The neural basis of tadpole transport in poison frogs

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Abstract

Parental care has evolved repeatedly and independently across animals. While the ecological and evolutionary significance of parental behavior is well recognized, underlying mechanisms remain poorly understood. We took advantage of behavioral diversity across closely related species of South American poison frogs (Family Dendrobatidae) to identify neural correlates of parental behavior shared across sexes and species. We characterized differences in neural induction, gene expression in active neurons, and activity of specific neuronal types in three species with distinct parental care patterns: male uniparental, female uniparental, and biparental. We identified the medial pallium and preoptic area as core brain regions associated with parental care, independent of sex and species. Identification of neurons active during parental care confirms a role for neuropeptides associated with parental care in other vertebrates as well as identifying novel candidates. Our work highlights the potential for comparative, mechanistic studies to build a more complete understanding of how shared principles and species-specific diversity govern parental care and other social behavior.

Background

Parental care is an important adaptation that allows exploitation of novel habitats, influences fitness and survival of parents and offspring, and serves as an evolutionary precursor to other affiliative behavior [1,2]. Specialized parental care strategies have evolved repeatedly and independently across animals, yet the mechanisms underlying parental behavior and its evolution remain poorly understood. The neural mechanisms promoting parental care in females are best understood in mammals [3]; however, female uniparental care evolved at the base of the mammalian lineage and therefore provides limited clues to the evolutionary origins of parenting behavior. Moreover, studies of male parental care come mostly from biparental systems [4,5] in which parental behavior cannot easily be dissociated from pair bonding. What is needed to further understand the mechanisms underlying parental behavior and its evolution are comparative studies across closely-related species that vary in parental care strategies.

Parental care can be conceptualized as a complex set of inter-related behaviors controlled by brain regions involved in the integration of sensory, social, motivational, and cognitive aspects of care [6]. Across vertebrates, these functions are largely performed by the social decision-making network (SDMN; [7]), a highly interconnected group of evolutionarily ancient and functionally conserved brain regions. Although studies on the neural mechanisms of parental behavior are sparse outside mammals, and particularly lacking in amphibians and reptiles, the SDMN provides an ideal starting point for this work as network nodes and connectivity are well understood, highly conserved, and behaviorally important ligand/receptor complexes have been extensively studied.

Dendrobatid poison frogs show remarkable diversity in parental care across closely related species, including male uniparental care, female uniparental care, and biparental care. Parental care in poison frogs involves egg attendance during embryo development, generally followed by transportation of tadpoles “piggyback” to pools of water upon hatching [8–10]. In some species, mothers regularly return to nourish growing tadpoles with unfertilized, trophic eggs until

metamorphosis [9–11]. Importantly, both male and female care occur with and without pair bonding in this clade [12], allowing the dissociation of pair bonding from parental care. The diversity of behavioral care strategies between closely-related poison frog species affords a unique opportunity to identify physiological, neural, and molecular contributions to parental care and its evolution.

In the current study, we take advantage of three closely related focal species with distinct care patterns: *Dendrobates tinctorius* (male uniparental care), *Ranitomeya imitator* (biparental care), and *Oophaga sylvatica* (female uniparental). By comparing neural activity in parental frogs as well as their non-caregiving partners, we identify core brain regions active during tadpole transport independent of sex, species, and pair-bonding. To identify neuronal types mediating tadpole transport, we characterize gene expression and activity patterns specifically in behaviorally relevant neurons within core brain regions. Our experiments are the first to explore neural and molecular mechanisms of parental care in amphibians and demonstrate the utility of mechanistic studies in closely related, behaviorally distinct species in identifying core neural correlates of parental behavior.

