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Neuroendocrine modulation of predator avoidance/prey capture tradeoffs: Role of tectal NPY2R receptors

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A B S T R A C T

The optic tectum rapidly inhibits food intake when a visual threat is present. Anatomical and electrophysiological evidence support a role for neuropeptide Y (NPY), originating from cells in the thalamus, in the tectal inhibition of prey capture. Here we test the hypothesis that tectal NPY receptor type 2 (NPY2R) influences prey-capture and predator-avoidance responses in the African clawed frog, *Xenopus laevis*. We tested two questions: 1) Does tectal NPY administration decrease food intake and alter prey-capture behavior? 2) Does tectal administration of a NPY2R antagonist increase food intake, alter prey-capture behavior, and alter predator avoidance behavior? NPY microinjected bilaterally into the tecta failed to significantly alter food intake at any dose tested, although predator presence significantly reduced food intake. However, NPY differentially altered discrete components of prey capture including increasing the latency to contact food and reducing the amount of time in contact with food. These effects were blocked by the NPY2R antagonist BIIE0246. Additionally, BIIE0246 elevated food intake on its own after bilateral tectal microinjection. Furthermore, BIIE0246 reversed the reduction of food intake caused by exposure to a predator. Overall, these findings indicate that tectal NPY2R activation causes frogs to consume food more quickly, which may be adaptive in predator-rich environments. Blocking tectal NPY2R increases baseline food intake and reduces or eliminates predator-induced changes in prey capture and food intake.

1. Introduction

A vast literature supports the notion that animals consider the risk of predation when making decisions about how to approach potential food objects and prey in their environment, often trading off risk against opportunity for obtaining energy (Cobas and Arbib, 1992; Anholt et al., 2000; reviewed recently by Harris and Carr, 2016). Most animals can assess potential threats in their environment and react in a way that reduces the possibility of being preyed upon (Lima and Dill, 1990; Lima, 1998). For example, when predators are abundant, larval frogs reduce the time spent active and reduce swimming speed in spite of food being available (Anholt et al., 2000), and this pattern suggests that the animals are simultaneously sensitive to risks from predation and gains from feeding (Anholt et al., 2000).

Most anuran amphibians locate food visually and recognize the prey via subcortical visual pathways (Carr, 2006). The optic tectum (OT) is critical for visual and mechanosensory detection of prey and threats. With respect to making visually guided approach/avoid decisions, the OT receives visual information from the retina via retinal ganglion cells (Maturana et al., 1960; Jacobson, 1962), integrates this information (Székely and Lázár, 1976; Deeg et al., 2009), and then commands appropriate adaptive responses (approach, avoid) via deep tectal neurons that project independently to premotor neurons in the brainstem (Kostyk and Grobstein, 1987; Ewert et al.,1990; Dicke and Roth, 1994). The OT plays a vital role in visually distinguishing between predator and prey as the ablation of the OT alone prevented visually-guided prey capture (Bechterew, 1884; Sperry, 1944) and completely hampered ducking, jumping or even eye closure responses to a dark object in *Rana* (Bechterew,1884; Ingle, 1973).

There is a lack of precise information about how visual predator cues are processed and detected by the OT. Work on anuran amphibians suggests that certain electrophysiologically identified neurons of the thalamus and pretectum (so-called TH3 and TH4 neurons) (Wietersheim and Ewert, 1978) detect key visual features of predators (Ewert 1967, 1968; Wietersheim and Ewert, 1978) and are responsible for inhibiting prey signals in the OT, thus shutting down feeding-related approach behavior when a predator is present (Ewert, 1980). In primates, visual threats are detected by neurons of the pulvinar thalamus which inhibit the superior colliculus (SC; mammalian homolog to the OT) (Van Le et al., 2013; Le et al., 2014; Carr, 2015; Soares et al., 2017). Whether these threat-detecting neurons in the primate pulvinar nucleus are homologous to threat-detecting neurons in the anuran thalamus remains an open question (Carr, 2015).

Although the neurochemistry of the predator detection pathway is not well studied, work from our laboratory (Shoukfeh et al., 2004) and others (Kozicz and Lázár, 1994; Chapman & Debski, 1995; Schwippert

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et al., 1998; Funke & Ewert, 2006) suggests that neuropeptide Y (NPY), an orexic peptide in mammals (Edwards et al., 1999; Gehlert, 1999; Kalra & Kalra, 2004), may be a major transmitter projecting from the pretectum to the OT to inhibit prey detection (Funke and Ewert, 2006; Schwippert and Ewert, 1995; Schwippert et al., 1998). NPYergic cells in the pretectum project to retinorecipient layer 9 in the OT (Kozicz & Lázár, 1994). Exposure of the OT to exogenous NPY reduces the neuronal response to electrical stimulation of the contralateral optic nerve (Schwippert and Ewert, 1995) or changes in ambient light (Schwippert et al., 1998) and prevents [14C]2-deoxyglucose uptake in the OT in response to prey presentation (Funke and Ewert, 2006), suggesting NPY dampens image-related OT response. A comparable mechanism exists (Gamlin et al., 1996) in pigeons. The innervation by NPY of the pretectum and tectum is also seen in different birds (Britto et al., 1989) and mammals (Borostyánkoi et al., 1999; Chronwall et al., 1985; Morin & Blanchard, 1997). It is suggested that NPY released in the tectum from pretectal projection neurons inhibits glutamate (Chen et al., 1997; Greber et al., 1994), thereby inhibiting excitatory retinotectal transmission (Funke & Ewert, 2006). Thus, these data suggest the possibility that in amphibians and birds NPY may suppress the tectal processing of visually-guided prey capture.

Although tectal NPY2R receptors appear to modulate field potentials induced by a light stimulus (Schwippert et al., 1998), their precise role in prey capture and predator avoidance is unknown. We hypothesized that activation of tectal NPY2R receptors reduces prey capture and promotes adaptive behavior in the presence of a predator. To test this hypothesis, we manipulated tectal NPY2R receptors pharmacologically while monitoring the results on prey capture and specific elements of approach and avoidance behavior in juvenile African clawed frogs *Xenopus laevis*.

