Behavioral/Cognitive

Mating Behavioral Function of Preoptic Galanin Neurons Is Shared between Fish with Alternative Male Reproductive Tactics and Tetrapods

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Understanding the contribution of neuropeptide-containing neurons to variation in social behavior remains critically important. Galanin has gained increased attention because of the demonstration that galanin neurons in the preoptic area (POA) promote mating and parental care in mammals. How widespread these mechanisms are among vertebrates essentially remains unexplored, especially among teleost fishes, which comprise nearly one-half of living vertebrate species. Teleosts with alternative reproductive tactics exhibit stereotyped patterns of social behavior that diverge widely between individuals within a sex. This includes midshipman that have two male morphs. Type I males mate using either acoustic courtship to attract females to enter a nest they guard or cuckoldry during which they steal fertilizations from a nest-holding male using a sneak or satellite spawning tactic, whereas type II males only cuckold. Using the neural activity marker phospho-S6, we show increased galanin neuron activation in courting type I males during mating that is not explained by their courtship vocalizations, parental care of eggs, or nest defense against cuckolders. This increase is not observed during mating in cuckolders of either morph or females (none of which show parental care). Together with their role in mating in male mammals, the results demonstrate an unexpectedly specific and deep-rooted, phylogenetically shared behavioral function for POA galanin neurons. The results also point to galanin-dependent circuitry as a potential substrate for the evolution of divergent phenotypes within one sex and provide new functional insights into how POA populations in teleosts compare to the POA and anterior hypothalamus of tetrapods.

Key words: **alternative reproductive tactics; galanin; hypothalamus; preoptic area; reproduction; social behavior**

Significance Statement

Studies of neuropeptide regulation of vertebrate social behavior have mainly focused on the vasopressin-oxytocin family. Recently, galanin has received attention as a regulator of social behavior largely because of studies demonstrating that galanin neurons in the preoptic area (POA) promote mating and parental care in mammals. Species with alternative reproductive tactics (ARTs) exhibit robust, consistent differences in behavioral phenotypes between individuals within a sex. Taking advantage of this trait, we show POA galanin neurons are specifically active during mating in one of two male reproductive tactics, but not other mating-related behaviors in a fish with ARTs. The results demonstrate a deep, phylogenetically shared role for POA galanin neurons in reproductive-related social behaviors with implications for the evolution of ARTs.

Introduction

Survival and reproductive success depend on the appropriate expression of a wide array of social behaviors, including aggression, courtship, and mating. Species exhibiting alternative reproductive tactics (ARTs) offer models to investigate neural mechanisms underlying widely divergent social behaviors between individuals of the same sex (Godwin, 2010; Feng and Bass, 2017). ARTs are most prevalent among teleost fish (Mank and Avise, 2006), the most species-rich vertebrate lineage (Nelson, 2006), which includes the midshipman (*Porichthys notatus*) that has two male morphs with distinct developmental histories (Bass, 1996). Type I males express a courting reproductive phenotype or a cuckold-

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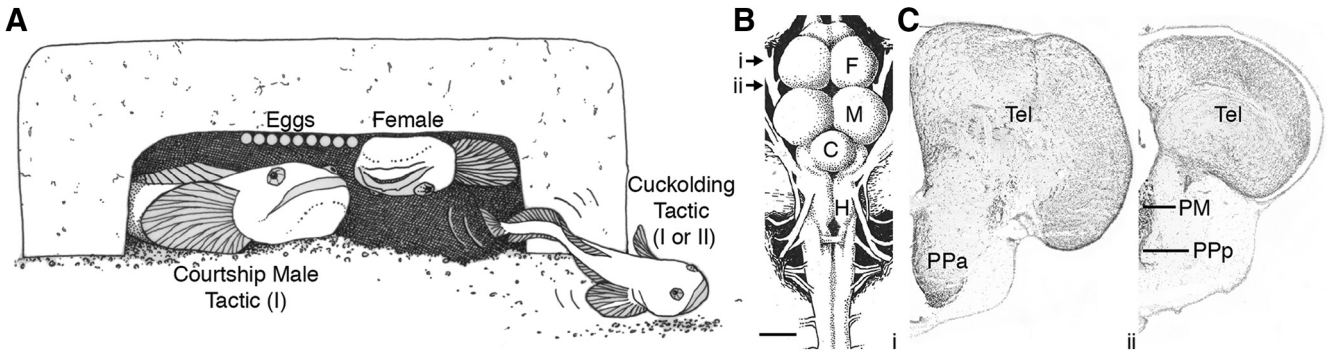

Figure 1. Midshipman reproductive behavior and preoptic area. ***A***, Line drawing of midshipman nest during mating. Courting type I male in nest with female laying eggs, and satellite mating male outside fanning sperm into the nest. Both type I and type II males satellite mate. ***B***, Overhead drawing of midshipman brain. Arrows indicate level of sections shown in ***Ci*** and ***Cii***. Scale bar, 1 mm. ***C***, Nissl (cresyl violet)-stained coronal sections through midshipman brain at rostral (***Ci***) and caudal (***Cii***) levels of the preoptic area. Images in Figures 3–6 taken at similar level as ***Ci***. C, Cerebellum; F, forebrain; H, hindbrain; M, midbrain; PM, magnocellular preoptic area; PPa, anterior parvocellular preoptic area; PPp, posterior parvocellular preoptic area; Tel, telencephalon.

ing phenotype in which they steal fertilizations at the nests of courting males, whereas smaller, sexually precocious type II males exclusively cuckold (Fig. 1*A*; Brantley and Bass, 1994; Lee and Bass, 2004, 2006). Courting type I males build nests, acoustically court females, provide parental care, and aggressively defend their nest and young from type I and type II male cuckolders (Arora, 1948; Brantley and Bass, 1994; McKibben and Bass, 1998). These differences, coupled with the type I male's behavioral flexibility to court or cuckold, provide an opportunity to determine the influence of both developmental history and behavioral context on the mechanisms of social behavior regulation.

To date, no studies identify molecularly-defined neuronal types that are differentially active during ART-specific behaviors found among both teleosts (Mank and Avise, 2006) and tetrapods (Zamudio and Sinervo, 2000; Maggioncalda and Sapolsky, 2002; Mank and Avise, 2006; Zamudio and Chan, 2008; Lamichhaney et al., 2016; Küpper et al., 2016). Neuropeptide-containing neurons in the preoptic area (POA) have emerged as key candidates, with many studies focusing on the oxytocin-vasopressin family of nonapeptides (Goodson and Bass, 2000, 2001; Bass and Grober, 2001; Donaldson and Young, 2008; Godwin and Thompson, 2012; Kelly and Goodson, 2014; Phelps et al., 2017).

Recently, the neuropeptide galanin has received much attention for its role as a regulator of social behavior (Dulac et al., 2014; Fischer and O'Connell, 2017), largely because of a series of studies demonstrating that galanin neurons in the medial POA promote parental care in mice of both sexes (Wu et al., 2014; Kohl et al., 2018; Moffitt et al., 2018). Earlier investigations of the POA additionally show a role for galanin in rat sexual behavior (Bloch et al., 1993, 1996) and identify populations of galanin neurons active during mating in male ferrets and mice (Bakker et al., 2002; Wu et al., 2014). Studies of teleosts also suggest a social behavior function for galanin. For example, our RNA-sequencing analyses of the midshipman POA (Fig. 1*B*,*C*) show elevated galanin transcript levels during mating in courting type I compared with cuckolding type II males, whereas cuckolding type I males have intermediate expression levels (Tripp et al., 2018). Sunfish (*Lepomis macrochirus*) that have ARTs similar to midshipman show higher galanin transcript expression in the whole brain of type I-like compared with type II-like males (Partridge et al., 2016) and a species of cichlid (*Astatotilapia burtoni*) that lacks ARTs shows increased whole-brain expression in dominant compared with reproductively suppressed, subordinate males (Renn et al., 2008).