Methods

Laboratory sample collection

Dendrobates tinctorius and *Ranitomeya imitator* frogs were housed in breeding pairs in the laboratory, allowing us to identify both parental individuals and their non-caregiving partners. To control for effects of experience, all pairs successfully reared at least one clutch from egg-laying through tadpole transport prior to the experiment. For the non-parental group, we collected frog pairs between parental bouts when they were not caring for eggs or tadpoles, collecting individuals of both the caregiving sex (non-transport; n=10 *D. tinctorius*, n=7 *R. imitator*) and their opposite sex partners (non-transport partner; n=9 *D. tinctorius*, n=8 *R. imitator*). For the tadpole

93 transport group, when we found transporting frogs, we collected both the tadpole transporting
94 individual (tadpole transporter; n=13 *D. tinctorius*, n=7 *R. imitator*) and its opposite sex, non-
95 transporting partner (transport partner; n=11 *D. tinctorius*, n=6 *R. imitator*). All brain tissue was
96 collected in an identical manner: frogs were captured, anesthetized with benzocaine gel, weighed
97 and measured, and euthanized by rapid decapitation. This entire process took less than 5
98 minutes. All procedures were approved by the Harvard University Animal Care and Use
99 Committee (protocol no. 12-10-1).
100
101 *Field sample collection*
102 *Oophaga sylvatica* (Puerto Quito-Santo Domingo population) were collected in field
103 enclosures in Ecuador in April and May of 2016. We collected non-parental control females (N=8)
104 from enclosures containing only mature females to ensure that frogs were not currently caring for
105 eggs or tadpoles. We collected tadpole transporting females (N=5) from enclosures containing
106 multiple males and females and therefore could not identify their non-caregiving male partners.
107 Frogs were captured, anesthetized with benzocaine gel, weighed and measured, and euthanized
108 by rapid decapitation. Procedures were approved by the Harvard University Animal Care and Use
109 Committee (protocol no. 15-03-239) and all samples were collected and imported in accordance
110 with Ecuadorian and US Law (collection permits: 005-15-IC-FAU-DNB/MA and 007-2016-IC-
111 FAU-DNB/MA; CITES export permit 16EC000007/VS issued by the Ministerio de Ambiente de
112 Ecuador).
113
114 *Immunohistochemistry*
115 Whole brains were placed into 4% paraformaldehyde at 4°C overnight and then
116 transferred to a 30% sucrose solution for cryoprotection. Once dehydrated, brains were
117 embedded in Tissue-Tek® O.C.T. Compound (Electron Microscopy Sciences, Hatfield, PA, USA),

rapidly frozen, and stored at -80°C until cryosectioning. We sectioned brains into four coronal series at 14µm, allowed slides to dry completely, and stored slides at -80°C.

To assess the level of neural activity across brain regions, we used an antibody for phosphorylated ribosomes (pS6; phospho-S6 Ser235/236; Cell Signaling, Danvers, MA, USA) and followed standard immunohistochemical procedures for 3',3'-diaminobenzadine (DAB) antibody staining (as in [13]). To ask whether neural activity was higher specifically in galanin neurons, we combined the pS6 antibody with a custom-made galanin antibody (peptide sequence: CGWTLNSAGYLLGPHAVDNHRSFNDKHGLA; Pocono Rabbit Farm & Laboratory, Inc, Canadensis, PA, USA) and followed standard immunohistochemical procedures for fluorescent double antibody labeling (as in [4]). Detailed methodological descriptions are in Supplemental Materials.

Microscopy and cell counts

Stained brain sections were photographed on a Leica DMRE connected to a QImaging Retiga 2000R camera at 20X magnification. We quantified labeled cells from photographs using FIJI image analysis software [14]. Brain regions were identified using a custom dendrobatid frog brain atlas (Supplemental Materials). We measured the area of candidate brain regions and counted all labeled cells in a single hemisphere for each brain region across multiple sections. We quantified cell number in the nucleus accumbens, the basolateral nucleus of the stria terminalis, the habenula, the lateral septum, the magnocellular preoptic area, the medial pallium (homolog of the mammalian hippocampus), the anterior preoptic area, the suprachiasmatic nucleus, the striatum, the posterior tuberculum (homolog of the mammalian midbrain dopamine cells representing the ventral tegmental area and substantia nigra), the ventral hypothalamus, and the ventral pallium.

