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Tectal CRFR1 receptors modulate food intake and feeding behavior in the South African clawed frog *Xenopus laevis*

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ABSTRACT

The optic tectum and superior colliculus rapidly inhibit food intake when a visual threat is present. Previous work indicates that CRF, acting on CRFR1 receptors, may play a role in tectal inhibition of feeding behavior and food intake. Here we test the hypothesis that tectal CRFR1 receptors modulate food intake and feeding behavior in juvenile *Xenopus laevis*. We performed five experiments to test the following questions: 1) Does tectal CRF injection decrease food intake/feeding behavior? 2) Does a selective CRFR1 antagonist block CRF effects on feeding/feeding behavior? 3) Does a reactive stressor decrease food intake/feeding behavior? 4) Does a selective CRFR1 antagonist block reactive stress-induced decrease in feeding/feeding behavior? 5) Does food deprivation increase food intake/feeding behavior? Tectal CRF injections reduced food intake and influenced exploratory behavior, hindlimb kicks, and time in contact with food. These effects were blocked by the selective R1 antagonist NBI-27914. Exposure to a reactive stressor decreased food intake and this effect was blocked by NBI-27914. Neither food intake or feeding behavior changed following 1 wk of food deprivation. Overall, we conclude that activation of tectal CRFR1 inhibits food intake in juvenile *X. laevis*. Furthermore, tectal CRFR1 receptors appear to be involved in the reduction of food intake that occurs in response to a reactive stressor.

1. Introduction

Most animals are under evolutionary selection pressure to efficiently catch prey to meet the energy requirements for growth and reproduction. Prey-capture-related behaviors can be broadly divided into two classes of response: the target-oriented or appetitive and the consummatory act (Tinbergen, 1948; Ewert, 1987), which in turn can be expanded into multiple behaviors (Avila and Frye, 1978; Duggan et al., 2016). Although the behaviors linked with prey capture (orientation, tracking, pursuit, snapping, wiping; Muto and Kawakami, 2013; Ewert, 1980) have been well studied across animal groups, the underlying neural circuits, and the homeostatic and neuroendocrine factors that modulate these circuits, are much less well known (Carr, 2015; Harris and Carr, 2016).

Decades of work in amphibians and other vertebrate groups has revealed a central role for the optic tectum (OT) in the sensorimotor integration required to detect and capture prey. The OT integrates both visual (Scalia, 1976; Ewert, 1980; Ewert et al., 2001; Carr, 2015; Liu et al., 2016) and mechanosensory (Deeg et al., 2009; Hiramoto and Cline, 2009; Deeg and Aizenman, 2011; Hamodi and Pratt, 2015; Felch et al., 2016; Hamodi et al., 2016) information in amphibians. Retinal fibers project to the superficial most layer of the OT (Lettvin, 1959) while mechanosensory inputs end in deeper layers (Hiramoto and Cline, 2009). Initiation of approach behavior begins in deep tectal neurons that project to pre-motor areas of the brainstem (Rubinson, 1968; Lázár, 1969; Weerasuriya and Ewert, 1981; Ingle, 1983; Lázár et al., 1983; Tóth et al., 1985; Ewert et al., 1985; Antal et al., 1986; Weerasuriya, 1989).

Glutamate is the principal neurotransmitter released by retinal afferents innervating the OT (Roberts and Yates, 1976; Langdon and Freeman, 1986, 1987; Debski et al., 1987; Nistri et al., 1990; Van Deusen and Meyer, 1990; Titmus et al., 1999), but there also is evidence that neuropeptides may modulate tectal contributions to feeding behavior. Several peptides have been identified in the anuran OT (Lázár, 2001), including CRF (Bhargava and Rao, 1993; Yao et al., 2004; Calle et al., 2005; Carr et al., 2010), NPY (Danger et al., 1985; Kozicz and Lazar, 1994; Chapman and Debski, 1995), and the melanocortins (Valverde et al., 2001), all of which are known to modulate food intake in other areas of the anuran brain (Carr et al., 2002; Crespi et al., 2004; Morimoto et al., 2011; Shimizu et al., 2013). Our laboratory (Carr et al., 2010; Carr et al., 2013; Carr, 2015; Prater et al., 2018) has reported that CRF, which is best known for its hypophysiotropic role in regulating ACTH secretion during stress (Norris and Carr, 2013), originating from tectal cells may act on tectal CRFR1 receptors to modulate tectal

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function. For example, we have shown that CRF is located in tectal neurons inhabiting layers 6 and 8 (Carr et al., 2010), and that CRF and CRFR1 protein content and transcript abundance changes in the OT in response to stressor exposure and food deprivation. Specifically, exposure to a stressor that inhibits food intake also elevates tectal CRF content, while food deprivation for 2 wk in subadults lowers tectal CRF levels (Prater et al., 2018). CRF is a known anorexigenic agent and it inhibits food intake (mammals, Dunn and Berridge, 1990; fish (Volkoff et al., 2005); amphibians, Crespi et al., 2004; Morimoto et al., 2011; birds (Denbow et al., 1999; Honda et al., 2014) when administered intracerebroventricular (icv) (Denbow et al., 1999; Contarino et al., 2000; Crespi et al., 2004; Morimoto et al., 2011), or microinjected into the PVN (by blocking NPY action, Heinrichs et al., 1993), the bed nucleus of the stria terminalis (Ciccocioppo et al., 2003) and basolateral amygdala (Jochman et al., 2005). A precise role for tectal CRF receptors in feeding behavior and food intake has not yet been demonstrated.

Here we test the hypothesis that tectal CRF receptors modulate food intake in juvenile *Xenopus laevis* by asking four questions: 1) Does activation of tectal CRF receptors decrease food intake? If so, then administration of exogenous CRF should act on the same receptors to decrease food intake and feeding behavior. 2) Does a selective CRFR1 antagonist block CRF effects on feeding and feeding behavior? 3) Does exposure to a reactive stressor (ether vapors), which increases tectal CRF concentrations (Prater et al., 2018), decrease food intake and alter feeding behavior, and if so, can we block these effects with a CRFR1 selective antagonist? 4) Does food deprivation increase food intake and, if so, can this be reversed with CRF? If CRF inhibits feeding behavior, then lowering endogenous CRF production in the tectum, by food deprivation, should increase food intake and feeding behavior.