2. Methods

2.1. Animals and care

Newly metamorphosed South African Clawed Frogs (*Xenopus laevis,* 0.3–1.5 g, from hereafter referred to as 'juveniles') were purchased from a commercial supplier (Xenopus Express). Juveniles were maintained in 8 L glass aquaria at a stocking density of 16 per 8 L deionized water containing 0.33 g/L Instant Ocean and kept at 19–22 °C on a 12L: 12D light regimen. Large adult female frogs for predator assays were reared in flow-through tanks (160 L) containing dechlorinated tap water at a stocking density of 15 per 160 L. Juveniles were fed floating pellets (Nasco, Fort Atkinson, WI, USA) while adults were fed sinking pellets from Nasco three times per week. Water was changed in the juvenile's tanks three times a week. All procedures were approved by the Texas Tech Animal Care and Use Committee.

2.2. Surgery and tectal microinjection procedure.

In newly metamorphosed frogs, the skull and overlying epithelium are transparent making it relatively easy to identify the OT for microinjection. Prior to surgery (48 h) juveniles were isolated in individual tanks containing 500 mL deionized water with 0.33 g/L Instant Ocean. On the day of surgery juveniles were lightly anesthetized in MS-222 (0.1 g/L dH2O and buffered with equal parts $NaHCO_3$) and the epithelium overlying the skull removed using a cautery pen. Small pilot holes were made in the skull cartilage with a 26 G needle overlying each tectal lobe. Frogs were then returned to their original isolation tank prior to receiving injections.

Twenty-four h after drilling pilot holes, frogs were anesthetized in MS-222 again and injected bilaterally with test agents or vehicle (see Experimental Design, Section 2.5) using a pulled glass capillary needle (1 μm diameter pulled, 20 μm in use) in a volume of 150 nL–200 nL via a microinjection rig (World Precisions Instruments, Inc.) after Prater et al. (2018). 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 where the neurons integrating visual input are located. For validation of this procedure see Prater et al. (2018) Fig. S1.

2.3. Prey-capture assay

The prey-capture assay was developed by Prater et al. (2018). Twenty-five h before experimentation, juveniles that had been fasted for 48 h previously were weighed, pilot holes created for microinjection (2.2 Surgery and tectal microinjection procedure), and frogs returned to their isolation tanks to recover. On the morning of the test, juveniles were removed from their isolation tank and placed into individual aquaria (15 cm L × 12 cmW × 13 cm D, 'test tank') filled with 0.5 L ddH20 and 0.15 g Instant Ocean and allowed to acclimate for 5–6 h. The test tanks were covered in black plastic. Sixty minutes prior to testing, juveniles were microinjected with test agents (2.2 Surgery and tectal microinjection procedure), vehicle, or received sham injections (see 2.5 Experimental design below) and returned to their individual test tank. 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 and food intake calculated by dividing the mass of liver eaten by body mass. A low light WV-CP504 Panasonic video camera (Kadoma, Japan) was used to record behavior in the dark with infrared lighting. All experiments were performed during lights off between 2 and 4 PM CST. There was no one in the recording room at the time of video recording. After testing, juveniles were placed back into their isolation tank. Prey capture behaviors (Table 1) were scored using JWatcher v. 1.0 (Blumstein and Daniel, 2007).

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.
	lunge	count	a slight rapid backward push with the forelimbs plus an obvious rapid forward push with the hind limbs to propel body within @ 8 cm of liver
#	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 while onsubstrate or at surface.
	hind limb kick	count	Frog brings hind feet to mouth when in contact with food or after wipe motion

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

2.4. Predator avoidance assay

This assay was based on the methods of Duggan et al. (2016) except that size-matched conspecific animals were not used. All experiments were performed in the dark with infrared lighting. A low light WV-CP504 Panasonic video camera (Kadoma, Japan) was used to record animal behavior onto DVDs. There was no one in the room at the time of video recording. Frogs received pilot holes for microinjection (see above) and were then isolated for 24 h in one half (test side) of a glass aquarium (50 L × 25 W × 30 H cm) covered in black with a clear divider (#TDMBX, Aqua Life, Hauppauge, NY) containing holes for free passage of water through the divider. Tanks were filled with 12 L of water containing 0.3 g Instant Ocean/L. The test side of the tank contained a hide constructed of PVC, painted black with the open ends (3.81 cm diameter) facing toward the divider. The test side was visually divided into equal thirds with the first third being nearest the predator and contained the food, the middle third contained the hide, and the back third was the farthest from the divider.

Juveniles received bilateral microinjections of test substances, returned to the test side, and were allowed to acclimate in the predator avoidance test tank for 10 min without a prey or predator stimulus. After 10 min a large conspecific female adult frog (predator) was added to the other side of the divider. Ten minutes after adding the predator, 1.2 g of chicken liver was glued to a lead washer (total size 4.45 cm in diameter, 0.64 cm high; painted black) and was then added to the first third of the tank side containing the juvenile frog. Behaviors were recorded for 30 min. In addition to the prey capture behaviors described in Table 1, we also scored time spent in the front, middle and back third of the tank using JWatcher v. 1.0 (Blumstein and Daniel, 2007). Frogs were returned to their home tanks after testing. Only data measured for the time with predator and prey presented together are reported.

2.5. Experimental design

In total, across three experiments, we used 128 juveniles to examine the effects of activating and inhibiting tectal NPY2R on prey capture and predator avoidance. Frogs were only used once and were euthanized after use for determination of gonadal sex.

In Experiment 1, we tested several doses of porcine NPY (pNPY) on prey-capture behavior. Juveniles (n = 8 per group, 40 total) were injected bilaterally via the tecta with one of three doses of pNPY (1.5, 15, or 150 ng/150 nL; Bachem, Torrance, CA, USA) dissolved in 0.6% saline, or with just 0.6% saline (150 nL). A separate group of animals served as sham-treated controls. In sham animals the glass needle was placed through the pilot hole in each tectal lobe but no injection was made. After injection (60 min) frogs were tested in the prey capture assay as described above.