Fluorescently stained brain sections were photographed at 20X magnification on a Leica DM4B compound microscope attached to a fluorescent light source. Each section was visualized

144 at three wavelengths (594nm, 488nm, 358nm) and images were pseudo-colored to reflect these
145 spectra. We used DAPI nuclear staining to identify brain regions as above and then quantified the
146 number of galanin positive cells, pS6 positive cells, and co-labeled cells from photographs of the
147 preoptic area using FIJI image analysis software [14]. We combined counts for all preoptic area
148 sub-regions due to the low overall number of galanin-positive neurons and because this more
149 closely reflected the neuroanatomical resolution of tissue punches used in phophoTRAP (see
150 below).
151
152 *Statistical analyses of cell counts*
153 We analyzed the relationship between parental behavior and pS6 neural activity to identify
154 brain regions whose activity differed during tadpole transport independent of sexes and species
155 (i.e. core parental care brain regions). We used generalized linear mixed models with a negative
156 binomial distribution appropriate for count data with unequal variances to test for differences in
157 pS6 positive cell number. For laboratory animals, behavioral group (tadpole transport vs non-
158 parental), sex, brain region, and their interactions were included as main effects predicting the
159 number of pS6-positive cells. For field sampled *O. sylvatica*, sex was omitted from the model as
160 we could not identify non-caregiving partners and collected only females. Individual was included
161 as a random effect, brain region area as a covariate to control for body size differences between
162 frogs, known size differences between brain regions, and rostral to caudal size/shape variation
163 within brain regions. We explored main effects of group, sex, and regional differences in further
164 detail using *post hoc* comparisons Tukey adjusted for multiple hypothesis testing.
165 We tested for differences in the number and activity of galanin neurons using generalized
166 linear mixed models. To compare the number of galanin neurons, we included behavioral group
167 (tadpole transport vs non-parental), sex, and their interactions as main effects predicting the
168 number of galanin positive cells using a negative binomial distribution appropriate for count data
169 with unequal variances. To analyze activity differences in preoptic area galanin neurons, we

included behavioral group, sex, and their interactions as main effects predicting the proportion of pS6 positive galanin (i.e. co-labeled) cells using a binomial distribution. All analyses were performed separately for each species using SAS Statistical Software (SAS 9.4; SAS Institute for Advanced Analytics).

PhosphoTRAP library construction & sequencing

We collected *D. tinctorius* males that were found transporting tadpoles to males that currently had tadpoles present in the leaf litter but had not yet transported them. Males were sacrificed as described above (N=9 per group). Brains were removed, embedded in Tissue-Tek® O.C.T. Compound, frozen on dry ice, and stored at -80°C for no more than 1 month. Once all animals had been collected, brains were sectioned at 100 µm on a cryostat and thaw mounted on SuperFrost Plus slides. A 0.96 mm tissue micro punch tool was used to isolate the medial pallium and rostral hypothalamus (anterior, medial, and magnocellular preoptic area and suprachiasmatic nucleus). To provide enough starting material for PhosphoTRAP, brain regions from three individuals were combined into a single sample, for a total of three biological replicates per group. PhosphoTRAP libraries for total (TOT) and immunoprecipitated (IP) RNA from each sample were constructed following [15] (details in Supplemental Materials). Libraries were then pooled in equimolar amounts and sequenced on an Illumina HiSeq 2500.