Though RNA-sequencing results are suggestive, it remains unknown whether galanin-expressing neurons play an active role in regulating specific behaviors in any non-mammal, including species exhibiting ARTs. Here, taking advantage of the extreme differences in male behavioral phenotypes, we use phosphorylated S6 protein (pS6) as a neural activity marker (Knight et al., 2012) to test the hypothesis that POA galanin (POA^{Gal}) neurons are differentially activated during mating in males exhibiting the courting type I tactic, but not during other type I reproductive-related behaviors such as parental care and territorial aggression, and not in cuckolders of either male morph or in females. Our results also provide new, behaviorally-based insights into unresolved comparisons between the POA of tetrapods and that of teleosts, which includes nonapeptide-containing populations proposed as homologues to hypothalamic nuclei among tetrapods (Forlano and Cone, 2007; Herget et al., 2014).

Materials and Methods

Animal subjects. Adult midshipman were collected from nests in California and Washington in May to August of 2016–2018 (Bass, 1996; McIver et al., 2014). Each morph has distinguishing external characteristics including relative size and coloration that aid their recognition in the field; morph type was later confirmed on the basis of gonad type/size and swim bladder with attached sonic muscle size and morphology (Bass and Marchaterre, 1989; Brantley and Bass, 1994). Fish were shipped overnight to Cornell University and housed in various sized aquaria (see behavior experiment descriptions for details of aquaria used in each experiment) with artificial seawater in environmental control rooms at 15–16°C with a 15/9 h light/dark schedule. Vocal behavior was monitored using hydrophones (Aquarian Audio H1a) as previously described (Feng and Bass, 2016). Behavior during mating, egg care, and nest defense experiments was recorded using video cameras (Canon Vixia HFR500) under red light. Analysis of videos was done using BORIS (Friard and Gamba, 2015). Animal procedures were approved by the Institutional Animal Care and Use Committee of Cornell University.

Experiment design. Each behavioral experiment described in detail in the following sections used pS6 expression as a marker for recent neural activity. Following earlier studies using the immediate early gene c-fos to identify neural activity in midshipman and medaka (Okuyama et al., 2011; Petersen et al., 2013), we examined pS6 protein expression 2 h after the onset of the behavior of interest in each experiment. This choice of time point was validated in two ways for the humming vocalization that is both necessary and sufficient for successful courtship by a type I male (Brantley and Bass, 1994; McKibben and Bass, 1998). Humming makes an ideal behavior for understanding the time course of pS6 expression in the midshipman brain, as it is controlled by a dedicated vocal central pattern generator including the vocal motor nucleus (VMN), which in-

nervates the vocal muscle and activates all calling behaviors (Bass and Baker, 1990; Chagnaud et al., 2011, 2012). Thus, activity in this nucleus is directly related only to vocalization, allowing for precise association of pS6 expression with time of behavior onset and offset.

For all behavioral experiments investigating pS6 expression in POA^{Gal} neurons following behavior, animals were housed in aquaria as described later. Behaving animals (e.g., spawning or humming) were collected 2 h after behavior onset. In the mating and courtship humming behavior experiments, control (non-mating or non-humming) animals were collected and killed in parallel with behaving animals. For parental care and nest defense behavior experiments control (without eggs or without cuckolders) animals were collected at similar times as behaving animals.

Timeline of pS6 expression in midshipman. As noted above, two groups of animals were used to validate the 2 h time point chosen to associate pS6 expression with courtship humming behavior. The first group included type I males collected from the courtship humming experiments described below. We compared pS6 expression in the VMN of males collected 2 h after humming onset to VMN pS6 expression in non-humming, control animals housed in the same aquarium (see Fig. 2*A*; for an example of hum recording, see Fig. 4*A*). For the second group of males, additional humming and non-humming, control type I males were collected. In this case, we compared VMN pS6 expression between humming and non-humming males housed alone, without females, in aquaria (61 × 56 × 30.5 cm) containing one artificial nest each. Vocal behavior was monitored using hydrophones as described in Experiment design. Males readily hum in captivity under similar conditions (Genova et al., 2012; McIver et al., 2014; Feng and Bass, 2016). Humming males were removed from their home aquarium 15 min after humming onset during the dark period and immediately isolated in 5 gallon, opaque buckets for 30 or 120 min during which time there is no humming. Non-humming controls were also removed from their home aquarium during the dark period and isolated for the same periods of time (Fig. 2*B*). Together, these experiments demonstrated that pS6 expression in a brain nucleus (VMN) that is directly associated with a discrete behavior (vocalization) is present 2 h after the onset of behavior (Fig. 2), the time course used for all behavioral experiments investigating pS6 expression in POA^{Gal} neurons.

Mating behavior experiment. Males were held in large (100–200 gallon) aquaria divided into segments by plastic mesh (Fig. 3*A*). Each segment was ~1 × 1 m and contained one large type I male (83.6–258.7 g, body weight; 17.8–26.0 cm, standard length), one smaller type I male (38.8–140.1 g, 14.2–21.4 cm), one type II male (6.5–18.2 g, 7.9–11.9 cm), and a single artificial nest made of a ceramic plate resting on a rim of bricks. Following the experimental design of prior studies, type I males were paired such that the larger male in the segment was expected to assume nest ownership and begin courting, whereas the smaller type I male would pursue the alternative tactic of cuckoldry (Lee and Bass, 2004, 2006; Tripp et al., 2018).

Females (21.0–45.3 g, 11.8–14.8 cm) were added to a pair of neighboring nest segments 30 min before the onset of dark (Fig. 3*A*), when the courtship advertisement call known as a "hum" and mating occur (Brantley and Bass, 1994; Feng and Bass, 2016). Fish activity was observed by the experimenters under red light. Once a female entered the nest of either courting male, the nest in the neighboring segment was covered with a plastic mesh cage to prevent mating. Fish were allowed to behave for 2 h after female entry into a nest. The mating type I male and female along with neighboring non-mating control type I male (i.e., male in nest covered by mesh cage) and non-mating control female were killed 2 h after the mating female entered the nest, and cuckolding type I and II males were killed along with their neighboring control animals (i.e., type I and II males blocked from accessing a nest) 2 h after they began cuckolding (for timeline, see Fig. 3*A*).

Courtship humming behavior experiment. Type I males were housed in aquarium segments as described in the mating behavior experiment (see Fig. 4*A*). Each segment contained a single nest with one type I male. Vocal behavior was continuously monitored with hydrophones as previously described (Feng and Bass, 2016). Male pairs were monitored remotely using TeamViewer (v12.0.78517) and Audacity (v2.1.3) beginning at the start of the dark period. Males that hummed (48.87–210.53 g, 15.5–25.0 cm) for at least 10 min were collected 2 h after the onset of humming (for timeline, see Fig. 4*A*). Control males (36.18–207.36 g, 15.9–24.4 cm) were non-humming neighbors of humming males and were collected and killed in parallel with humming males. Hydrophone recordings confirmed that control males did not hum during the 2 h experiment. Total humming duration was quantified using Raven Pro 1.5 (Cornell Laboratory of Ornithology) following previously described methods (Feng and Bass, 2016).

Parental care behavior experiment. To generate stimulus nests with fertilized eggs, type I males were housed singly in aquaria (61 × 56 × 30.5 cm) containing one artificial nest each. Gravid females were added to aquaria and allowed to mate. Parental males and females were removed after mating and replaced by experimental type I males. Males that mated were tested in unfamiliar nests, except in one case where the nest-holding male was tested in its own nest after being removed from the aquarium and then returned 24 h later to determine whether the activation of POA^{Gal} neurons differed between males in nests with eggs they fertilized and males in nests with eggs fertilized by other males. Experimental males were added to aquaria containing nests that either had fertilized eggs (54.62–155.80 g, 16.7–23.9 cm) or no eggs (67.25–117.64 g, 17.4–21.2 cm), and then were allowed to establish residency in nests (see Fig. 5*A*). We expected that experimental type I males would take over nests and provide care given that nest takeover by type I males has been observed in captivity (Brantley and Bass, 1994) and is common in nature (Cogliati et al., 2013; Bose et al., 2016a), and that type I males will provide care for eggs that are not their own (Bose et al., 2016b). Nesting males were killed 2 h after onset of the dark period (for timeline, see Fig. 5*A*), when midshipman are most active (Feng and Bass, 2016). Behaviors in the nest during the experiment were video recorded.