2. Methods

2.1. Animals and care

Newly metamorphosed South African clawed frogs (*X. laevis*, < 2.0 g, n = 126) were obtained commercially (Xenopus Express, Inc., Brooksville, FL, USA). *X. laevis* were reared in deionized water containing 0.33 g/L Instant Ocean® in a large glass tank (8 L) at a stocking density of 20 frogs. Room temperature was 19–22 °C with a 12 L:12D light regimen. Frogs were fed 1 piece of NASCO floating *Xenopus* chow/animal three times per week prior to testing, and the tank and water were cleaned three times per week. 48 h prior to testing, the frogs were placed individually in plastic tanks (15 cm L × 12 cm W × 13 cm D) with 500 mL of deionized water and 0.15 g of Instant Ocean ®. Twenty-four hours prior to testing, frogs were weighed, and body mass was recorded. All procedures were approved by the Texas Tech Animal Care and Use Committee. Individual frogs were used only once.

2.2. Surgery

In newly metamorphosed frogs, the skull and overlying epithelium are transparent making it relatively easy to identify the OT for microinjection. Frogs assigned to an experiment involving tectal microinjections were lightly anesthetized in tricane methanesulfonate (MS-222, 0.1 g/L dH2O and buffered with equal parts $NaHCO_3$) and the overlying epithelium removed using a cautery pen. Small holes were made with a 26 G needle in the skull cartilage overlying each tectal lobe. Animals were then returned to their home cage.

2.3. Microinjections

Twenty-four h after drilling pilot holes, frogs were anesthetized in MS-222 again and injected bilaterally with test agents or vehicle using a pulled capillary tube (1 μm diameter) in a volume of 150 nL via a microinjection rig (World Precisions Instruments, Inc.). Glass capillary needles were prepared using a Flaming/Brown micropipette puller (P-97, Sutter Instruments). Injections were made in the most superficial layers of the OT. Accuracy was checked on a subset of animals (n = 6) by routine paraffin histology and hematoxylin and eosin staining (Fig. S1).

2.4. Experiment 1

Ovine CRF (oCRF, Anaspec, Freemont, CA, USA) was dissolved in sterile 0.6% NaCl and administered bilaterally into the tecta at a dose of 0.15 μg/150 nL (volume and concentration based on Baram et al., 1997). Mean body mass was 0.51 ± 0.06 g for the oCRF treated frogs (n = 9), 0.47 ± 0.04 g for the vehicle treated frogs (n = 9), and 0.60 ± 0.09 g for sham frogs (n = 11). Sham-treated frogs received the surgical treatments but the glass capillary was just touched to the tectal surface. oCRF was used as it shows low affinity for the CRF binding protein (Valverde et al., 2001) and high affinity to the xCRFR1 receptor (Dautzenberg and Hauger, 2002).

2.5. Experiment 2

We used the CRFR1 selective antagonist NBI- 27914 to block CRFR1 receptors in the OT. This antagonist displaces radiolabeled CRF binding to tectal CRF receptors and blocks CRF-induced changes in transcriptional activity in tectal slices in vitro (Carr et al., 2013). NBI-27914 (Tocris, Minneapolis, MN, USA) was dissolved in a vehicle of ethanol, Tween 80, and 0.6% saline (1:2:7) as suggested by studies in laboratory mammals (Million et al., 2013). Frogs first received either 0.6% saline (150 nL) or oCRF (0.15 μg in 150 nL 0.6% saline) using the procedure described above. Frogs then were immediately injected with either antagonist vehicle (150 nL) or NBI-27914 (0.15 μg/150 nL). Mean body mass measurements were 0.42 ± 0.02 g for the vehicle/vehicle treatment (n = 8), 0.37 ± 0.04 g for oCRF/vehicle frogs (n = 6), 0.54 ± 0.05 g for the saline/NBI - 27,914 treated frogs (n = 6), and 0.53 ± 0.04 g for the oCRF/NBI-27914 treated frogs (n = 6).

2.6. Experiment 3

Juveniles (n = 12; M_b = 1.407 ± 0.159 g) were placed into a bell jar containing a separate smaller beaker that held ether-soaked cotton balls (approximately 50 mL of ether). Frogs were exposed to ether vapors for 1 min. The control group (n = 12, M_b = 1.461 ± 0.151 g) was not treated.

2.7. Experiment 4

In a separate experiment, frogs were injected with NBI-27914 or vehicle 15 min prior to the 1-min ether exposure procedure described in Section 2.6. Frogs were assigned to one of four groups: NBI-27914 vehicle (n = 8, M_b = 0.352 ± 0.030 g) and no stressor; NBI-27914 (n = 8, M_b = 0.393 ± 0.039 g) and no stressor; vehicle (n = 8, M_b = 0.419 ± 0.022 g) followed by stressor exposure; NBI-27914 (n = 8, M_b = 0.409 ± 0.030 g) followed by stressor exposure.

2.8. Experiment 5

One group of frogs (n = 8, M_b = 0.69 ± 0.060 g) was deprived of food for 1 wk before testing. Another group of frogs (n = 7, M_b = 0.89 ± 0.07 g) were fed regularly (Section 2.1). Frogs were weighed prior to group assignment, body weights ranked, and systematically assigned to one of the two groups, normal food rations or food deprived.

2.9. Measurement of feeding behavior

All experiments were performed during the dark cycle with the assistance of infrared lighting. At $t = -24$ h, frogs were weighed and

Table 1
Ethogram for the quantification of prey capture in Juvenile *Xenopus laevis*.

ME	Behavior	Measure	Description
#	Latency to move	Duration	Time to move after addition of liver
&	Latency to contact	Duration	Time until 1/3 of frog's body contacts liver
%#	Wipe	Duration	Frog brings forelimbs to mouth
%#	Sweep	Duration	Forelimb sweeping for food
&	Contact with food	Duration	Frog is touching or holding the food, first 1/3 of frog body in contact with food
%#	Locomotion	Duration	Frog is actively swimming/locomoting
%#	Exploring	Duration	Tank bumping, wall pushing
%	Inactive	Duration	Frog not moving
	Hindlimb kick	Count	Frog brings hindfeet to mouth when in contact with food or after wipe motion

*ME is mutually exclusive. If they share a symbol, they do not happen at the same time.
Based upon (Avila and Frye, 1978).

placed into individual tanks (15 cm L × 12 cm W × 13 cm D) filled with 0.5 L ddH_20 and 0.15 g Instant Ocean. On the day of testing, frogs assigned to experiments 1, 2, and 4 were injected ($t = -60$ min) then returned to their tank prior to recordings. Frogs assigned to experiments 3 and 4 were exposed to ether vapors (t = −60 min) then returned to their tank prior to recording. Frogs assigned to experiment 5 remained in their respective tanks. At $t = 0$, 0.6 g of chicken liver (Pilgrim's Pride Corporation, Greenly, CO) was dropped into the tank and, after 60 min ($t = 60$ min), the remaining liver was weighed. Food intake was calculated as a percentage of body mass. The entire 60 min trial was recorded with a low-light video camera (Panasonic WV – CP604, Kadoma, Japan). After recording, individual feeding behavior (Table 1) were scored using JWatcher 1.0, as per the handbook's instructions (Blumstein and Daniel, 2007). Feeding behavior for n = 2 frogs were not recorded due to technical difficulties in Experiment 4.