PhosphoTRAP analysis

To analyze phosphoTRAP data we first quantified gene expression by mapping sequenced reads back to a brain tissue specific *D. tinctorius* transcriptome (Fischer & O'Connell, *unpublished*) and estimated their abundance using Kallisto [16]. As gene expression is known to differ across brain regions [17], we performed all subsequent analysis steps separately for the Mp and POA. Analysis methods are described in detail in the Supplemental Materials. Briefly, we normalized read counts using DESeq2 [18] and quantified transcript enrichment/depletion in

active neurons as a log-fold difference between transcript counts from immunoprecipitated (IP) and total (TOT) mRNA for each sample. We then calculated differential fold enrichment between parental and non-parental individuals by dividing mean log-fold expression values from the two behavioral groups. We refer to this final metric as the log-fold difference ratio between tadpole transport and non-transport behavioral groups.

Our primary objective was to utilize phosphoTRAP data to identify Mp and POA cell types whose activity differed between tadpole transport and non-parental individuals. To this end, we restricted further analysis to a subset of 158 transcripts representing cell types with known roles in parental care (Table S1). We identified transcripts as significantly enriched/depleted based on a combination of log-fold enrichment thresholds (>4) and permutation testing (Supplemental Materials). Permutation testing and visualization were done using R Statistical Software (version 3.5.0; the R Foundation for Statistical Computing).

Results

Neural induction during tadpole transport

We compared neural activity patterns in tadpole transporters and their non-transporting partners across three closely related poison frog species with distinct parental care strategies (Fig. 1A). Differences in neural activity depended on behavioral group, sex, and brain region (Fig. 1B; Table 1) and associations between behavioral group and neural induction were brain region specific (Table 1; group*region: *D. tinctorius*: $F_{1,2515}=5.00$, $p<0.0001$; *R. imitator*: $F_{12,557}=6.85$, $p<0.0001$; *O. sylvatica*: $F_{12,557}=5.53$, $p<0.0001$). We found overall differences between the transporting and non-transporting sex in male uniparental *D. tinctorius* (sex*group*region: $F_{1,2515}=3.89$, $p<0.0001$) but not biparental *R. imitator* (Table 1; Fig. 1B). Indeed, *post hoc* analyses of region-specific differences revealed greater similarity between sexes in biparental and monogamous *R. imitator* than male uniparental *D. tinctorius* (Fig. 1B,C; Table S2).

Comparing neural activity patterns associated with parental care across species allowed us to identify brain regions important in parental care independent of sex and species (i.e. core parental care brain regions). We observed parallel increases in neural activity in tadpole transporting individuals in two core brain regions across all species: the preoptic area (POA) and the medial pallium (Mp; homolog of the mammalian hippocampus. In the POA, patterns differed by subdivision, with female-specific effects in the magnocellular POA and male-specific effects in the anterior POA (Fig. 1C). We also observed increased neural activity in the Mp of the non-caregiving partners of tadpole-transporters (Fig. 1C).

Gene expression in behaviorally relevant neurons

Following identification of core brain regions active during tadpole transport across species and sexes, we sought to identify behaviorally relevant neuronal types within these regions. We found 25 transcripts with significant log-fold expression enrichment/depletion in the POA and 32 transcripts with significant log-fold expression enrichment/depletion in the Mp, seven of which were overlapping between brain regions (Fig. 2; Table S3). Of the overlapping transcripts, four had log-fold expression differences in the same direction (galanin, prolactin receptor, neuropeptide Y receptor 2, brain specific angiogenesis inhibitor associated protein 2) and three had log-fold expression differences in opposite directions (aquaporin 4, dopamine receptor 1B, leptin receptor) between brain regions (Fig. 2).

Galanin neuron number and activity

Recent demonstrations of the importance of galanin in mediating parental care in mice [19,20] and the enrichment of galanin transcripts in neurons active during tadpole transport led us to ask whether activity differences specifically in POA galanin neurons were associated with parental care. Parental *R. imitator* had significantly more galanin neurons than did non-parental *R. imitator,* independent of sex (behavioral group: $F_{1,404}$=4.58, p=0.0329), but there were no