In Experiment 2, frogs (five treatment groups, n = 8 per group, 40 total) were microinjected bilaterally into the tecta first with 150 ng/150 nL pNPY or vehicle (0.6% saline) followed by BIIE0246 (1.12 ng/200 nL; Tocris, Minneapolis, MN, USA), a selective NPYR2 antagonist, dissolved in 50% DMSO/dH2O or 50% DMSO/dH2O alone as a vehicle control. Separate groups of animals served as surgical shams. In surgical shams the microinjection needle was lowered to touch, but not enter the tecta. After injection (60 min) frogs were tested in the prey capture assay as described above.

In Experiment 3, juveniles were microinjected bilaterally into the tecta with BIIE0246 (1.12 ng/200 nL, n = 16) or vehicle (50% DMSO, 200 nL, n = 16) while a separate group served as surgical shams (n = 16). After injection (60 min) juveniles were tested in the predator avoidance assay as described above. Half of the animals were exposed to an empty tank on the other side of the divider (n = 24) while half of the animals were exposed to a predator (see 2.4 above) on the other half of the divider (n = 24). The large female *X. laevis* that were used as predators were used more than once but never twice in 24 h.

2.6. Gonadal sex identification.

After behavioral trials, juveniles were euthanized with MS-222 (5 g/L dH2O) buffered with equal parts $NaHCO_3$ and preserved in Bouin's fixative for 48 h followed by long-term storage in 70% EtOH. Frogs were then dissected, and gonads were photographed using a Nikon SMZ1500 confocal microscope and identified as male or female using criteria described in Carr et al. (2003). Sex ratios of the juvenile test frogs were 21 F:14 M (5 unidentified) for Experiment 1, 19 F:21 M for Experiment 2, and 19 F:25 M (4 unidentified) for Experiment 3.

2.7. Statistical analyses

For Experiments 1 and 2 food intake and prey capture behaviors were analyzed with one-way ANOVA and Fisher's least significant difference test (LSD) using a 5 ×1 factorial design. Data were checked by Kolmogorov-Smirnov for normality, and Bartlett's test for independence (homoscedasticity). Each behavior score was used as a dependent variable and treatment (Experiments 1 and 2) and treatment used as an independent variable. Dependent variables were durational scores (latency to move, latency to contact, exploring, contact with food, inactive, locomotion, sweep, wipe; Table 1) and counts (hindlimb kick, lunge). Contact with food and exploring data were transformed by log or square root transformation to improve normality.

Food intake data from Experiment 3 intake were analyzed with two-way ANOVA and LSD test using a 3 × 2 factorial design; two independent variables, predator presence (two levels) and test agent (three levels). A total of thirteen individual behaviors [Counts: air gulps, hiding; Scans (30 s): time spent in food section, tube section, back section, facing tank divider; Duration (s): latency to contact food, exploring, time in contact with food, inactive, locomotion, sweep, and wipe] were analyzed as additional dependent variables through two-way ANOVA after square-root transformation of the data. Data were checked by Kolmogorov-Smirnov test for normality, Levene's test for equality of error variance, and an F test for heteroskedasticity. Individual behavior scores were used as response variables and treatment and predator groups used as fixed factors. Within group differences were analyzed using the LSD test for drug treatment. Significant main effects were analyzed by post hoc tests when a significant interaction between predator presence and drug treatment was not found (Wei et al., 2012).

Time spent in the food section failed to meet the criteria for parametric ANOVA even after data transformation and was thus analyzed using a generalized linear model (McCullagh and Nelder, 1989) in SPSS (IBM Inc., Armonk, N.Y.). Time spent in the food section was used as a response variable and treatment and predator groups used as fixed factors. The model was selected based on the output of goodness of fit statistics and low scores of value/df. Best fit for time in the food section was achieved using the Poisson loglinear model. Normality testing of the standard deviance residuals confirmed that the data were normally distributed.

Data are presented as mean ± SEM and their p value; alpha was set to 0.05. All statistical analyses were performed using SPSS (v. 21–25, IBM Inc., Armonk, N.Y.).

3. Results

3.1. Experiment 1

One-way analysis of variation revealed no statistically significant effect of treatment on liver consumption ($F_{4,\ 35} = 1.87$, $p = 0.3857$, Fig. 1A).

Data for all behavioral measurements and statistical results are presented in Table S1. There was a statistically-significant treatment effect on latency to contact food (Fig. 1B, $F_{4,35} = 3.871$, $p = 0.010$). A post-hoc LSD test showed that juveniles microinjected with largest