2.10. Tissue collection

Following behavioral testing, juvenile frogs were euthanized with MS-222 (1 g/L dH2O buffered with equal parts sodium bicarbonate), a small slit made in their abdomens, and the frogs placed into Bouin's fixative or 10% neutral buffered formalin (EMD Chemicals, Inc., Gibbstown, USA). Gonadal sex was determined as previously described (Carr et al., 2003). Juveniles were dissected to reveal internal genitalia and photographed with a Nikon SMZ1500 microscope equipped with a Nikon DXM 1200F CCD.

2.11. Verification of capillary needle placement

Micropipettes were guided into the pre-drilled openings above the tecta and 150 nL of black recording ink (GRASS®, Quincy, MA, USA) injected into each lobe. Brains were removed, processed for routine paraffin embedding, and sectioned at 10 µm. Slide mounted sections were stained with Harris' hematoxylin and eosin and photographed using a Nikon Eclipse 55i microscope and Nikon Digital Sight camera (Nikon ®). Injection location was confirmed in all 6 specimens examined.

2.12. Statistical analysis

Prior to analysis, normality was assessed with Shapiro-Wilk's test. If normality was violated, the data were $\log_{10}$ or square-root transformed. Prior to $\log_{10}$ transformation, data with the value zero were assigned a value of 0.25 and all data were multiplied by 100 (McCune and Grace, 2002). Welch's correction was applied to data that was not homogenous as determined by Levene's test. Student's two-tailed *t*-test was used for comparison of two independent groups with one independent variable of continuous data and effect sizes are reported (Cohen's *d*, Experiment 3 and Experiment 5). Count data (Table 1) were analyzed by Mann-Whitney tests and effect sizes are reported (*r*). Experiments with more than two groups and one independent variable were analyzed by one-way analysis of variance (ANOVA) followed by Fisher's LSD multiple comparison test for durational data (Experiment 1). Durational data (Table 1) with two independent variables were analyzed by two–way ANOVA and count data were square-root transformed to stabilize variance (Experiment 2 and Experiment 4). Outliers were only removed if they prevented the data from reaching homogeneity of variance and normality (n = 4, ether exposed frogs, boxplots were used to detect outliers and were > 3 standard deviations outside of the mean). Removal of outliers did not change significant results but allowed homogeneity of variance and normality in the data set (Weisberg, 2014). Two main effects and an interaction were analyzed in two-way ANOVA, and effect size estimates (partial eta squared, η^2) are reported to measure the degree of association. It should be noted that contact with food for the ether/antagonist study failed Levene's test even with transformation, and wiping behavior failed Shapiro-Wilk in the same experiment. All statistical analyses and graphing were performed with SPSS (v. 11, SPSS Inc., Chicago, IL) and GraphPad Prism 7.

Additional comparisons (pre- and post- mass in food deprivation study) were also analyzed by Student's *t*-test. Pre- and post-masses were used to determine if food deprivation caused a reduction in mass. Prior to exploring sex as a covariate, the data in each experiment were investigated with a principal component analysis.

3. Results

In experiment 1, food intake changed with injection type ($F_{(2,28)} = 9.52$, $p < 0.001$, $\eta^2 = 0.405$; Fig. 1A). oCRF injected into the tecta significantly decreased food intake when compared to both sham ($p < 0.001$) and vehicle injections ($p < 0.001$, Fig. 1). Food intake in the sham and vehicle groups did not differ ($p = 0.52$, Fig.1A).

Individual prey-capture behaviors (Table 1) that were significantly altered by oCRF administration are shown in Fig. 1. All behavioral results are available in Table S1. oCRF decreased time in contact with food (Fig. 1B, $F_{(2,26)} = 6.66$, $p < 0.01$, $\eta^2 = 0.339$; sham vs. oCRF, $p < 0.01$; saline vs. oCRF, $p = 0.04$; saline vs sham, $p = 0.44$) and number of hindlimb kicks (Fig. 1C, $\chi^2 = 11.24$, $p < 0.01$; sham vs. oCRF $p < 0.01$; saline vs oCRF $p < 0.01$; sham vs saline $p = 0.88$). oCRF caused an increase in exploratory behavior compared to shams but not compared to saline treated frogs (Fig. 1D, $F_{(2,\ 26)} = 3.88$, $p = 0.033$, $\eta^2 = 0.230$; oCRF vs. sham, $p = 0.03$; oCRF vs. saline, $p = 0.15$; sham vs. saline, $p = 0.77$). Frogs spent different amounts of time inactive ($F_{(2,26)} = 3.74$, $p = 0.04$, $\eta^2 = 0.223$) but post-hoc tests failed to find significance (oCRF vs. sham, $p = 0.06$; sham vs. saline, $p = 0.09$; oCRF vs. saline, $p = 0.97$) although there was a trend in oCRF increasing inactivity. Locomotion ($F_{(2,26)} = 0.19$, $p = 0.83$, $\eta^2 = 0.014$), latency to move ($F_{(2,26)} = 1.30$, $p = 0.29$, $\eta^2 = 0.091$), latency to contact food ($F_{(2,26)} = 1.90$, $p = 0.35$, $\eta^2 = 0.128$), sweeping ($F_{(2,26)} = 2.40$, $p = 0.11$, $\eta^2 = 0.156$) and wiping ($F_{(2,26)} = 0.29$, $p = 0.75$, $\eta^2 = 0.022$) stayed consistent across treatments.