248 differences in galanin neuron number in *D. tinctorius* or *O. sylvatica* (Fig. S2). Both *D. tinctorius*
249 and *R. imitator* showed differences in galanin neuron activity associated with parental care, but
250 not in the same manner: in *D. tinctorius* the proportion of active galanin neurons was greater in
251 the female partners of non-transport males than any other group (sex*behavioral group:
252 $F_{1,40}$=12.73, p=0.0010; Fig. 3). In contrast, in *R. imitator* the proportion of active galanin neurons
253 was greater during tadpole transport in both males and females (behavioral group: $F_{1,26}$=8.15,
254 p=0.0083; Fig. 3). We observed no differences in the proportion of active galanin neurons
255 between tadpole-transporting and non-transporting *O. sylvatica* females.
256
257
258 **Discussion**
259 Parental care requires the coordination of hormonal, neural, and molecular changes, many
260 of which remain poorly understood. We took advantage of shared parental behavior across three
261 poison frog species with distinct parental care strategies, combining lab and field data to
262 disentangle sex- and species- specific mechanisms from core neural mechanisms at the levels of
263 brain regions, gene expression, and neuron type. We identified the medial pallium and preoptic
264 area as core brain regions associated with parental care and demonstrated expression changes
265 in genes associated with parental care in other vertebrates. Mechanistic studies in closely related,
266 behaviorally variable poison frogs offer a unique opportunity to distinguish shared principles and
267 neural diversity in the mechanisms mediating the maintenance and evolution of parental care.
268
269 *Core brain regions for parental care*
270 By comparing patterns of neural activity across closely related species with distinct
271 parental care strategies, we were able to identify core brain regions in which increased neural
272 induction during parental care was sex and species independent. We observed increased neural
273 induction in the medial pallium (Mp) and one or more subdivisions of the preoptic area (POA)

during parental care in all focal species. The POA's widespread connections with other brain regions and high density of neuromodulators make it ideally positioned to modulate complex social behavior, including parental care. Although data outside mammals is sparse, POA activity is associated with parental behavior across vertebrates, including mammals [3], birds [3,21], fish [22], and now frogs. In brief, the POA appears to be a core node in parental care circuitry across vertebrates. Importantly, parental care has evolved independently across these clades, indicating convergence across behavioral and neural levels.

In contrast with the POA, the Mp is not commonly associated with parental care. Although the precise function of the hippocampus and its non-mammalian homologs remains an area of active research, this brain region is classically implicated in memory, and specifically spatial memory [23,24]. Poison frogs inhabit complex rain forest environments in which tadpole deposition sites are a limited resource of variable quality. Behavioral studies in poison frogs document the use of cognitive spatial maps [25] and demonstrate the importance of spatial memory for navigating back to high-quality tadpole deposition pools [26] and for relocating offspring in egg provisioning species [27]. Increased neural induction in the Mp during tadpole transport is therefore in line with the unique ecological and evolutionary pressures associated with parental care in poison frogs. Indeed, spatial cognition is an important, but rarely examined, component of parental care [28,29], and comparisons of hippocampal involvement in parental care across species may yield interesting results given the functional conservation of this structure across vertebrates [30].

Shared parental care circuitry across sexes

The strength of our comparative design is highlighted by identification of inter-specific neural activity patterns between sexes. Neural activity during tadpole transport differed between males and females in uniparental *D. tinctorius*, but not biparental *R. imitator*. Females are not directly involved in tadpole transport in either species; however, biparental *R. imitator* females

provide parental care in the form of egg attendance prior to tadpole transport and tadpole provisioning following transport [12,30,31]. Thus, similar patterns of neural activity in male and female biparental *R. imitator* could arise either because both sexes are in a "parental state" that modulates long-term circuit activity or because even indirect involvement in tadpole transport activates parental circuitry (i.e. female frogs have to know where their tadpoles are transported in order to return to feed them). In either case, similarities in neural activity patterns associated with parental care across sexes suggest that parental care circuitry is conserved across sexes.