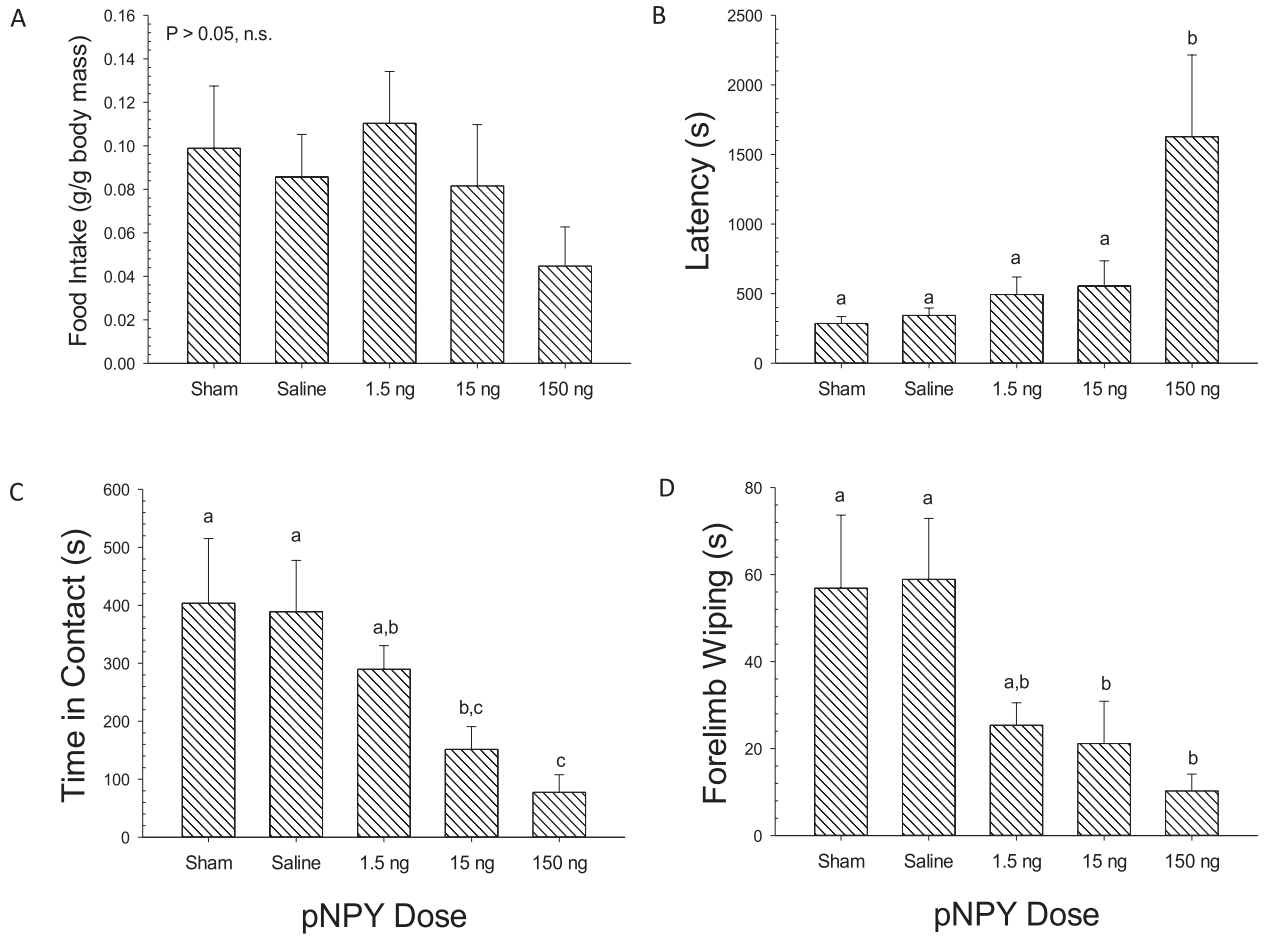

Fig. 1. Dose-related effects of porcine NPY on food intake (Fig. 1A), latency to contact food (Fig. 1B), time in contact with food (Fig. 1C), and time spent in forelimb wipes (Fig. 1D) in juvenile *X. laevis*. Bars with different superscripts are statistically different based upon one-way ANOVA followed by Fisher's LSD test ($p < 0.05$). Bars represent the mean + S.E.M. of 8 animals per group.

doses of pNPY (150 ng) took significantly longer to contact food compared to sham controls ($p = 0.001$), juveniles microinjected with saline ($p = 0.002$), the smallest dose of pNPY (1.5 ng) ($p = 0.005$, Fig. 1B), or the medium dose of pNPY ($p = 0.010$, Fig. 1B). Treatment reduced the amount of time spent in contact with the food (Fig. 1C, $F4, 35 = 7.699$; $p = 0.006$). Post-hoc comparisons using the LSD test indicated that juveniles treated with the largest dose of pNPY spent significantly less time in contact with food than the sham ($p < 0.001$), saline-treated ($p < 0.001$, Fig. 1C) and low dose treated juveniles. Juveniles receiving the mid dose pNPY spent significantly less time in contact with food compared to sham ($p = 0.020$) and the vehicle-injected juveniles ($p = 0.020$, Fig. 1C).

When eating, most anurans will wipe their mouth, most likely to push prey into the stomach but also to manipulate stomach eversion in the event of ingesting toxins (Naitoh and Wassersug, 1996). One-way ANOVA showed a significant main effect of treatment on time spent wiping (Fig. 1D, $F4, 35 = 3.960$, $p = 0.009$). Juveniles treated with the largest dose of pNPY reduced wiping duration relative to saline ($p = 0.005$) and sham ($p < 0.05$) (Fig. 1D). Juveniles treated with the medium dose, but not the low dose, of pNPY also reduced wiping duration relative to shams ($p = 0.020$) and vehicle injected ($p = 0.020$).

There were no treatment differences with respect to exploring tank edges ($F_{4,35} = 2.171$, $p > 0.05$), time spent inactive ($F_{4, 35} = 1.687$, $p > 0.05$), locomotion ($F_{4, 35} = 0.6174$, $p > 0.05$), sweeps ($F_{4, 35} = 1.814$, $p > 0.05$), latency to move ($F_{4, 35} = 0.6373$, $p > 0.05$) or number of hindlimb kicks ($F_{4, 35} = 1.787$, $p > 0.05$). There were not enough data to test for main effects on lunge.

3.2. Experiment 2

Although variance differed significantly based upon treatment ($F_{4,35} = 5.95$, $p = 0.001$), pNPY treatment alone did not alter food intake, confirming the results from Experiment 1 (Fig. 2A).

Data for all behavioral measurements and statistical results from Experiment 2 are presented in Table S2. There was a significant effect of treatment on latency to contact food ($F_{4,35} = 6.034$, $p < 0.001$, Fig. 2B). pNPY injection significantly increased the latency to contact food relative to shams ($p < 0.001$), DMSO ($p < 0.002$), BIIE0246 ($p < 0.001$), and juvenile frogs that received both NPY and BIIEO246 ($p = 0.002$). There was a significant overall effect on time in contact with food ($F_{4,35} = 20.9$, $p < 0.001$; Fig. 2C), Post-hoc comparisons using the LSD indicated that pNPY (150 ng/150 µl) decreased the time in contact with food compared to shams ($p < 0.001$), vehicle-treated ($p < 0.001$), BIIE0246 alone ($p < 0.001$) and BIIE0246 in combination with pNPY ($p < 0.001$). Treatment affected the duration of forelimb wipes ($F_{4,35} = 10.6$, $p < 0.001$, Fig. 2D). pNPY decreased significantly the duration of wipes compared to sham ($p < 0.001$), DMSO ($p < 0.001$), BIIE0246 alone ($p < 0.001$) and juvenile frogs treated with both pNPY and BIIE0246 ($p < 0.001$). Interestingly, we also found that treatment had a significant effect ($F_{4,35} = 8.420$, $p < 0.001$, Table S2) on inactivity. pNPY increased inactivity relative to shams ($p < 0.001$), vehicle-injected ($p = 0.019$), BIIE0246 alone ($p < 0.001$) and juvenile frogs treated with both pNPY and BIIE0246 ($p = 0.007$).