In experiment 2, data were analyzed by a two-way ANOVA and main and interaction effects are reported. Main effects included oCRF injection (+/ for oCRF and −/ for vehicle) and antagonist injection (/+ for NBI-27914 and /− for vehicle). There was a significant interaction between oCRF and antagonist injection for food intake ($F_{(1,26)} = 5.67$, $p = 0.026$, $\eta^2 = 0.205$) but no main effects (oCRF main effect: $F_{(1,26)} = 1.69$, $p = 0.208$, $\eta^2 = 0.074$; antagonist main effect: $F_{(1,26)} = 2.37$, $p = 0.139$, $\eta^2 = 0.101$). However, post-hoc tests revealed oCRF injection alone decreased food intake for *X. laevis* (+/− vs. −/−, $p = 0.03$; Fig. 2A) and NBI-27914 reversed the food intake reduction (+/+ vs. −/−, $p = 0.996$; Fig. 2A) caused by oCRF treatment. NBI-27914 alone did not cause an increase in food intake (−/− vs. −/+, $p = 0.84$; Fig. 2A).

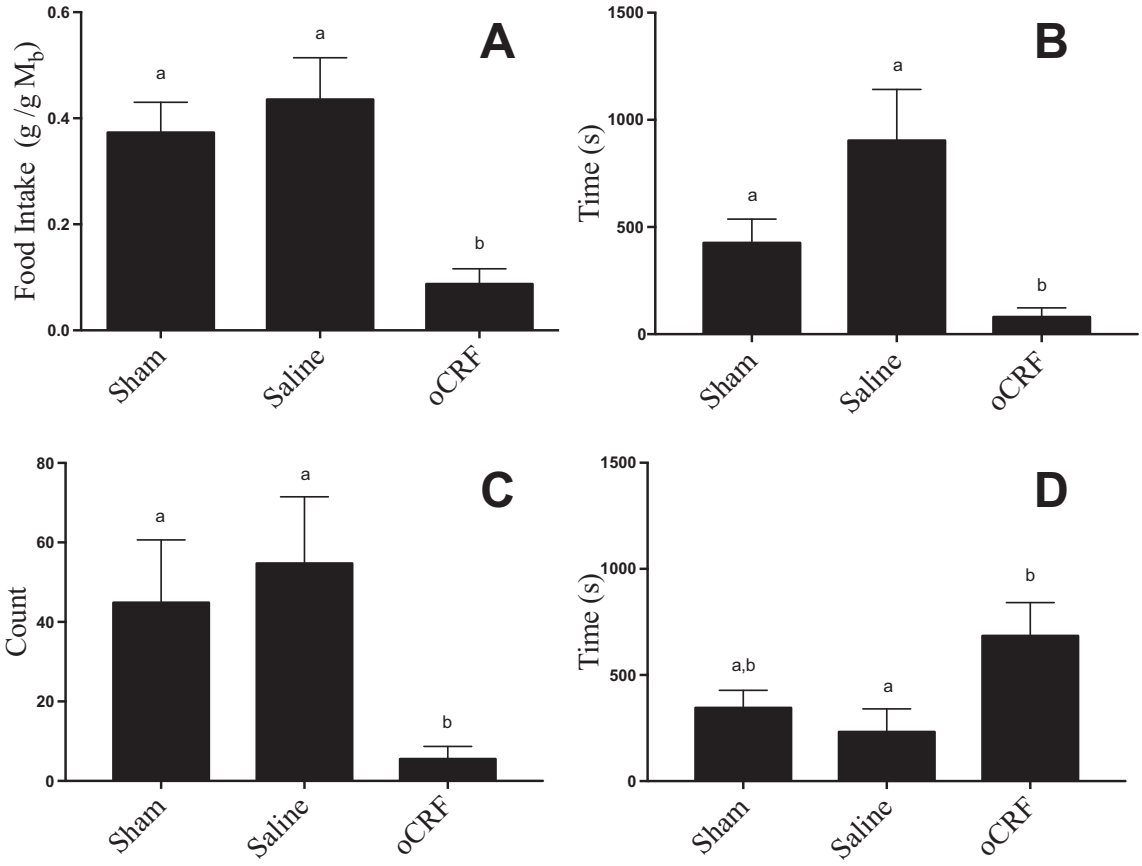

Fig. 1. The effects of bilateral tectal oCRF injection on food intake (A), time in contact with food (B), hindlimb kicks (C), and exploratory behavior (D) in juvenile *X. laevis*. Bars represent mean ± S.E.M. of n = 9–11 animals. Bars with different superscripts are statistically different based upon one-way ANOVA and Fisher's LSD post-hoc test for durational data (Figs. 1A, B, D) and Kruskal-Wallis for count data (Fig. 1 C)($p < 0.05$).

Table S2 summarizes all of the behavioral results for experiment 2. The oCRF and antagonist vehicle combination did not significantly affect contact with food (Fig. 2B; interaction: $F_{(1,27)} = 0.963$, $p = 0.337$, $\eta^2 = 0.042$; oCRF main effect: $F_{(1,27)} = 1.62$, $p = 0.217$, $\eta^2 = 0.068$; antagonist main effect: $F_{(1,27)} = 0.064$, $p = 0.803$, $\eta^2 = 0.003$) or hindlimb kicks (Fig. 2C; approached an interaction: $F_{(1,27)} = 0.258$, $p = 0.123$, $\eta^2 = 0.109$; oCRF main effect: $F_{(1,27)} = 0.030$, $p = 0.863$, $\eta^2 = 0.001$; antagonist main effect: $F_{(1,27)} = 0.049$, $p = 0.827$, $\eta^2 = 0.002$). The only feeding behavior affected by treatment was exploratory behavior (Fig. 2D). Exploratory behavior was affected by an interaction ($F_{(1,27)} = 10.76$, $p = 0.004$, $\eta^2 = 0.339$) between the two main effects, oCRF ($F_{(1,27)} = 0.037$, $p = 0.850$, $\eta^2 = 0.002$) and antagonist ($F_{(1,27)} = 4.03$, $p = 0.058$, $\eta^2 = 0.161$). oCRF treated frogs (+/− vs −/+) explored more compared to their vehicle injected counterparts (Fig. 2D). Neither oCRF nor antagonist had an effect on inactivity (interaction: $F_{(1,27)} = 0.055$, $p = 0.817$, $\eta^2 = 0.002$; oCRF main effect: $F_{(1,27)} = 0.007$, $p = 0.936$, $\eta^2 = 0.000$; antagonist main effect: $F_{(1,27)} = 1.277$, $p = 0.271$, $\eta^2 = 0.055$), latency to contact (interaction: $F_{(1,27)} = 0.400$, $p = 0.534$, $\eta^2 = 0.018$; oCRF main effect: $F_{(1,27)} = 1.49$, $p = 0.235$, $\eta^2 = 0.063$; antagonist main effect: $F_{(1,27)} = 1.12$, $p = 0.302$, $\eta^2 = 0.048$), latency to move (interaction: $F_{(1,27)} = 0.00$, $p = 0.995$, $\eta^2 = 0.000$; oCRF main effect: $F_{(1,27)} = 0.00$, $p = 0.994$, $\eta^2 = 0.00$; antagonist main effect: $F_{(1,27)} = 0.425$, $p = 0.521$, $\eta^2 = 0.018$), locomotion (interaction: $F_{(1,27)} = 0.031$, $p = 0.861$, $\eta^2 = 0.001$; oCRF main effect: $F_{(1,27)} = 0.263$, $p = 0.613$, $\eta^2 = 0.011$; antagonist main effect: $F_{(1,27)} = 0.514$, $p = 0.481$, $\eta^2 = 0.022$), wiping (interaction: $F_{(1,27)} = 2.33$, $p = 0.141$, $\eta^2 = 0.092$; oCRF main effect: $F_{(1,27)} = 0.379$, $p = 0.544$, $\eta^2 = 0.016$; antagonist main effect: $F_{(1,27)} = 0.398$, $p = 0.534$, $\eta^2 = 0.017$).