In addition to broad sex similarities in *R. imitator*, we also observed increased neural activity in the Mp of non-caregiving *D. tinctorius* females. While they are not the typically caregiving sex, females of *D. tinctorius* and related species will occasionally perform tadpole transport [32,33]. This behavioral flexibility demonstrates that parental circuits are present and can be activated under certain circumstances in females, and we suggest an increase in Mp neural activity is related to females' monitoring of their partners' behavior and ability to perform tadpole transport in the absence of their male partners. In other words, females may monitor male behavior in order to assess when and if they need to take over parental behaviors to ensure the survival of their offspring. The diversity of behavioral care strategies between species combined with this behavioral flexibility within species in poison frogs affords a unique opportunity to further disentangle the evolution of sex-specific parental care circuits in future.

Expression variation in behaviorally relevant neurons

Using *D. tinctorius* males, we characterized gene expression differences specifically in neurons active within the POA and Mp during parental care, focusing our analyses on genes previously identified as markers of neuronal types involved in parental care [34]. Of particular interest in the POA were increased expression of the vasopressin 1b receptor, a gonadotropin-releasing hormone receptor, and a number of stress response related genes (Urocortin-3, CART, CRF binding protein). Links between vasopressin and parental care have been demonstrated in

rodents [35,36] and vasopressin and gonadotropin releasing hormone may also influence parental care indirectly through their regulation of other molecules with known roles in parental care (e.g. oxytocin, prolactin) [3]. Stress hormones are known to increase in response to the behavioral and metabolic demands of parental care [37,38] providing a link between parental behavior and the observed upregulation of stress-related signaling pathways.

Notable in the Mp were increased expression of vasopressin and androgen receptor transcripts. As described above, vasopressin signaling is widely implicated in parental care, and has been specifically linked to space use and behavioral and life-history trade-offs in parental prairie voles [28,29]. Space use and navigational abilities differ between males and females in many species, and it has been proposed that greater navigational abilities in males are a side effect of increased androgen signaling [39]. Increased androgen signaling during parental care in poison frogs could facilitate the heightened spatial cognition important during tadpole transport. Increasing signaling via region specific receptor expression overcomes the lower testosterone levels typically observed in parental males [40].

In addition to changes specific to either the POA or Mp, we observed a number of transcripts with significant expression differences in both regions. Among them were dopamine and prolactin receptors, and a number of molecules and receptors most commonly implicated in feeding behavior (galanin, leptin receptor, NPY receptor). Dopamine and prolactin play known roles in parental care [40–42], while other shared transcripts (and some of those unique to a single brain region) are traditionally associated with feeding behavior. There is growing recognition that molecules traditionally classified as feeding-related play important roles in mediating social behavior, providing exciting opportunities to explore the repeated targeting of feeding related mechanisms in the convergent evolution of parental care [43].

Galanin and parental care

Initially described in relation to feeding behavior, recent work uncovered a role for POA galanin neurons in driving parental care in both male and female mice [19,20]. We found a positive association between parental care and galanin neuron number and activity in biparental *R. imitator*, but not in male uniparental *D. tinctorius,* nor female uniparental *O. sylvatica*. Indeed, the only significant difference outside *R. imitator*, was a relative increase in galanin neuron activity in the female partners of non-transporting male *D. tinctorius*, and we note that the percent of active galanin neurons was overall low in all species.

While recent work demonstrates a sex-independent, behavior-specific link between galanin neuron activity and parental care [19,20], the earliest work on POA galanin in rodents showed that microinjection of galanin into the POA of male rats facilitated copulatory behavior [44], and work in fish similarly suggests an association between male courtship behavior and galanin signaling [45,46]. Thus, species in which the role of galanin in social behavior has been explored vary in parental care strategy: rats are female uniparental, only some male mice exhibit male care, and fish include both male uniparental and female uniparental species. Together with our findings across frog species with distinct care patterns, these observations suggest that the role of galanin signaling in parental care may be mediated – both acutely and evolutionarily – by life history differences related to parental care, interactions among partners, and male courtship strategy. In brief, galanin appears to have been repeatedly evolutionarily co-opted to modulate social behavior, but the type(s) of social behavior influenced by galanin signaling are complex, mediated by the behavioral variation and evolution history, and providing fertile ground for future comparative research.