Nest defense behavior experiment. To test whether pS6 expression in POA^{Gal} neurons of courting type I males was dependent on nest defense against attempted cuckolders, we allowed courting type I males to mate either in the presence or absence of cuckolding males. Type I males were held singly in aquaria (see parental care behavioral experiment above) with one artificial nest. Males that mated without cuckolders (69.21–70.76 g, each 17.5 cm) were housed alone, while males that mated with cuckolders (43.06–108.65 g, 15.1–20.1 cm) were housed with one smaller type I male and one type II male. Once the courting male began humming, one or two females were added to the nest, and mating behavior was observed. Courting type I males were collected and killed 2 h after a female entered and remained in the nest (for timeline, see Fig. 6*A*).

Immunohistochemistry. Directly following removal from tanks, animals were deeply anesthetized in 0.025% benzocaine (Sigma-Aldrich), and then perfused with ice-cold marine teleost Ringer's solution (http://comm.archive.mbl.edu/BiologicalBulletin/COMPENDIUM/CompTab6.html#TAB6B-F) followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Following perfusion, brains were removed and postfixed in 4% paraformaldehyde for 1 h at 4°C, and then transferred to 0.1 M PB for storage at 4°C. Brains were cryoprotected in 25% sucrose in 0.1 M PB overnight at 4°C, and then frozen in Tissue Plus OCT compound (Tissue-Tek) at −80°C. Brains were sectioned at 25 μm in three series. Tissue sections were thaw mounted onto Superfrost Plus slides (ThermoFisher Scientific), allowed to dry overnight at room temperature, and then were stored at −80°C.

Immunohistochemistry was performed on brain tissues collected from behaving (e.g., mating, humming) animals and controls in parallel. Before labeling, slides containing tissue sections were returned to room temperature and allowed to dry. Slides were washed three times for 10 min in phosphate buffered saline (PBS), followed by a 2 h incubation in blocking solution of 0.2% bovine serum albumen (Sigma-Aldrich), 0.3% Triton X-100 (Sigma-Aldrich), and 10% normal goat serum (NGS; ThermoFisher Scientific) in PBS, then 18 h in guinea pig anti-galanin (1:250, custom raised against midshipman galanin peptide; Pocono Rabbit Farm and Laboratory; RRID:AB_2783794) and rabbit anti-pS6 (1:250, Cell Signaling Technology, catalog #4858; RRID:AB_916156) primary antibodies in blocking solution at room temperature. Following primary antibody incubation, slides were washed three times in PBS, and then incubated 2 h in goat anti-guinea pig secondary antibody conjugated to AlexaFluor 488 (1:500; Life Technologies, catalog #A-11073; RRID:AB_2534117) and donkey anti-rabbit secondary antibody conjugated to

Table 1. Total preoptic galanin and pS6 + preoptic galanin neurons from all experimental groups

Experiment	Group	Mean Gal neurons	Mean pS6 + Gal neurons
Spawning	Type I courting mating	390	255
	Type I courting control	417	4.8
	Type I cuckolding mating	374.6	2
	Type I cuckolding control	440	6.8
	Type II cuckolding mating	201.5	9
	Type II cuckolding control	172.25	5.25
	Female mating	30	1
	Female control	44	1.67
Humming	Humming	393.75	30.63
	Control	329.13	3.75
Egg care	Eggs	341.856	0.14
	Control	340.83	0.17
Nest defense	No cuckolders	489.5	291
	With cuckolders	443.5	316.5

Note that in instances where tissue sections were lost or damaged, cells from those sections were not counted.

AlexaFluor 568 (1:200; Life Technologies, catalog #A10042; RRID: AB_2534017) in PBS+10% NGS. After secondary antibody incubation, slides were washed in PBS three times for 10 min, followed by one 10 min wash in double distilled water, and then coverslipped with ProLong Gold with DAPI (ThermoFisher Scientific). Brains from animals collected after isolation for pS6 time course were labeled for pS6 only.

After coverslipping, slides were allowed to dry at room temperature overnight, then edges were sealed with nail polish. Slides were stored at 4°C. Specificity of anti-galanin antibody was confirmed by performing immunohistochemistry following pre-adsorption of antibody with 50 μM galanin peptide (Pocono) or omission of primary antibody. A prior study has demonstrated specificity of anti-pS6 primary antibody in teleost fish. Western blot confirmed anti-pS6 specificity for midshipman (Butler et al., 2018).

Image acquisition and processing. POA images were acquired on a Zeiss LSM 880 confocal microscope (Cornell University Biotechnology Resource Center Imaging Facility, NIH S10OD018516). The POA of each brain was imaged bilaterally at 20× with a 10-level *z*-stack (5 μm optical section, 2.5 μm step). Tile scanning with 20% overlap and medium (0.70) stitching threshold was used when necessary to capture the entire POA in an image. Quantification of pS6-expressing galanin neurons was done using FIJI (Schindelin et al., 2012). Green and red channels of each image were merged, and then *z*-stacks were projected using the maximum intensity function. Cell bodies were first identified using the following criteria: (1) a clearly defined perimeter and (2) an identifiable nucleus and/or neurite. Observers blind to the condition of the animal then determined whether the POA^{Gal} neuron also expressed pS6. Criteria for pS6 expression were either visually identifiable overlap of green and red signal (i.e., yellow signal) or red signal visible within the perimeter of the green cell body. Counting of neurons expressing pS6 was performed by the same observer blind to animal condition. See Table 1 for average numbers of POA^{Gal} and pS6-expressing POA^{Gal} neurons from each experimental group.

Images of the VMN for pS6 expression timeline experiments were collected on a Nikon Eclipse microscope with 20× objective. For animals collected during the courtship humming experiment, total neurons and number of pS6+ neurons in the VMN were counted by an observer blinded to the condition of each animal. In the post-vocalization isolation experiment, general patterns of pS6 expression in the VMN were characterized by an experienced observer. Images were cropped and resized for figures using Adobe Photoshop CS6. To increase accessibility, red images were converted to magenta for figures using FIJI.

Statistics. Statistical tests were conducted using R v3.3.3. Because the total number of POA^{Gal} neurons varies by morph and body size, all comparisons were made using the proportion of POA^{Gal} neurons that expressed pS6 (number of pS6+ POA^{Gal} neurons observed/total POA^{Gal} neurons observed). Comparisons of the proportion of pS6-labeled POA^{Gal} neurons in the mating experiment were made by one-way ANOVA and *post hoc* comparisons made with Tukey's HSD test. Comparisons of the proportion of pS6-labeled POA^{Gal} neurons and pS6-labeled VMN neurons in the courtship humming experiment were made using a Welch two sample *t*-test to correct for unequal variance between groups. Comparison of the proportion of pS6-labeled POA^{Gal} neurons during egg care was made using two sample *t*-test. Correlations of humming and parenting behaviors with the proportion of pS6-labeled POA^{Gal} neurons were tested using Pearson's correlation coefficient. Because of limited sample size, only descriptive statistics were used to describe data from the nest defense experiment.