In experiment 3, ether–exposed frogs ate less than controls ($t = 2.48$, $p = 0.02$, $d = 1.01$; Fig. 3). Ether exposure did not impact any of the measured prey-capture behaviors: exploratory behavior ($t = 1.83$, $p = 0.12$, $d = 2.59$); latency to contact ($t = 1.05$, $p = 0.32$, $d = 0.06$); time in contact with food ($t = 0.66$, $p = 0.52$, $d = 0.941$); hindlimb kicks (Mann-Whitney $U = 15$, $p = 0.70$, $r = 0.00$); inactivity ($t = 1.40$, $p = 0.21$, $d = 1.98$); locomotion ($t = 0.76$, $p = 0.47$, $d = 1.07$); latency to move ($t = 1.23$, $p = 0.25$, $d = 1.18$); sweeping ($t = 0.30$, $p = 0.77$, $d = 0.310$); or wiping ($t = 0.91$, $p = 0.39$, $d = 1.28$). A summary of behavioral data for experiment 3 is shown in Table S3.

In experiment 4, treatment groups (control, −/) and ether, +/) were pre-injected with either NBI-27914 (/+) or vehicle (/−). *X. laevis* ate less when exposed to ether vapors and this effect was prevented when pre-treated with antagonist prior to exposure (interaction: $F_{(1,25)} = 10.3$, $p = 0.004$, $\eta^2 = 0.292$; ether main effect: $F_{(1,25)} = 5.13$, $p = 0.033$, $\eta^2 = 0.170$; antagonist main effect: $F_{(1,25)} = 5.04$, $p = 0.034$, $\eta^2 = 0.168$; (+/−) vs. (−/−), $p < 0.001$; (+/+) vs. (+/−), $p = 0.001$). Antagonist alone did not increase food intake (−/+) vs. (−/−), $p = 0.508$; Fig. 4A), which was consistent with our findings in Experiment 2.

Table S4 summarizes the feeding behavior for Experiment 4. Exploratory pairwise comparisons revealed that antagonist increased contact with food compared to vehicle for ether exposed *X. laevis*. (+/+ vs. +/−, $p = 0.027$, interaction: $F_{(1,25)} = 1.04$, $p = 0.317$, $\eta^2 = 0.040$; ether main effect: $F_{(1,25)} = 0.088$, $p = 0.769$, $\eta^2 = 0.003$; antagonist main effect: $F_{(1,25)} = 2.57$, $p = 0.121$, $\eta^2 = 0.093$, Fig. 4B) relative to ether-exposed vehicle control animals. Both antagonist and ether exposure had an interaction ($F_{(1,25)} = 7.43$, $p = 0.012$, $\eta^2 = 0.229$) and main effect on hindlimb kicks (ether main effect: $F_{(1,25)} = 5.96$, $p = 0.022$, $\eta^2 = 0.193$; antagonist main effect:

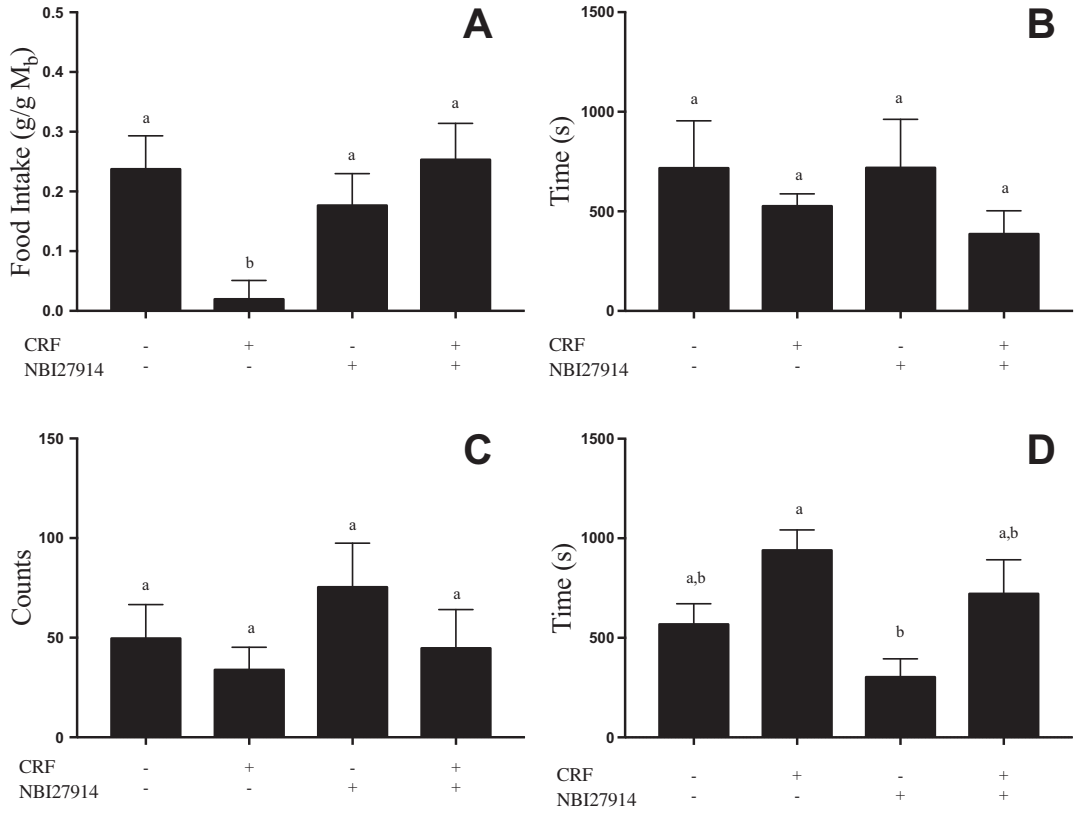

Fig. 2. Food intake (A), time in contact with food (B), hindlimb kicks (C), and exploratory behavior (D) following bilateral tectal administration of oCRF in the presence or absence of NBI-27914 in juvenile *X. laevis*. Bars represent mean ± SEM of n = 6–8 animals. Superscripts were determined by two – way ANOVA followed by Fisher's LSD post – hoc tests. Error bars represent ± SEM.

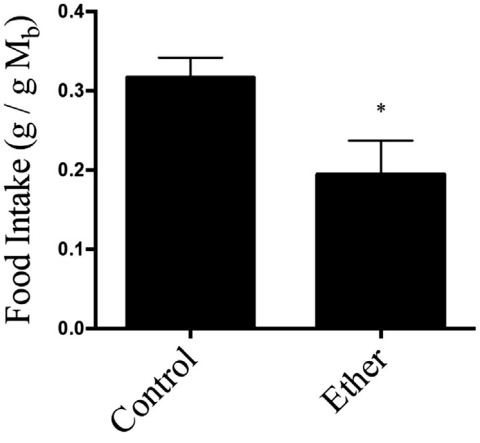

Fig. 3. Effect of ether vapor exposure on food intake in *X. laevis*. Bars represent mean ± SEM of n = 12 animals. Bars with asterisks are statistically different based upon Student's two-tailed *t* – test ($p < 0.05$).

$F_{(1,25)} = 5.64$, $p = 0.026$, $\eta^2 = 0.184$). However, inspection revealed that antagonist alone (−/+) caused an increase in hindlimb kicks in untreated frogs (−/+ compared to −/−, $p = 0.002$), and ether exposure did not cause a decrease in hindlimb kicks (+/− compared to −/−, $p = 0.833$). Locomotion decreased after ether exposure, but antagonist alone did not increase locomotion (interaction: $F_{(1,25)} = 1.80$, $p = 0.192$, $\eta^2 = 0.067$; ether main effect: $F_{(1,25)} = 14.0$, $p = 0.001$, $\eta^2 = 0.359$; antagonist main effect: $F_{(1,25)} = 1.20$, $p = 0.283$, $\eta^2 = 0.046$; +/− vs. −/−, $p = 0.084$; +/− compared to +/+, $p = 0.090$, −/+ compared to −/−, $p = 0.868$). Similar to locomotion, changes in latency to move was in the comparison of frogs that were injected with antagonist and either exposed to ether or untreated but not with frogs that were injected with vehicle and either exposed to ether or untreated (interaction: $F_{(1,25)} = 0.763$, $p = 0.391$, $\eta^2 = 0.030$; ether main effect: $F_{(1,25)} = 5.95$, $p = 0.022$, $\eta^2 = 0.192$; antagonist main effect: $F_{(1,25)} = 0.600$, $p = 0.446$, $\eta^2 = 0.023$; +/+ vs. −/+, $p = 0.035$; +/− compared to −/−, $p = 0.253$). Neither ether nor antagonist had an effect on latency to contact food (interaction: $F_{(1,25)} = 0.682$, $p = 0.417$, $\eta^2 = 0.027$; ether main effect: $F_{(1,25)} = 0.099$, $p = 0.756$, $\eta^2 = 0.004$; antagonist main effect: $F_{(1,25)} = 0.094$, $p = 0.762$, $\eta^2 = 0.004$); exploring (interaction: $F_{(1,25)} = 0.722$, $p = 0.403$, $\eta^2 = 0.028$; ether main effect: $F_{(1,25)} = 0.261$, $p = 0.614$, $\eta^2 = 0.010$; antagonist main effect: $F_{(1,25)} = 1.46$, $p = 0.239$, $\eta^2 = 0.055$); inactivity (interaction: $F_{(1,25)} = 1.06$, $p = 0.312$, $\eta^2 = 0.041$; ether main effect: $F_{(1,25)} = 0.498$, $p = 0.487$, $\eta^2 = 0.020$; antagonist main effect: $F_{(1,25)} = 0.661$, $p = 0.424$, $\eta^2 = 0.026$); wiping (interaction: $F_{(1,25)} = 3.35$, $p = 0.077$, $\eta^2 = 0.100$; ether main effect: $F_{(1,25)} = 0.543$, $p = 0.467$, $\eta^2 = 0.018$; antagonist main effect: $F_{(1,25)} = 1.65$, $p = 0.209$, $\eta^2 = 0.052$); or sweeping (interaction: $F_{(1,25)} = 0.102$, $p = 0.752$, $\eta^2 = 0.004$; ether main effect: $F_{(1,25)} = 0.050$, $p = 0.824$, $\eta^2 = 0.002$; antagonist main effect: $F_{(1,25)} = 1.86$, $p = 0.184$, $\eta^2 = 0.069$).