Conclusions

Our findings lay the foundation for exciting work using poison frogs as a model to explore neural and molecular mechanisms of parental care, sex-specific behavioral patterns, and the integration of social and environmental cues to coordinate complex social behavior. We identified

core brain regions associated with tadpole transport across dendrobatid poison frogs with distinct care strategies. Moreover, we confirmed a role in amphibians for hormones and neuropeptides associated with parental care in other vertebrates. While increased POA activity was associated with parental care across species, activity specifically of galanin neurons differed between species, suggesting that shared brain regions may nonetheless rely on unique neuronal types to mediate similar behavior. Comparative studies in closely related, but behaviorally distinct, species provide opportunities to build a more holistic understanding of how shared principles and species-specific diversity govern parental care.

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Data availability

Cell counts, read counts from phosphoTRAP, R code for phosphoTRAP analysis, and the *D. tinctorius* brain atlas are available as Supplemental Materials associated with the manuscript. Raw sequencing reads will be made available through the NCBI SRA repository upon publication.

403

404 **Authors' contributions**

405 LAO conceived of the study; LAO, KS and LAC designed and coordinated the study; EKF,
406 ABR, NAM, and EET collected samples; EKF and LAO performed molecular work and data
407 analysis; EKF and LAO wrote the manuscript with input from all authors; All authors gave final
408 approval for publication and agree to be held accountable for the work performed therein.

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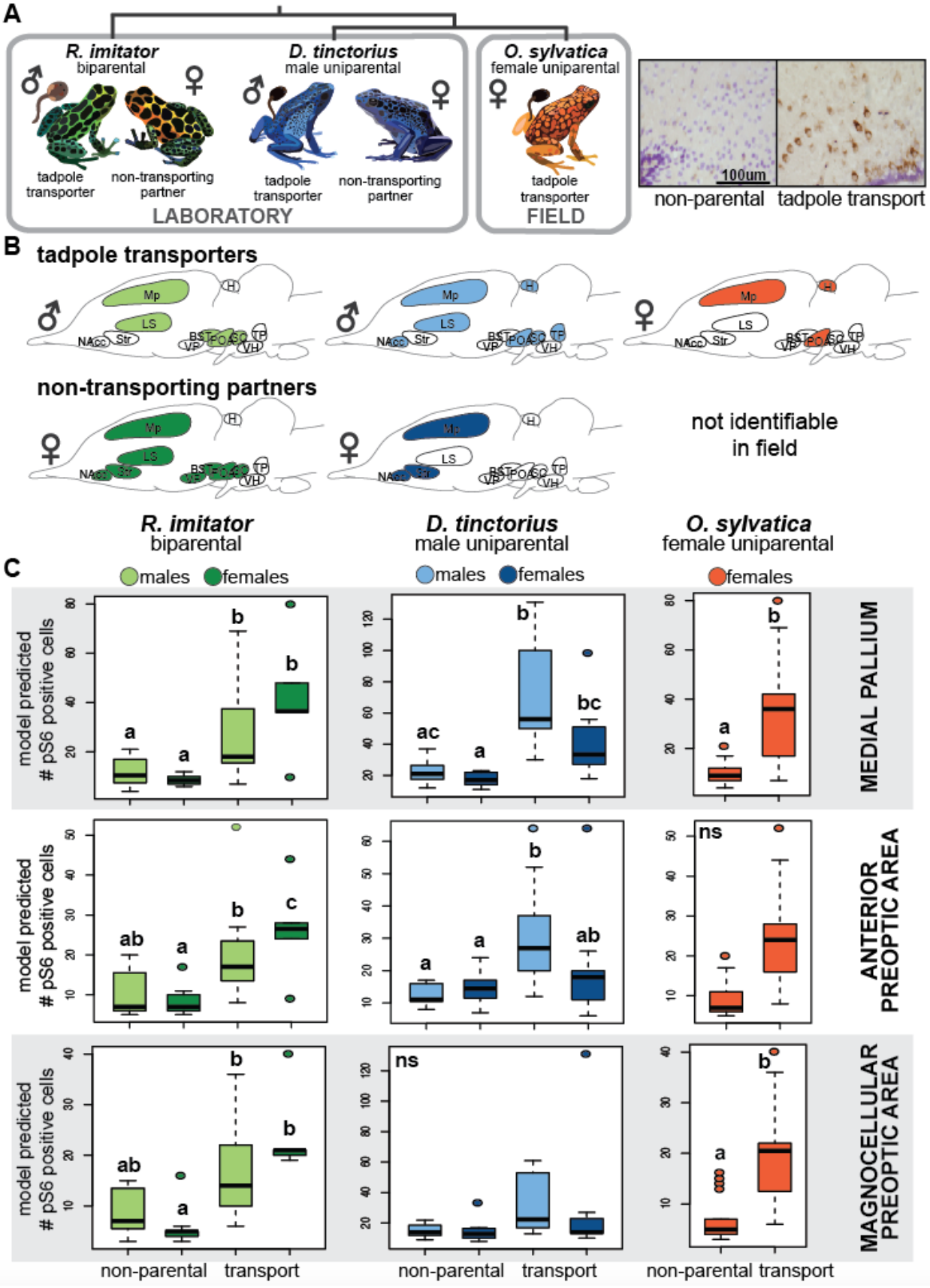
Tables & Figures