Administration of the NPY2R selective non-peptide antagonist BIIE0246 (1.12 ng) not only reversed the behavioral effects of pNPY

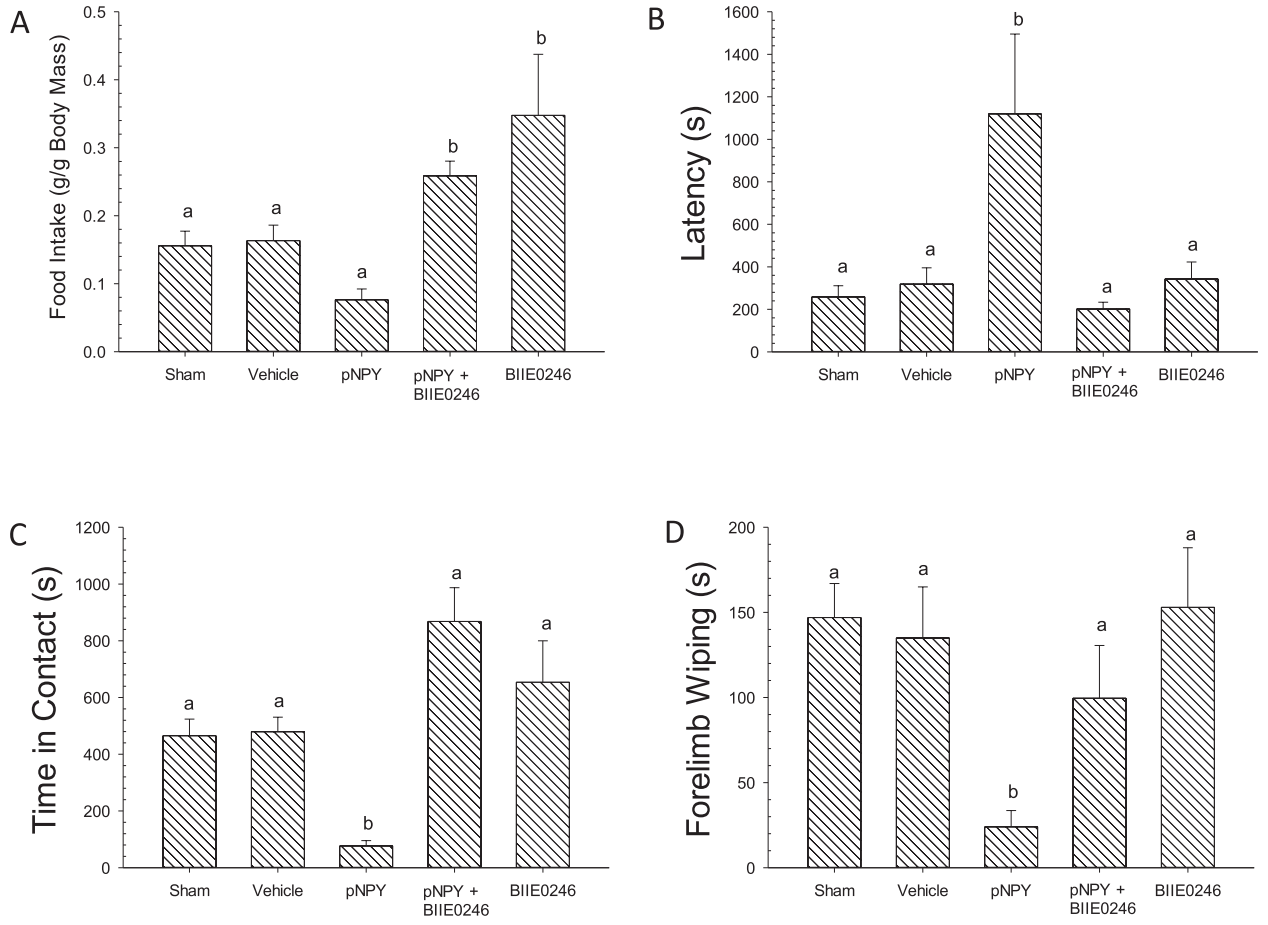

Fig. 2. Effects of porcine NPY (150 ng/150 nL) alone, or after pretreatment with BIIE0246 (1.12 ng/200 nL), on food intake (Fig. 2A), latency to contact food (Fig. 2B), time in contact with food (Fig. 2C), and time spent in forelimb wipes (Fig. 2D) in juvenile *X. laevis*. Bars with different superscripts are statistically different based upon one-way ANOVA followed by Fisher's LSD test ($p < 0.05$). Bars represent the mean + S.E.M. of 8 animals per group.

after tectal administration but had interesting effects on its own (Fig. 2). One-way ANOVA followed by LSD tests revealed that BIIE0246 significantly increased food intake over sham ($p < 0.010$), vehicle ($p < 0.010$), and NPY-treated ($p < 0.001$) animals (Fig. 2A). BIIE0246 (versus BIIE0246 + pNPY) reversed the effect of pNPY on latency to contact ($p > 0.05$, Fig. 2B), time in contact with food ($p > 0.05$, Fig. 2C), and forelimb wipes ($p > 0.05$, Fig. 2D). There were no treatment effects on any of the other prey-capture behaviors.