Results

POA^{Gal} neurons

In our transcriptomic study of midshipman, we refer to the POA as the POA-anterior hypothalamus given this region includes nonapeptidergic cell nuclei present in the anterior hypothalamus of tetrapods (see Introduction and Discussion). For simplicity and to facilitate comparisons with tetrapods, we use the POA designation here. Like other teleosts (Herget et al., 2014), this region includes three major divisions: anterior and posterior parvocellular nuclei flanking the anterior commissure and a magnocellular nucleus caudal to the commissure (Fig. 1*B*; Foran et al., 1997; Foran and Bass, 1998). The POA contains the primary population of galanin neurons in the midshipman brain (Tripp and Bass, 2020). Here, most POA^{Gal} neurons were observed in the anterior parvocellular POA, though some are in the posterior parvocellular and magnocellular POA. See Table 1 for mean numbers of POA^{Gal} and pS6-expressing POA^{Gal} neurons from each experimental group.

Timeline of pS6 expression in midshipman brain

To confirm the time course of pS6 expression in the midshipman brain following behavior, we collected animals 2 h after the onset of courtship humming, and examined pS6 expression in the VMN that directly innervates vocal muscle (Bass and Baker, 1990). A significantly ($t_{(11.249)} = -3.472$, $p = 0.005057$, Welch two-sample *t* test) greater proportion of VMN neurons expressed pS6 in males collected following humming ($n = 8$) compared with non-humming ($n = 8$) controls (Fig. 2*A*).

Additionally, to observe the time course of pS6 expression following behavior, we isolated animals following short periods of humming (Fig. 2*B*). In three animals isolated for 30 min following humming, pS6 label in VMN neurons was faint or absent. However, in one animal isolated for 2 h after humming, strong label is present in VMN. In contrast, for non-humming animals isolated for either 30 minutes ($n = 3$) or 2 h ($n = 1$), pS6 label was absent or faint in VMN. Together, these experiments demonstrate that pS6 expression in the midshipman brain directly associated with a behavior is strong 2 h following the onset of behavior, the time point animals were collected for the other studies reported on here, and that expression is robust for at least 2 h after behavior offset.

POA^{Gal} activity increases only in courting type I males during mating

Because we previously reported increased expression of galanin transcripts in the POA of courting type I males compared with cuckolding type II males during mating (Tripp et al., 2018), we predicted that POA^{Gal} neurons are active only in courting type I males during mating. Additionally, based on prior studies of mammals, we predicted that there would be no increase in POA^{Gal} neuron activation in females (Bakker et al., 2002; Wu et al., 2014). To test these hypotheses, we created a courting/cuckolding mating behavior paradigm where groups of type I and type II males were held in paired divided aquaria, with each chamber

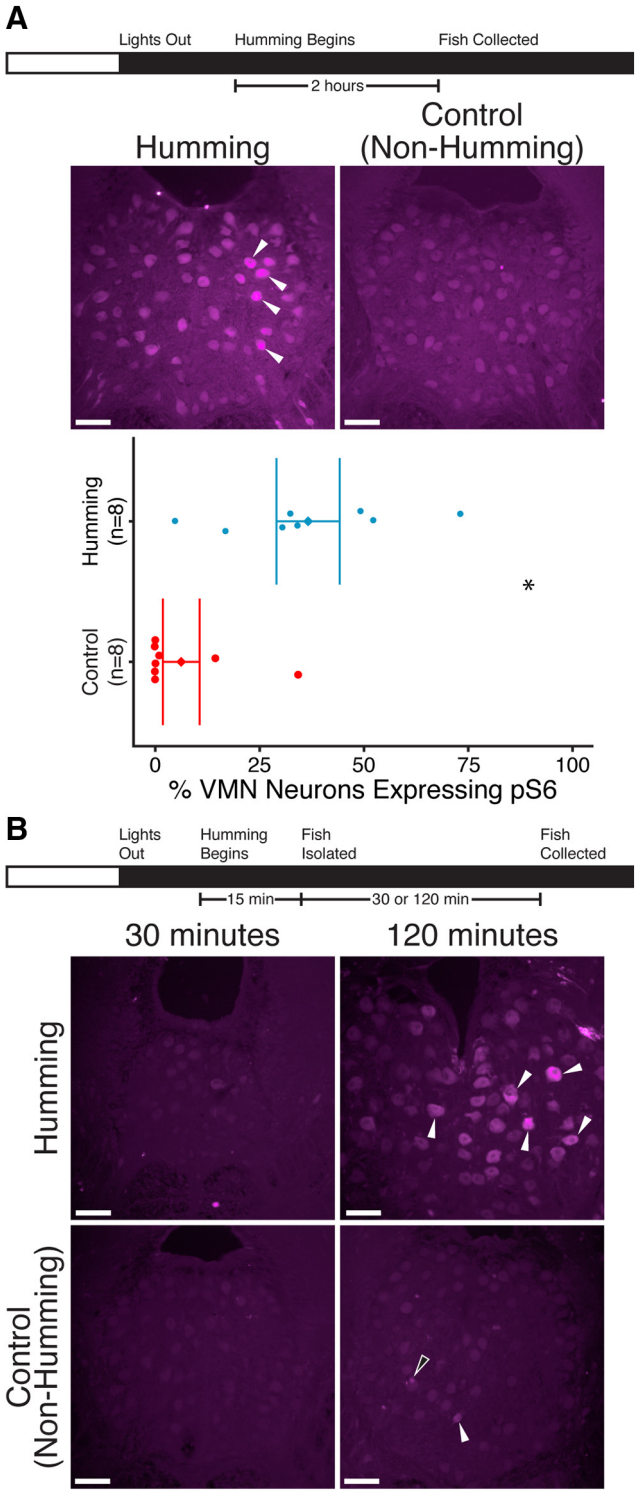

Figure 2. Timing of pS6 expression. ***A***, pS6 expression is present 2 h after onset of humming behavior. Top, Timeline of humming behavioral experiment. Middle, Representative images of pS6 expression in VMN of humming (left) and control non-humming (right) animals collected 2 h after onset of humming fish's vocalization; animals from the courtship humming experiment (Fig. 4). Filled arrowheads indicate examples of strongly labeled VMN neurons in humming fish. pS6-labeled vocal pacemaker (premotor) neurons also visible immediately ventrolateral to VMN in humming male (3 somata in lower right corner). Bottom, Humming fish from the courtship humming experiment show a significant increase in the proportion of VMN neurons expressing pS6 2 h after the onset of humming, compared with non-humming control fish collected at the same time ($t_{(11.249)} = -3.472$, $p = 0.005057$, Welch two-sample t test). *$p < 0.05$. Error bars show mean (diamonds) ± SEM. ***B***, pS6 expression persists at least 120 min after offset of humming behavior. Top, Timeline of post-humming isolation experiment. Bottom, Expression of pS6 is mostly absent in the VMN of male fish housed alone and then isolated for 30 min after humming (top left). Expression is, however, robust in fish isolated for 120 min after humming (top right). Filled arrowheads indicate examples of strongly labeled neurons. Expression is mostly absent in matched, non-humming control animals (bottom row). Open arrowhead in 120 min control image indicates imaging artifact. Scale bars. 100 μm.

containing a single artificial nest to which gravid females could be introduced (Fig. 3*A*). A female was added to each chamber and when one female entered a nest, the other nest was blocked using a plastic mesh cage to prevent mating. Courting type I males ($n = 5$), cuckolding type I males ($n = 5$), cuckolding type II males ($n = 5$), and females ($n = 3$) were collected from nests where mating occurred, along with paired control animals from neighboring nests that were prevented from mating, over seven total spawning trials (Fig. 3*A*). Fewer females were collected, as some that remained gravid were kept to be reused in additional spawning experiments. Each mating animal and its paired control were collected 2 h after they began mating (for rationale, see Materials and Methods). Midshipman cuckoldry consists of either satellite mating in which a cuckolding male inserts its tail into the nest and fans sperm onto eggs, or sneak mating in which the cuckolder enters the nest, behaviorally mimics a female, and attempts to fertilize eggs as they are laid (Brantley and Bass, 1994; Lee and Bass, 2006). To reduce variation within groups, we collected only satellite type I and satellite type II cuckolding males in this experiment, which were more frequently observed than sneaking males of either morph.