In experiment 5, food deprivation (1 wk) did not cause an increase in food intake ($t = 0.52$, $p = 0.68$, $d = 0.099$) although post-deprivation body-masses for food-deprived frogs ($M_b = 0.69 \pm 0.06$) were less than controls ($M_b = 0.89 \pm 0.06$; Student's *t* – test, $p = 0.045$) indicating that 1 wk of food deprivation was sufficient to alter energy homeostasis at this developmental stage. Food deprived animals were consistent in their behavior compared to controls (latency to contact: $t = 0.10$, $p = 0.93$, $d = 0.134$; exploring: $t = 0.67$, $p = 0.52$, $d = 1.05$; contact with food: $t = 0.11$, $p = 0.91$, $d = 0.115$; hindlimb kicks: Mann-Whitney $U = 10$, $p = 0.24$, $r = 0.107$; inactive: $t = 1.40$,

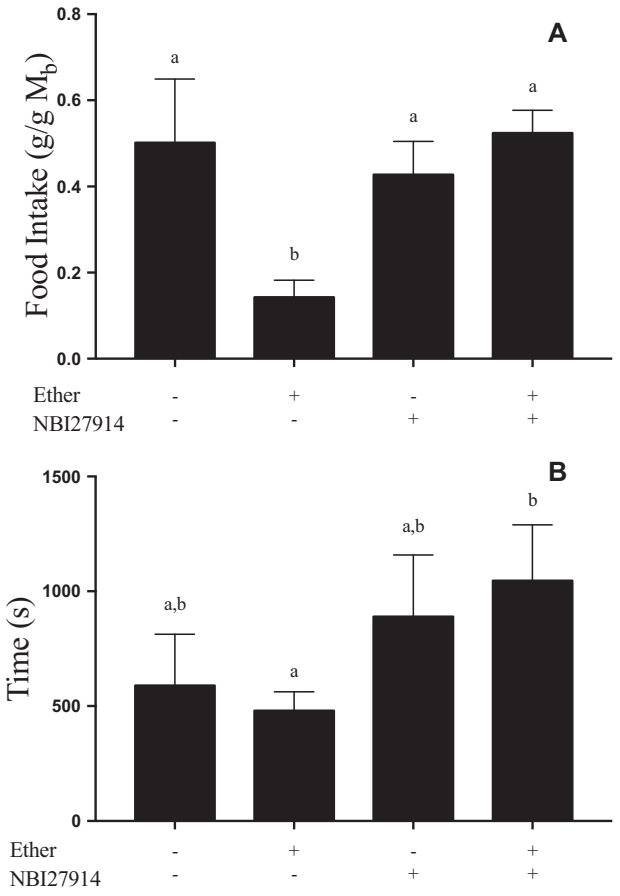

Fig. 4. Food intake (A) and time in contact with food (B) differences following ether exposure (+) or no ether exposure (−) and NBI-27914 (+) or vehicle injection (−) into the tecta of juvenile *Xenopus laevis*. Bars represent mean ± SEM of n = 6–8 animals. Bars with different superscripts are statistically different based upon two-way ANOVA and Fisher's LSD post – hoc test ($p < 0.05$).

$p = 0.21$, $d = 0.567$; locomotion: $t = 0.76$, $p = 0.48$, $d = 0.403$; latency to move: $t = 1.2$, $p = 0.25$, $d = 0.403$; sweeping: $t = 0.30$, $p = 0.77$, $d = 2.10$; wiping: $t = 0.91$, $p = 0.39$, $d = 0.770$). A summary of the prey-capture behaviors for experiment 5 is shown in Table S5.

Sex determination revealed that the metamorph frogs used in experiments 2 and 3 were biased towards females (only 6 and 9 males, respectively) based on assessment of gonadal gross morphology, so investigative statistics were not done for these experiments. Behavioral data in experiments 1, 4, and 5 were further analyzed via principal component analysis using R (version 1.0.136; R Core Team, 2013) to investigate any behaviors that could be influenced by phenotypic sex as determined by gonadal morphology as there are no secondary sex characteristics apparent at this stage. Factors included phenotypic sex, food intake, latency to contact, exploring, contact with food, gulping, hindlimb kicks, inactivity, locomotion, latency to move, sweeping, and wiping. Experiment 1, 4, and 5 had a balanced sex ratio and principal components analysis (not shown) revealed that the majority of the variability was due to behavior with no indication of sex having an influence on any of the behaviors.

4. Discussion

In this set of experiments, we tested the hypothesis that tectal CRF inhibits feeding behavior in *X. laevis*. We showed that 1) CRF injected into the OT decreased food intake and that this effect can be blocked by pre-treatment with a selective CRFR1 antagonist, 2) exposure to ether vapors (a reactive stressor, Olsen et al., 1999; Emmert and Herman, 1999), known to elevate tectal CRF content (Prater et al., 2018), reduced food intake and this effect was blocked with a selective CRFR1 antagonist, and 3) lack of food for one week failed to alter food intake in juvenile *X. laevis*. Thus, our overall hypothesis that tectal CRFR1 receptors modulate food intake was supported.

Tectal oCRF injection may have decreased food intake by decreasing the number of hindlimb kicks and time in contact with food. Intracerebroventricular administration of CRF generally affects locomotion in many different vertebrate species, including amphibians (Lowry et al., 1990; Lowry and Moore, 1991; Lowry et al., 1993; Lowry et al., 1996; Lowry and Moore, 2006). Interestingly, CRF modulation of amphibian locomotion involves receptors in the brainstem, including premotor areas thought to receive tectal efferents that control prey capture and predator avoidance (Ewert et al., 1990; Schwippert et al., 1990; Lowry et al., 1996). Hindlimb kicks are used to tear food, are elicited after a strong stimulation in the mouth, and also may be regulated by the retina-tecto/tegmento-bulbar/spinal pathway (Ewert et al., 2001; Hutchinson, 1964). In our study we also measured three aspects of movement not related to targeted feeding behavior, but which inevitably occur during the 3600 s test window: exploring, general locomotion, and inactivity. Of these, exploratory behavior was the only behavior to show an effect of treatment, although post-hoc tests could not eliminate the possibility that injection itself caused this effect as oCRF treatment showed no differences compared to saline injected animals. The fact that locomotion was not affected by oCRF administration suggests that CRF did not make the frogs lethargic or sick.

The fact that oCRF both reduced hindlimb kicks and increased exploratory behavior is reminiscent of the pattern of behavioral effects observed in rats after CRF administration to the hypothalamus (Monnikes et al., 1992). Monnikes et al. (1992) reported dual effects of CRF on locomotion that were dose dependent. At low doses CRF increased locomotion while at higher doses the peptide increased inactivity and produced freezing behavior (Monnikes et al., 1992), which is the ultimate stage in mammalian fear responses (De Franceschi et al., 2016), and would happen if capture by a predator was imminent. In our study we observed a treatment effect on inactivity, although post-hoc testing was unable to show clear differences between individual levels of treatment. Such a dual effect is more difficult to explain in our experiment because only a single dose of CRF was used. However, the diffusion of the peptide through the upper tectal layers that were targeted by the injections may have affected multiple efferent pathways from the OT.