3.3. Experiment 3

Two-way analysis of variance revealed a statistically significant main treatment effect on liver consumption ($F_{2,42} = 10.23$, $p < 0.001$, Fig. 3A). Post-hoc comparisons using the LSD test indicated that animals treated with BIIE0246 (1.12 ng/200 nL) consumed significantly more liver than sham animals ($p = 0.002$) or animals injected with vehicle (50% DMSO) ($p < 0.001$). The main effect of predator was statistically significant as well ($F_{1,\ 42} = 18.17$, $p < 0.001$, Fig. 3A). Frogs exposed to a predator ate significantly less than frogs that were not exposed to any predator. However, the interaction effect (treatment*predator) was not statistically significant ($F_{2,\ 42} = 0.591$, $p > 0.05$). Although frogs ate more in the antagonist treated group, the antagonist did not completely prevent the predator induced decrease in food intake based upon comparison of means (BIIE0246 without v. with predator, post-hoc Student's two-tailed *t*-test, $p = 0.046$, Wei et al., 2012) (Fig. 3A)

Data on behavioral endpoints and statistical analyses are present in Tables S3–S5. As with food intake, treatment and predator effects on behavioral endpoints were analyzed through a two-way ANOVA preceded by square root transformation of the data. Both main effects were statistically significant ($F_{2,42} = 5.150$, $p = 0.010$ for treatment; $F_{1,42} = 7.51$, $p = 0.009$ for predator) for latency to contact food (Fig. 3B), although there was not a statistically significant interaction between the two independent variables ($F_{2,42} = 0.659$, $p = 0.523$). Multiple comparisons testing showed frogs receiving treatment BIIE0246 took significantly less time to contact food ($p < 0.05$, LSD) than vehicle and sham groups. Frogs exposed to the predator took longer to contact food (Fig. 3B) across sham and vehicle treatments. Antagonist treatment completely blocked the predator-induced increase in latency to contact food (post-hoc two-tailed *t*-test, $p = 0.384$, Fig. 3B).

Both main effects were statistically significant ($F_{2,42} = 23.3$, $p < 0.001$ for treatment; $F_{1,42} = 73.2$, $p < 0.001$ for predator) for time in contact with food (Fig. 3C). Post-hoc comparisons showed that BIIE0246 significantly increased ($p < 0.001$, LSD) time in contact with food relative to vehicle and sham groups. Predator presence decreased the time in contact with food across treatment groups (Fig. 3C). The interaction between drug treatment and predator was not statistically significant ($p = 0.948$). Antagonist treatment did not completely block the predator-induced reduction in time in contact with food, as post-hoc Student's *t*-test revealed a significant difference between predator and no predator means in the antagonist group ($p < 0.001$, Fig. 3C).

A main effect of treatment ($F_{2,42} = 10.7$, $p < 0.001$) and predator ($F_{1,42} = 63.3$, $p < 0.001$) were observed for time inactive after square root transformation. The main effect of treatment on inactivity was not observed with pNPY alone in Experiment 1 but reported for Experiment

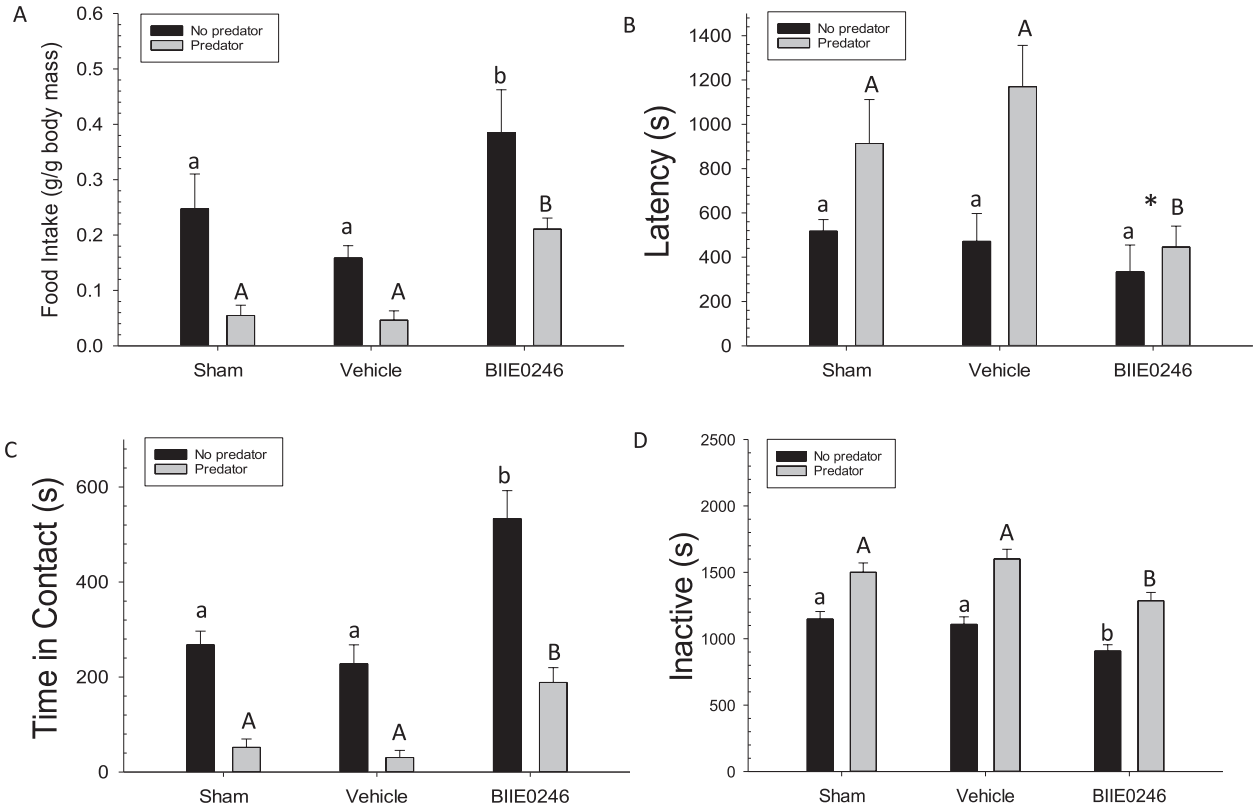

Fig. 3. Effects of BIIE0246 (1.12 ng/200 nL) on food intake (Fig. 3A), latency to contact food (Fig. 3B), time in contact with food (Fig. 3C), and time spent inactive (Fig. 3D) in juvenile *X. laevis* in the absence or presence of a predator. Predator and treatment main effects were significant for all behaviors (Tables S3–S5). Post-hoc analysis of injection main effects are indicated by superscripts. Bars with different superscripts within a predator levels are significantly different. Lower case subscripts indicate the no predator level, upper case superscripts indicate the predator level. Asterisk indicates that the means are not statistically different based upon post-hoc Student's two-tailed *t*-test (Wei et al., 2012). Bars represent the mean + S.E.M. of 8 animals per group.