The brains of all animals in this experiment and those reported below were dissected immediately after collection, and prepared for immunohistochemistry with tissue sections labeled by antibodies raised against galanin and pS6 (see Materials and Methods). One pair of type II male brains (mating and control) was damaged during immunohistochemistry and was removed from further analyses. The proportion of POA^{Gal} neurons expressing pS6 is increased in type I males engaged in the courting tactic, but not type I or type II males engaged in the cuckolding tactic, or mating females (Fig. 3*B*,*C*). ANOVA comparison shows a statistically significant difference among groups ($F_{(7,26)} = 34.22$, $p = 1.5 \times 10^{-11}$). Tukey *post hoc* comparison reveals that courting type I males engaged in mating have a significantly higher proportion of POA^{Gal} neurons expressing pS6 than all other mating and control groups ($p < 1.00 \times 10^{-7}$), whereas there are no significant differences between all other mating and control groups ($p > 0.99$ for all other comparisons).

The type I male courting tactic is a suite of several related behaviors that contribute to reproductive success (Brantley and Bass, 1994). Because these behaviors can occur in quick succession (e.g., courtship vocalization, known as humming, immediately precedes female entry to the nest), or may be interleaved with mating itself (e.g., bouts of aggression toward cuckolding males), it is possible that one of these component behaviors explains the POA^{Gal} activation seen here in the courting male phenotype. To determine whether POA^{Gal} activity is specific to mating, rather than other related component behaviors, we next tested whether these cells were similarly activated during the other principle behaviors that comprise the type I male courting phenotype: courtship humming, care for eggs, and nest defense against attempted cuckolders (Brantley and Bass, 1994) of either male morph.

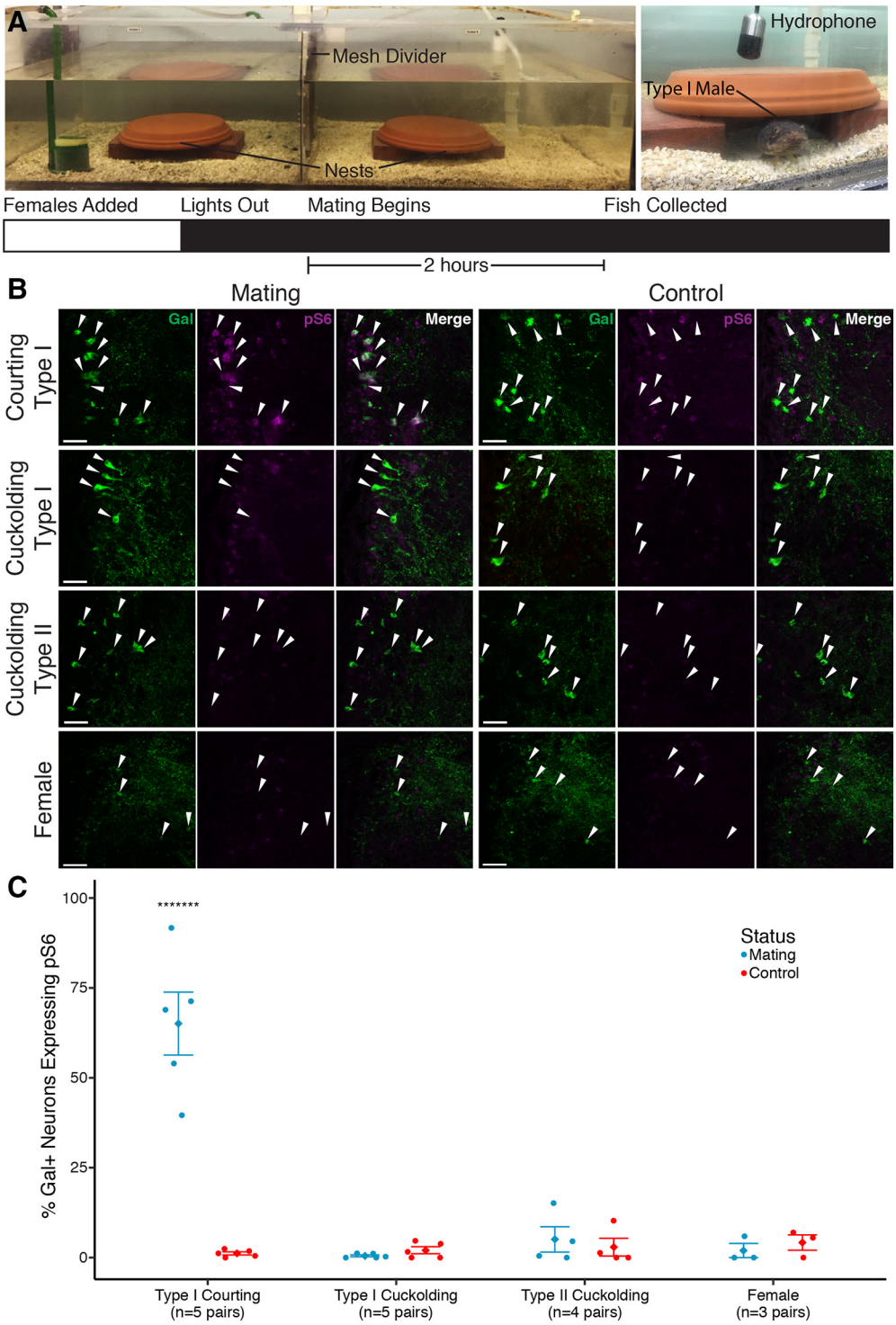

Figure 3. Mating behavior experiment. ***A***, Divided tanks used for mating experiment (left) and close up image of type I male in an artificial nest with nearby hydrophone for sound recording (right) with experiment timeline (below). Photographs taken during lights on to enable easy visualization of setup. ***B***, pS6 expression in galanin-expressing POA (POA^{Gal}) neurons of mating (left) and non-mating controls (right) for courting type I males, cuckolding type I males, cuckolding type II males, and females (non-mating control courting type I alone inside nest covered by mesh cage; control cuckolding type I and II males and females blocked from accessing a nest). Left images show galanin label in green, middle images show pS6 label in magenta, with merged images on the right. White arrowheads indicate location of galanin cell bodies. Scale bars, 50 μm. ***C***, Proportion of POA^{Gal} neurons expressing pS6 in mating (blue) and control (red) courting type I males, cuckolding type I males, cuckolding type II males, and females. ANOVA $F_{(7,26)} = 34.22$, $p = 1.5\times 10^{-11}$. *******$p < 1\times 10^{-7}$ Tukey's test. Error bars show mean (diamonds) ± SEM.

