effect on any ventilation measure analyzed using EEG electrodes, including ventilatory frequency, amplitude, or total ventilation (frequency × amplitude) [21]. This suggests that the differences we detect result from the injection procedure. Ventilation in CRF-injected fish is likely not depressed to the level of vehicle-only fish because the excitatory effects of CRF are immediate and not because the groups respond differently to the injection procedure.

4.4.2. Intracerebroventricular injection affects regaining equilibrium

Recovery from anesthesia is highly stereotyped: ventilation returns first, followed by equilibrium, and finally normal locomotion [29]. Although all groups have equivalent IoV, IoV does not predict the timing of equilibrium recovery for vehicle or CRF-injected fish (Fig. 3). Corticotropin-releasing factor-injected fish required significantly more time to RE than control fish. Vehicle-only fish, on average, RE after control fish and before CRF-injected fish (Fig. 2C). In future studies, we suggest that if fish are allowed to recover unaided, as they were in this study, RE can be used as a standardized measure of individual recovery. Additionally, because RE is associated with cortisol levels for CRF-injected fish, the timing of RE can be used to predict whether the stress response has been activated and, therefore, whether central CRF is under additional negative feedback regulation.

5. Conclusion

As a whole, these physiological and behavioral data (i.e., IoV, VR, RE), which are easily collected during the recovery stage following any anesthetic administration/surgical procedure, can provide valuable insight when evaluating the effects of icv CRF (or any other neuroactive substance) in more complex experimental contexts. It is critical to employ a behavioral and non-invasive measure of injection efficacy and procedural confounds so that an individual's behavior following injection can be appropriately attributed to action of the neuromodulator rather than an artifact of the injection process. For example, a previous study shows that icv injection of both vehicle and CRF can decrease rates of social interactions among rainbow trout [18]. Additionally, neuromodulator action may become confounded with additional experimental manipulations following injection that may independently yield changes in behavior. For example, the role of CRF in social processes is of great interest, yet many common behavioral effects of CRF may easily become confounded with behaviors that differ as a result of social manipulation (e.g., social status), such as rates of locomotion [23,24,44]; patterns of social or socially-relevant behaviors (e.g., anxiety) [15,18,45–47]; energy balance/homeostasis [4,22,25,48]; and even VR [49]. Given the complexity of social behavior, and the dynamic ways in which CRF has been shown to influence behavior during social interactions [18], using physiological and behavioral recovery data to distinguish among behavioral, social, or other environmental effects, and the direct effects of central CRF administration, will be vital to accurately assessing how CRF may represent and/or affect social status in vertebrates, and more specifically, sexual allocation in the bluebanded goby.

Acknowledgments

We would like to thank Ravi Batra and Kim Connor for observing recovery, David Sinkiewicz for assistance with EIA, Dr. Erica Crespi for use of injection equipment, Dr. Anne Murphy for statistical advice, and Devaleena Pradhan, David Sinkiewicz, Victoria Smith, and the staff at the USC Wrigley Institute for Environmental Studies for technical support. This work was supported by a National Science Foundation grant to M.S.G. (IOB-0548567), the GSU Department of Biology, and the GSU Brains & Behavior Program.

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