2. Frogs receiving BIIE0246 were significantly ($p < 0.01$) less active than either control group. Frogs exposed to a predator remained inactive longer than frogs exposed to no predator. The interaction between the two independent variables was not significant ($p = 0.48$). Predator presence still strongly increased time spent inactive in the antagonist-treated animals based upon comparison of means (Student's *t*-test, $p < 0.001$, Fig. 3D).

Statistical analysis revealed main effects on the time that test frogs spent in the section of the test tank containing food. There was a main effect of BIIE0246 treatment on time spent in the section with food (Wald χ^2 [2, N = 48] = 24.9, $p < 0.001$). The animals treated with BIIE0246 spent significantly ($p < 0.001$) more time in the food section of the tank than vehicle treated but not sham treated animals. The main effect for predator was also significant (Wald χ^2 [1, N = 48] = 115, $p < 0.001$). Animals exposed to a predator spent less time in the food section than animals without predator group.

Both predator presence and drug treatment significantly affected air gulping. Both main effects (predator, $F_{1,42} = 21.1$, $p < 0.001$; treatment, $F_{2,42} = 3.41$, $p < 0.05$) were statistically significant but there was not a significant interaction between the two independent variables. However, post-hoc testing revealed that while BIIE0246 treated frogs differed from untreated animals ($p = 0.016$), they did not differ from vehicle controls. Predator presence decreased air gulping.

There were three behaviors that were significant for a main effect of predator presence but not drug treatment; wiping ($F_{1,42} = 11.02$, $p = 0.020$), locomotion ($F_{1,42} = 6.691$, $p = 0.013$) and sweeping ($F_{1,42} = 27.50$, $p < 0.001$). Predator presence significantly decreased wipe duration, locomotion, and time spent sweeping. Interestingly, the predator effect on sweeping was dependent upon an interaction with treatment ($p = 0.001$, predator × treatment interaction, Table S5). Comparison of means by post-hoc Student's two-tailed *t*-test revealed that predator presence decreased sweeping in the sham ($p < 0.001$) and vehicle ($p = 0.023$) groups but not the BIIE0246 group ($p = 0.797$). Thus, BIIE0245 completely blocked the predator induced decrease in sweeping behavior. Reduced sweeping, along with an increased latency to contact food (above), were the only two antipredator behaviors completely blocked by BIIE0246 treatment.

Frogs exposed to predators also spent more time in the tank section farthest away from the predator ($F_{1,42} = 35.4$, $p < 0.001$). While a main effect for treatment on time spent in the back tank section was observed ($F_{2,42} = 3.29$, $p = 0.047$), pairwise comparisons showed that while there was a significant difference between BIIE0246 treated juvenile frogs and sham juvenile frogs ($p = 0.018$), the difference between the antagonist and vehicle treated was not significant. There was not a significant interaction ($p = 0.179$). Although there was a main effect of predator on time spent in the tube section of the test tank ($p = 0.032$, juvenile frogs avoided this section during predator exposure), there was no main effect of treatment and no interaction between treatment ($p = 0.767$) and predator ($p = 0.062$). The time spent hiding was not analyzed statistically as there were not enough data to test. Neither independent variable influenced exploring behavior (Tables S3–S5).

4. Discussion

Although previously collected data from anesthetized and paralyzed toads implicate tectal Y2 receptors in modulating visually guided behavior (Schwippert and Ewert, 1995; Schwippert et al., 1998; Funke and Ewert, 2006), a role for these receptors in normally behaving animals has not been tested to date. Our findings indicate that while

bilateral administration of pNPY at three doses did not significantly affect overall food intake, this peptide consistently impacted key elements of prey capture behavior, leading animals to consume the same amount of food in a shorter period of time. To our knowledge, this is the first report of tectally administered NPY on food intake in any vertebrate. NPY is a potent orexigenic peptide when administered into the arcuate nucleus or brain ventricles in most vertebrates examined so far (Volkoff et al., 2009; Matsuda et al., 2012; Yokobori et al., 2012; Pandit et al., 2013; Boswell and Dunn, 2017) including anurans (Crespi et al., 2004; Shimizu et al., 2013), possibly via a hypothalamic NPY1R (Shimizu et al., 2013). Furthermore, there is abundant evidence that NPY arcuate neurons play a critical role in the regulation of appetite in mammals (Norris and Carr, 2013; Pandit et al., 2013). Arcuate NPY neurons co-express agouti-regulatory protein, and are critically involved in the signaling of leptin and other peripheral regulatory hormones. However, our data suggest the role of NPY in the OT may be more nuanced as pNPY administration did not increase overall food consumption, but blocking NPYR2 receptors did.

Changes in foraging behavior in response to predators has been extensively noted in the ecology literature and may include increased vigilance, more time hiding, or discrete changes in prey capture/foraging behavior that reduce the vulnerability of an animal to predators (Harris and Carr, 2016). Foraging/predator avoidance tradeoffs are a key feature of optimal foraging theory, and it is possible to mathematically model tradeoffs in such a way that an animal can simultaneously maximize food intake and increase survival probability (McNamara and Houston, 1992). This theoretical construct is relevant for the data found here, as pNPY caused no effect in overall food intake but did alter key prey-capture behaviors that in theory might decrease the frog's vulnerability to a predator. At the largest dose, pNPY decreased the duration of wipes, increased the latency to contact food, and decreased the amount of time in contact with the food. These observations, taken together with the finding that overall food intake did not change, suggests that frogs were more hesitant to approach the food and, when they did contact the food, they ate more quickly after administration of the largest pNPY dose. Such changes in behavior after pNPY administration may reduce the vulnerability of the frogs to predators, as reduced time in contact with the food may allow more time for vigilance or defensive behaviors. Moreover, hesitancy to contact prey has been reported in other species in the presence of a predator (Abramsky et al., 2002; Lehtiniemi, 2005; Freitas & Volpato, 2008; Tang et al., 2017).