POA^{Gal} activity does not increase during courtship vocalization

Courtship vocalization (humming) immediately precedes mating and contributes to female nest localization and mating (Brantley and Bass, 1994; McKibben and Bass, 1998; Zeddies et al., 2010). Because there are increases in both galanin transcript expression in the POA (Tripp et al., 2018) and, as shown in the mating behavior experiment, in POA^{Gal} activity in courting type I males, galanin expression and neuron activity may have been related to humming specifically, but not mating itself. To test whether the observed POA^{Gal} activation in type I males is associ-

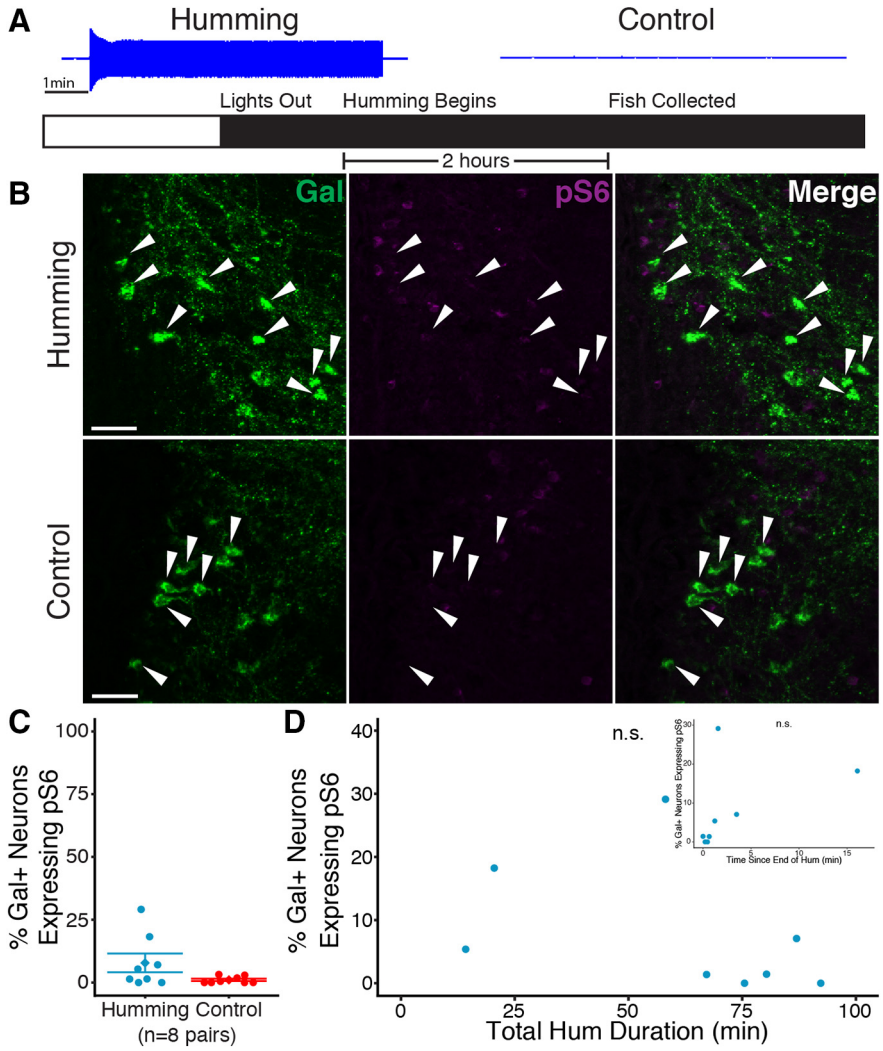

Figure 4. Courtship humming behavioral experiment. ***A***, Example of recording from a humming male (left) and a non-humming male (right) and timeline of experiment. ***B***, pS6 expression in galanin-expressing POA (POA^{Gal}) neurons of humming and non-humming control animals. Left images show galanin label in green, middle images show pS6 label in magenta, with merged images on the right. White arrowheads indicate location of galanin cell bodies. Scale bars, 50 μm. ***C***, Proportion of POA^{Gal} neurons expressing pS6 in humming (blue) and non-humming control (red) type I males ($t_{(7.2434)} = -1.8073$, $p = 0.1122$). Error bars show mean (diamonds) ± SEM. ***D***, Relationship between pS6 expression in POA^{Gal} neurons and total hum duration during experiment for humming males ($p = 0.3021$, $r = -0.418533$). Inset, The proportion of POA^{Gal} neurons expressing pS6 is not correlated with length of time between end of humming and removal from nest for humming type I males. Pearson's correlation $p = 0.2451$, $r = 0.465508$. n.s., not significant.

ated with courtship humming, we recorded male vocalization using hydrophones, and collected eight males 2 h after the onset of humming (Fig. 4*A*) along with eight non-humming control males housed in the same divided aquarium (see description in Fig. 3*A* legend). There is no significant increase in the proportion of POA^{Gal} neurons expressing pS6 in type I males humming in the absence of females compared with non-humming control males ($t_{(7.2434)} = -1.8073$, $p = 0.1122$, Welch two-sample t test; Fig. 4*B*,*C*). Additionally, for humming males, pS6 expression in POA^{Gal} neurons is not significantly correlated with time spent humming during the 2 h following hum onset ($p = 0.3021$, $r = -0.418533$; Fig. 4*D*), or with the latency between hum offset and collection from the nest ($p = 0.2451$, $r = 0.465508$; Fig. 4*D*, inset).

POA^{Gal} activity does not increase during egg care

Immediately after mating, females depart from the nest and type I males begin egg care which includes fanning and brushing of eggs, and protecting them from predators (Arora, 1948; Brantley and Bass, 1994; Sisneros et al., 2009; Bose et al., 2016a). To test whether the observed POA^{Gal} activation is associated with egg care behavior that begins following mating, we collected singly housed type I males from nests containing eggs ($n = 7$), or control nests without eggs ($n = 6$) 2 h after onset of the dark period, when midshipman are most active (Feng and Bass, 2016; Fig. 5*A*). Observation of videos taken during the egg care experiment show that four of seven males given nests with eggs performed both fanning and brushing during the 2 h of observation. Additionally, two males were observed brushing but not fanning, and only one male did neither during the experiment. The proportion of POA^{Gal} neurons expressing pS6 in type I males in nests with eggs is not significantly increased compared with males in nests without eggs ($t_{(11)} = 0.66927$, $p = 0.5171$, two-sample t test; Fig. 5*B*,*C*). There is also no significant correlation between pS6 expression in POA^{Gal} neurons and bouts of egg brushing, defined as putting mouth to eggs ($p = 0.6706$, $r = -0.1978782$; Fig. 5*D*), or with the amount of time spent fanning fins ($p = 0.06185$, $r = 0.7312215$; Fig. 5*E*). The proportion of POA^{Gal} neurons was <1% for all animals, including the single male that was returned to a nest containing eggs it had fertilized. This result was surprising, given the importance of medial POA galanin neurons in mouse parental care (Wu et al., 2014; Kohl et al., 2018).

POA^{Gal} activation does not require territory defense

Courting males in our courting/cuckolding mating paradigm engage in territorial defense against cuckolders in addition to courtship toward and mating with a female (Brantley and Bass, 1994; Lee and Bass, 2004, 2006). To determine whether the increased POA^{Gal} activity observed during type I male mating was due to nest defense against cuckolders, we conducted another mating experiment in which courting type I males mate either with or without potential cuckolders present in the aquarium. We collected courting type I males mating with and without cuckolders present 2 h after a female entered and remained in the nest (Fig. 6*A*). Because we were no longer able to collect males in reproductive condition from their natural habitat due to the lack of sufficiently low tides exposing nests at the end of the breeding season (Brantley and Bass, 1994; Sisneros et al., 2004), we were only able to obtain $n = 2$ mating males for each group in this experiment. Because of this small sample size, which would result in low power in any statistical test, we use only descriptive statistics for pS6 expression in these males. Consistent with prior studies (Brantley and Bass, 1994; Lee and Bass, 2004, 2006), for both males collected during mating with cuckolders, video analysis confirms cuckolding attempts by other males and aggressive behaviors by courting males during the 2 h experiment.