NBI-27914 administered bilaterally via the tectal lobes reversed the effects of oCRF on food intake, indicating that the effects of oCRF on food intake are mediated in part by tectal CRFR1 receptors. Interestingly, treatment with NBI-27914 alone (experiments 2 and 4) did not alter food intake, suggesting that elevated, but not baseline, release of OT CRF impacts feeding. These results differ from previous studies in juvenile *X. laevis* where i.c.v. injection of CRF antagonist increased feeding (Crespi et al., 2004), suggesting a specific role of OT CRF on feeding. The findings with individual feeding behavior are not so clear, as oCRF in combination with NBI-27914 vehicle delivery failed to alter time in contact with food or hindlimb kicks based upon two-way ANOVA. In Western spadefoot toads (*Spea hammondii*) foraging behavior was decreased by i.c.v. oCRF injection and increased with a non-selective CRFR1 antagonist (alpha-helical CRF 9-41; Ki of 17 nM at human CRFR1) (Crespi and Denver, 2004), but results differed by developmental stage. Crespi and Denver found that i.c.v. injection of antagonist alone in premetamorphic tadpoles reduced locomotion but did not affect foraging, similar to our antagonist and food-intake findings in experiments 3 and 4. However, foraging was increased after antagonist injection in prometamorphic tadpoles (Crespi and Denver, 2004), suggesting developmental stage is an important factor (Crespi et al., 2004). The differences between our work and that of Crespi et al. (2004) could be due to differences between injection sites. In theory

i.c.v. injection would distribute the antagonist to CRF receptors in a number of hypothalamic and thalamic locations surrounding the third ventricle whereas the peptide was delivered to the OT in our study. If feeding behavior is tonically inhibited by CRF receptors in the hypothalamus or thalamus, our targeted injections would likely not have reached those receptors. Secondly, alpha-helical CRF is non-selective and targets both CRFR1 and CRFR2 receptors. In fact, inhibitory constants (Ki) for alpha-helical CRF 9-41 binding at the human CRFR2 receptors are lower than for CRFR1 receptors (Ki = 17 and 5 nM at human CRFR1 and rat CRFR2α, respectively) (Perrin and Vale, 1999). Thus, the baseline regulation of feeding behavior revealed by Crespi et al. (2004) may have involved CRFR2 receptors, whereas we used a highly selective CRFR1 antagonist in our study.

NBI-27914 appeared to have an inhibitory effect on exploratory behavior but only compared to the group that was injected with oCRF and NBI-27914 vehicle. NBI-27914 failed to have this effect when co-injected with oCRF. This finding, and the lack of oCRF effect on time in contact with food or hindlimb kicks when co-injected with NBI-27914 vehicle, indicates that non-peptide antagonists such as NBI-27914 and antalarmin (which require hydrophobic solvents for delivery), may not be ideal for sub-microliter microinjection volumes into small brain areas, at least in anurans. We chose NBI-27914 (Baram et al., 1997) for this study because of its ability to displace radiolabeled oCRF from tectal CRF binding sites in *X. laevis* OT (Carr et al., 2013). Since the discovery of NBI-27914 (Baram et al., 1997), selective CRF R1 antagonists with more (CP 376395 hydrochloride; Chen et al., 2008) or complete (NBI-35965; Million et al., 2003) water solubility have become available and may be better suited for CNS microinjection studies in laboratory animals. Our findings also bring into question the degree to which time in contact with food and hindlimb kicks contribute to oCRF-induced reductions in food intake, as these behaviors were not significantly altered by oCRF treatment in experiment 2. One possibility is that these behaviors are far downstream of the apical targets for tectal CRFR1 modulation of food intake. However, more details are required on the mode of CRF action in the OT before any such conclusions are drawn.

The influence of stress on sensorimotor systems is surprisingly understudied. Central CRF neurons have been implicated in stress in anurans (Denver, 2009), suggesting that their role in the CNS regulation of stress has been conserved for several hundred million years at least. Moreover, a previous study by our laboratory (Prater et al., 2018), suggests that tectal CRF neurons are sensitive to a reactive stressor, ether vapors. Prater et al. (2018) were the first to show that ether vapor exposure reduced feeding behavior and increased OT CRF concentration in sexually immature *X. laevis*. Here, using much smaller frogs (< 2 g), we observed the same inhibitory effect of ether vapor exposure on food intake. This suggests that the ability to reduce food intake in response to a novel reactive stressor is present throughout much of the post-metamorphic development in this species.

When fasting, animals undergo a suite of behavioral and physiological changes. Initially animals may reduce activity to conserve energy but will increase foraging behavior when deprived of food for longer periods of time (Wang et al., 2006). For example, when zebrafish are deprived of food for 7 d post-fertilization they are more likely to approach food and less likely to engage in escape behavior (Filosa et al., 2016). This satiety-state modulation of visually guided behavior depends upon neuronal decision making in the OT (Filosa et al., 2016) and may be driven by CRF. For example, in *X. laevis*, ICV injection of CRF causes a decrease in food intake while NPY, corticosterone, and CRF antagonist cause increases in food intake (Crespi et al., 2004). CRF mRNA content (combined mid-posterior hypothalamus, tectum, and pretectum) also decreases 6 h after a meal and remains low through 31 d of food deprivation in juvenile *X. laevis* (Crespi et al., 2004). However, 2-wk of food deprivation did not influence CRF mRNA content of the OT but decreased CRF peptide content of the OT in larger sub-adult *X. laevis* (Prater et al., 2018). Following 1-wk food deprivation in this experiment, *X. laevis* did not increase food intake or change behavior, although there was loss of body mass. However, *X. laevis* has several known adaptations to starvation (Merkle and Hanke, 1988) including reductions in oxygen consumption, increased lipolysis, and changes in plasma hormone levels such that stage III of starvation is not observed until at least 12 mo of food deprivation. As such, it is not entirely unexpected that 1 wk of food deprivation did not increase food intake. While longer bouts of food deprivation may ultimately have led to changes in food intake, IACUC restrictions prevent such a study at present, at least in animals this small.

In summary, our data indicate that activation of tectal CRF R1 receptors inhibits food intake in the anuran *X. laevis,* whereas the effects on individual prey-capture behaviors seem to be more variable. Exposure to a reactive stressor appears to activate tectal CRFR1 receptors and reduce food intake, possibly through the release of CRF from intrinsic tectal neurons (Carr et al., 2010; Carr et al., 2013). One-week of food deprivation had no effect on food intake or prey-capture behaviors. Our data support a role for tectal CRF R1 receptors in modulating food intake in response to a stressor.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2018.07.013.

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Conflicts of interest

None.

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