Our data indicate that the effects of pNPY on prey capture behavior resulted from activation of tectal Y2 receptors, as the effects of pNPY were blocked by the Y2 selective antagonist BIIE0246. In fact, tectally administered BIIE0246 not only reversed the effects of pNPY on prey capture but elevated food intake on its own. Y2 receptors have a well-established role in inhibiting food intake (Parker and Balasubramaniam, 2008), but this is the first evidence to our knowledge for such a role in the tectum. Y2 receptors in the arcuate nucleus inhibit food intake in mammals (Abbott et al., 2005) presumably by acting as presynaptic autoreceptors (Broberger et al., 1997; King et al., 2000) and inhibiting NPY release. It is unlikely that the cognate Y2 receptors targeted in this study have the same role, as blocking these receptors with BIIE0246 led to increased food intake. If the Y2 receptors were presynaptic autoreceptors, BIIE0246 would have disinhibited endogenous NPY secretion leading to increased NPY release, thereby accentuating of the effects observed with pNPY alone. Our data do suggest that NPY released in the OT provides a basal inhibitory regulation over food intake in the absence of other threatening stimuli. The exact location of the tectal Y2 receptors modulating approach and avoidance are currently unknown, but the possibility exists that they may negatively modulate glutamate release from retinal ganglion cells that project to the superficial OT (Gábriel and Straznicky, 1995). Y2 receptors inhibit glutamate release in the mammalian hippocampus (Greber et al., 1994). The likely source of NPY afferents innervating the OT are thalamotectal inhibitory neurons (Kozicz and Lázár, 1994; Chapman and Debski, 1995) that inhibit prey capture (Ewert, 1984). In the tongue-less *X. laevis,* prey capture likely involves olfactory, visual, and lateral lines cues (Elepfandt, 1996) and only the last two sensory modalities involve the OT (Claas and Dean, 2006; Hiramoto and Cline, 2009). Which of these modalities is engaged after blocking tectal Y2 receptors requires further study.

Exposure to a predator decreased food intake in sham, DMSO and BIIE0246 treated frogs, confirming results previously reported by Duggan et al. (2016). As seen in Experiment 2, administration of the selective NPY2R antagonist BIIEO246 bilaterally into the OT significantly increased food intake relative to sham and vehicle-treated frogs. This finding strongly suggests that predator exposure increases NPY release in the OT, as antagonism of Y2 receptors reversed the effect of predator exposure. These findings are qualitatively similar to those reported after NPY R2 blockade by BIIEO246 in the arcuate nucleus of male Wistar rats (Abbott et al., 2005). Abbott et al. (2005) showed pretreatment with BIIE0246 administered directly into the arcuate nuclei increased food intake even in satiated rats. Such results are consistent with the hyperphagic phenotype of NPY2R knockout mice phenotype (Sainsbury et al., 2002).

Frogs treated with BIIEO246 took less time to contact food in the presence of predator and spent more time eating in the presence or absence of predator. Duggan et al. (2016) were the first to report that *X. laevis* contacted food more quickly and spent more time eating in the absence of predator. Our results suggest that NPY2R antagonism disinhibited the predator alarm by blocking NPY action in the OT, thus releasing feeding behavior even when a predator was present. In addition, frogs injected with BIIE0246 spent less time avoiding the predator and spent more time in the food and tube sections. Studies on *X. laevis* and zebra fish (*Danio rerio*) reported that untreated animals avoid the predator stimulus by remaining in the area of the tank furthest from predator (Duggan et al., 2016; Luca and Gerlai 2012). Furthermore, studies on several other fish species found that prey species recognize visual and chemical cues associated with predators, and maintain a greater distance from predators (Lehtiniemi, 2005; Freitas & Volpato, 2008; Tang et al., 2017) in the presence of such cues. Our data raise the possibility that BIIE0246 blocks some predator recognition circuits in the OT mediated by NPY2R receptors, and thus frogs treated with the antagonist are more likely to approach the predator. Interestingly this effect of BIIE0246 resembles the taming response elicited by full superior colliculi lesions in infant capuchin monkeys (Maior et al., 2011). Monkeys with intact superior colliculi never approach a snack item (banana) when it's in the proximity of a mock natural predator (rubber snake). However, monkeys with full superior colliculi lesions have no fear of the predator stimulus and readily approach food even in the presence of the predator stimulus (Maior et al., 2011).

The aquatic lifestyle of *X. laevis* is somewhat unique among anurans, and thus antipredator behavior in *X. laevis* (Green, 2009; Chum et al., 2013) possesses some of the same features seen in fish species (Lehtiniemi, 2005; Freitas & Volpato, 2008; Tang et al., 2017) including increased hiding and inactivity and remaining submerged for longer periods of time. Zebrafish (*Danio rerio*) and goldfish (*Carassius auratus*) preferred lower part of the tanks when exposed to predator (Khor et al. 2013; Matsuda et al., 2012) and corticotropin-releasing factor (CRF), a anxiogenic peptide, treatment increased the time taken to move from lower parts to the water surface (Matsuda et al., 2012). In *X. laevis* remaining submerged longer when a threat is present would be expected to indirectly affect air gulp frequency. In fact, when threatened *X. laevis* have been reported to 'burst breathe' at the surface, maximizing gas exchange efficiency in fewer trips to the surface (Chum et al., 2013). Thus, a treatment-induced decrease in the number of air-gulps may represent increased anxiety in *Xenopus laevis* (Chum et al., 2013). Duggan et al. (2016) showed the median number of air-gulps decreased in the presence of predator, although the result was not statistically significant. Here we show that predator exposure in our hands significantly reduced the number of air gulps in juvenile frogs,

although BIIE0246 had no effect.

In summary, we report that a) tectal Y2 receptors suppress prey capture under basal conditions, b) activation of Y2 receptors accelerates feeding and modulates discrete aspects of prey capture, and c) tectal Y2 receptors are likely activated in response to a predator and function to suppress prey-capture. Thus, the tectal NPY system may play an adaptive role in predator-induced reductions in prey-capture.

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Appendix A. Supplementary data

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