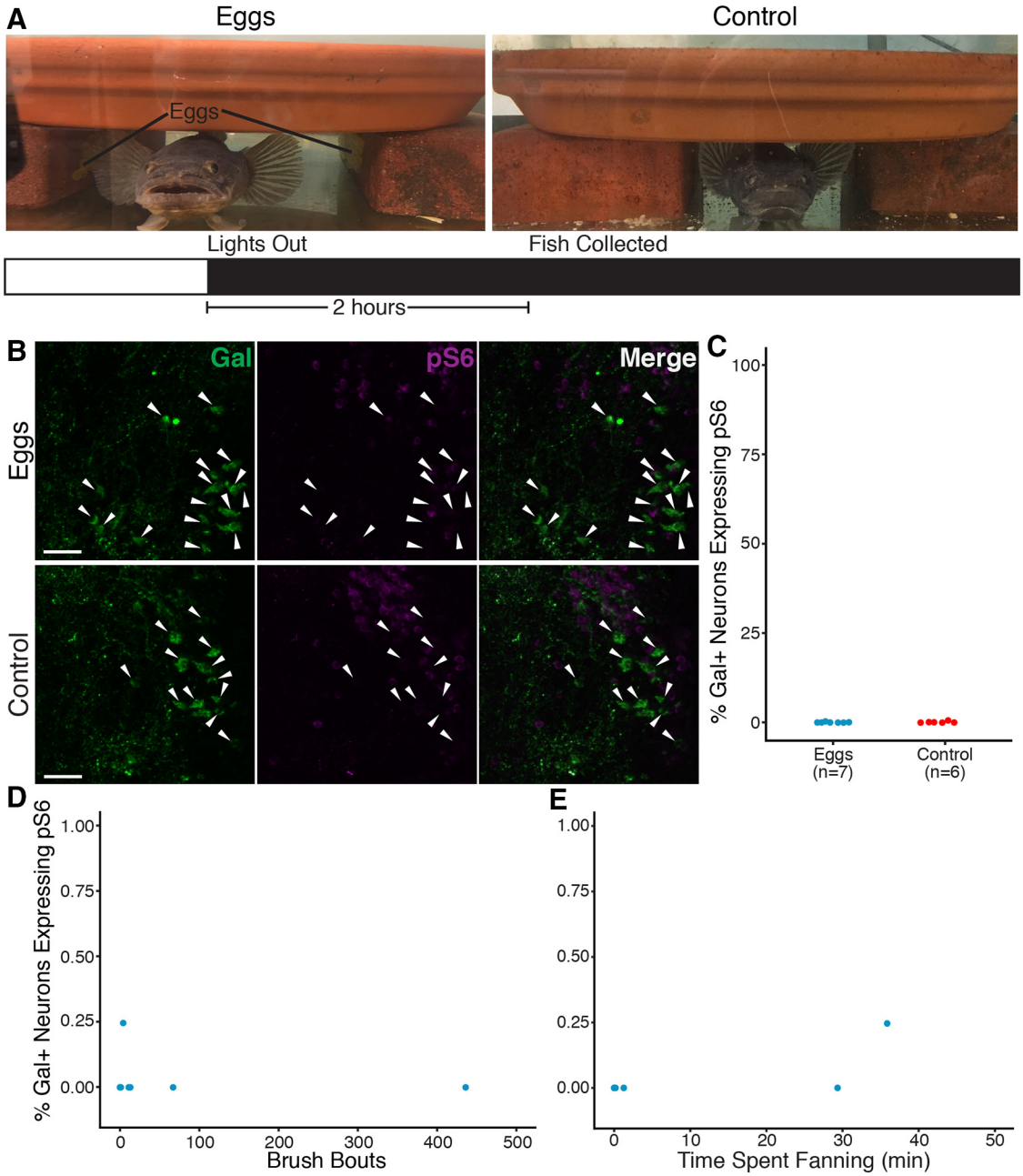

Figure 5. Egg care behavioral experiment. ***A***, Experiment design. Example nests with eggs (left) and control nest without eggs (right) and timeline of experiment (below). Photographs taken during lights on to enable easy visualization of setup. ***B***, pS6 expression in POA^{Gal} neurons of males in nests with eggs and control animals without eggs. Left images show Gal label in green, middle images show pS6 label in magenta, with merged images on the right. White arrowheads indicate location of galanin cell bodies. Scale bars, 50 μm. ***C***, Proportion of POA^{Gal} neurons expressing pS6 in males in nests with eggs (blue) and control nests without eggs (red). Independent *t* test $t_{(11)} = 0.66927$, $p = 0.5171$. No error bars shown due to extremely low variation within groups. Four of seven males in nests with eggs fanned eggs; all of these also brushed eggs. Two additional nesting males with eggs only brushed them. One male neither brushed nor fanned the eggs. Proportion of POA^{Gal} neurons expressing pS6 is not correlated with (***D***) the number of bouts of brushing eggs with mouth (Pearson's correlation $p = 0.6706$, $r = -0.1978782$). On the x-axis, two of the points overlap above 0 and two overlap to the right of 0 at 11 and 13 bouts, or (***E***) the amount of time spent fanning fins in the nest (Pearson's correlation $p = 0.06185$, $r = 0.7312215$). On the x-axis, four of the points overlap directly above 0; one of these is just to the right of 0 at 0.18 min fanning.

Both groups of males had similar levels of pS6 expression in POA^{Gal} neurons (Fig. 6*B*,*C*). Courting type I males mating without cuckolders present have pS6 expression in 66.78 ± 36.93% (mean ± SD) of POA^{Gal} neurons, whereas males mating with cuckolders present show pS6 expression in 70.44 ± 26.90% of POA^{Gal} neurons. Strikingly, for each of the mating males in this experiment, the proportion of POA^{Gal} neurons expressing pS6 (Fig. 6*C*) is within essentially the same range as that reported in the first experiment (Fig. 3*C*) for courting type I males, each of which had at least one cuckolder at their nest during mating. One male that mated without cuckolders present has a highly similar, but 1% greater, proportion of POA^{Gal} neurons expressing pS6 than the maximum expression seen in the first experiment. Although the possibility remains that there are subtle differences in POA^{Gal} activity between males defending against cuckolders and males without cuckolders present, our results indicate that the presence of cuckolders during mating is not necessary for increased POA^{Gal} neuron activity.

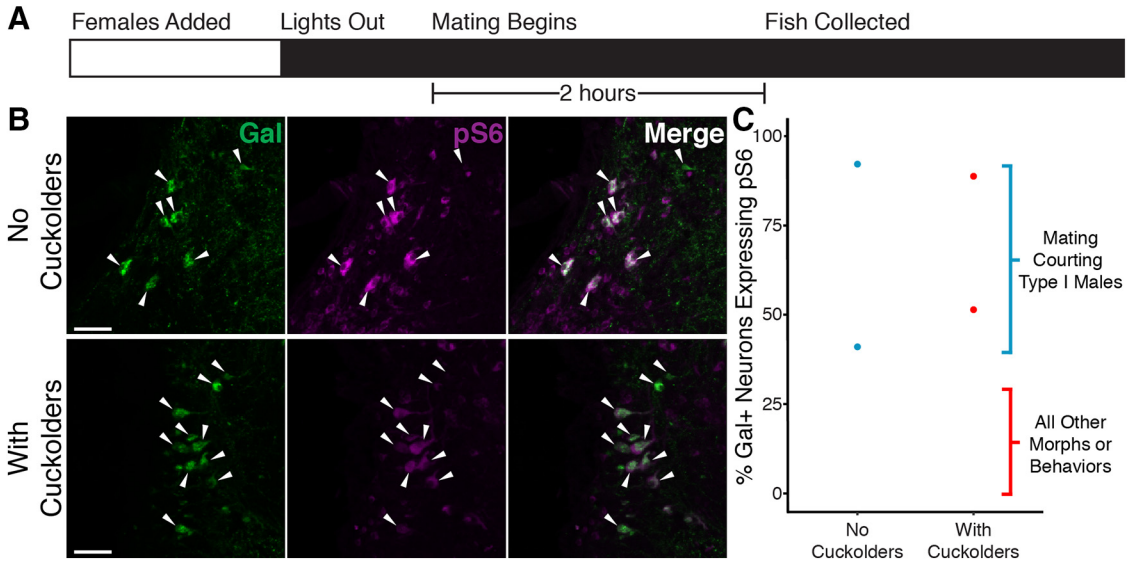

Figure 6. Nest defense behavioral experiment. ***A***, Timeline of experiment. ***B***, pS6 expression in POA ^{Gal} neurons of males mating with no cuckolders and with cuckolders present. Left images show galanin label in green, middle images show pS6 label in magenta, with merged images on the right. White arrowheads indicate location of galanin cell bodies. Scale bars, 50 μm. ***C***, Proportion of POA ^{Gal} neurons expressing pS6 in males mating without cuckolders (blue points) and with cuckolders present (red points). Blue brackets indicate the range of POA ^{Gal} neurons expressing pS6 in courting type I males that mated in the earlier experiment (Fig. 3*C*). The red brackets indicate the range of POA ^{Gal} neurons expressing pS6 in all other animals in all other experiments (Figs. 3*C*, 4*C*, 5*C*). No error bars shown due to small sample sizes.

Discussion

Galanin is a widely studied peptide (Hokfelt, 2010), but relatively few experiments, so far limited to mammals, investigate the active involvement of galanin-expressing neurons in regulating social behavior (Park and Baum, 1999; Bakker et al., 2002; Wu et al., 2014; Kohl et al., 2018). Our experiments take advantage of the expression of male ARTs in teleost fish to investigate the behavioral function of galanin-expressing neurons on a broader evolutionary landscape. The results are significant for several reasons. First, although studies of gene expression in the brain of teleosts (including our own of midshipman) suggest a role for galanin in male mating behavior, the current investigation is the first to demonstrate that galanin-expressing neurons are active during mating in teleosts as they are in mammals (Bakker et al., 2002; Wu et al., 2014). By identifying a mating behavior role for POA ^{Gal} neurons in fish, we demonstrate a phylogenetically shared function for these neurons between members of the two major clades of bony vertebrates: actinopterygians that include teleosts and sarcopterygians that include tetrapods (Nelson, 2006). Second, these findings show how the transcriptomic variation that we identified in the POA (Tripp et al., 2018) translates into neuronal mechanisms to help explain "consistent interindividual differences in behavior" (after Bengston et al., 2018). Third, by identifying differences in the activity of POA ^{Gal} neurons both within and between male morphs during reproductive behaviors, the results indicate a potential role for galanin-driven circuitry in the evolution of intrasexual behavioral plasticity among species with ARTs. Last, the above results together reveal a previously unrecognized, function-based relationship between a POA neuropeptidergic population in teleosts and the more highly differentiated tetrapod POA.

Male morph-specific phenotypes

Together, our results show a role for a specific cell type, POA ^{Gal} neurons, in the courtship mating tactic of type I male midshipman fish. The results are consistent with reports of increased expression of the immediate early gene c-fos in POA/POA-anterior hypothalamus ^{Gal} neurons following mating in male, but not female, mice and ferrets (Bakker et al., 2002; Wu et al., 2014). This further links the neuroendocrine basis for differential regulation of individual, context-dependent reproductive-related behaviors in species with ARTs to those without ARTs.

Despite the behavioral flexibility exhibited by type I males that can switch between courting and cuckolding dependent on social context (Lee and Bass, 2004), our prior studies emphasize the importance of developmental history in determining male morph-specific phenotypes, largely highlighting traits related to vocal mechanisms that distinguish the type I male morph, but are shared between the type II male morph and females (Feng and Bass, 2017). The present results demonstrate the importance of behavioral context, in this case the activation of one neuropeptide cell type during a single reproductive-related tactic (courtship) that is absent during the alternative tactic (cuckoldry), regardless of developmental morph. As highlighted earlier, differential activation of these neurons among individuals within a morph during alternative mating tactics points to a potential role of POA ^{Gal} driven circuits in the evolution of behavioral plasticity at the individual level for species with ARTs.

The increase in POA ^{Gal} activity seen here in courting males may be related to interactions with the mating female at the entrance of and within the nest. These include blocking the entrance to prevent females from leaving once they enter, lateral pressing against the female before egg-laying, biting and maneuvering the female within the nest during egg-laying and sometimes forcing females into the nest (Brantley and Bass, 1994). The precise role in relation to these more nuanced mating interactions remains unclear. For example, POA ^{Gal} neurons may be regulating the performance of these behaviors, or alternatively may be promoting mating interactions in general over other components of the courtship tactic (e.g., humming or nest defense). Because we used red light to limit disrupting nocturnal courting male behavior, we were unable to illuminate and record components of spawning behavior within the nest's interior.

Comparisons to mammals

Our results inform evolutionary comparisons between cell populations in the POA of teleosts, the most species-rich vertebrate group that are within the actinopterygian clade, with those in the more highly differentiated POA and anterior hypothalamus of mammals and other tetrapods that largely comprise the sister group of sarcopterygians. Developmental studies identify the amniote POA as part of the telencephalon, separate from the hypothalamus (Domínguez et al., 2013; Puelles et al., 2013), whereas the teleost POA is a distinct "morphogenetic entity" lying between the telencephalon and hypothalamus (Affaticati et al., 2015). In teleosts, this region includes nonapeptide-synthesizing neurons that are proposed homologues of the oxytocin and vasopressin synthesizing paraventricular and supraoptic nuclei in the amniote anterior hypothalamus (Forlano and Cone, 2007; Herget et al., 2014). How teleost POA populations other than those synthesizing nonapeptides compare to those in amniotes remains largely unknown. The current study is an important function-based step toward resolving this issue; that the predominant location of galanin-containing neurons active during male mating interactions is in the anterior parvocellular POA of midshipman supports its comparison to the medial preoptic nucleus of mammals that includes galanin-expressing neurons active during parental care (Wu et al., 2014).

The results of our egg care experiment were quite surprising, given the role of POA^{Gal} neurons in mouse parental care (Wu et al., 2014; Kohl et al., 2018). We expected to see POA^{Gal} activity related to egg care because courting type I males are the sole providers of parental care (Arora, 1948; Brantley and Bass, 1994), including care for eggs they did not fertilize after taking over a nest (Bose et al., 2016b). We observe both fanning and brushing in males given nests with eggs, but neither behavior appears to be sufficient to drive POA^{Gal} pS6 expression nor is either behavior correlated with it, suggesting that POA^{Gal} neurons are not driving egg care behaviors. These results, however, are consistent with a more recent study investigating the role of POA^{Gal} neurons in parental care behavior in poison dart frogs, which found POA^{Gal} activation during parental care only in a species with biparental care, and not in species with either maternal or paternal care only (Fischer et al., 2019). Because midshipman provide paternal care only, our results are consistent with this lack of activation observed in uniparental species of frogs.

Based on the results of the current study, we are unable to determine whether the observed POA^{Gal} activity results in galanin release, or whether these neurons are regulating behavior through release of other transmitters. However, evidence from other studies suggests that galanin peptide is playing a role in male reproductive behavior. First, in midshipman, transcripts encoding galanin are upregulated in courting type I males during mating (Tripp et al., 2018). Additionally, microinjection of galanin into the medial preoptic nucleus (a subdivision of the POA) of rats facilitates sexual behaviors (Bloch et al., 1993, 1996). Together, these studies show that galanin is involved in the regulation of reproductive behavior and suggest that the activity of POA^{Gal} neurons in the present study is related to galanin release.

Concluding comments

We show that a population of galanin-expressing neurons in the POA increases activity in courting type I male midshipman during mating interactions with females, but not other type I male mating-related behaviors or cuckolding by either type I or type II males, nor mating females. Thus, neural circuitry driven by POA^{Gal} neurons may be a potential substrate for the evolution of tactic-specific behaviors among species with ARTs. From an even broader evolutionary perspective, this work demonstrates that POA^{Gal} neurons have a shared role between widely distant vertebrate lineages in social behaviors that directly contribute to individual fitness, including male mating in fish and mammals and mammalian parental care in tetrapods. The results further provide function-based evidence allowing for a better understanding of widely divergent patterns between fish and tetrapods in the organization of molecularly identified cell groups in the POA that are major focal points for studies of the neural substrates of social behavior plasticity across multiple vertebrate lineages.

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