Table 1. Summary of main statistical effects for neural induction differences.

		df	F value	p value
R. imitator	group	1,1731	13.83	0.0002
	sex	1,1731	0.64	0.4242
	region	12,1731	87.48	<0.0001
	sex*group	1,1731	1.25	0.2630
	group*region	12,1731	6.85	<0.0001
	sex*region	12,1731	2.69	0.0013
	sex*group*region	12,1731	1.45	0.1344
D. tinctorius	group	1,2519	9.73	0.0018
	sex	1,2519	1.36	0.2443
	region	12,2519	80.15	<0.0001
	sex*group	1,2519	1.76	0.1844
	group*region	12,2519	5.00	<0.0001
	sex*region	12,2519	3.39	<0.0001
	sex*group*region	12,2519	3.89	<0.0001
O. sylvatica	group	1,557	1.02	0.3126
	region	12,557	9.40	<0.0001
	group*region	12,557	5.53	<0.0001



526 **Figure 1. Patterns of neural induction associated with parental care. (A)** Overview of
527 experimental design. Our comparative approach allowed us to identify brain regions important in
528 parental care independent of sex and species. **(B)** Overview of brain regions showing differences
529 in neural activity between parental and non-parental individuals (shaded) for tadpole transporting
530 sex and their non-transporting partners. Small symbols indicate the sex of transporting and non-
531 transporting partner individuals. Comparing across species, we identified Mp and POA as active
532 during tadpole transport regardless of sex and species (i.e. as core parental care brain regions).
533 **(C)** Detailed results for core brain regions. Letters above the box plots indicate significant group
534 differences ($p<0.05$). Representative micrographs of pS6 staining (brown) with cresyl violet
535 nuclear stain (purple) from the mPOA are shown at top right. Abbreviations: BST = basolateral
536 nucleus of the stria terminalis, H = habenula, Ls = lateral septum, Mp = medial pallium (homolog
537 of the mammalian hippocampus), NAcc = nucleus accumbens, aPOA = anterior preoptic area,
538 mPOA = magnocellular preoptic area, SC = the suprachiasmatic nucleus, Str = striatum, TP =
539 posterior tuberculum, VH = ventral hypothalamus, VP = ventral pallium.

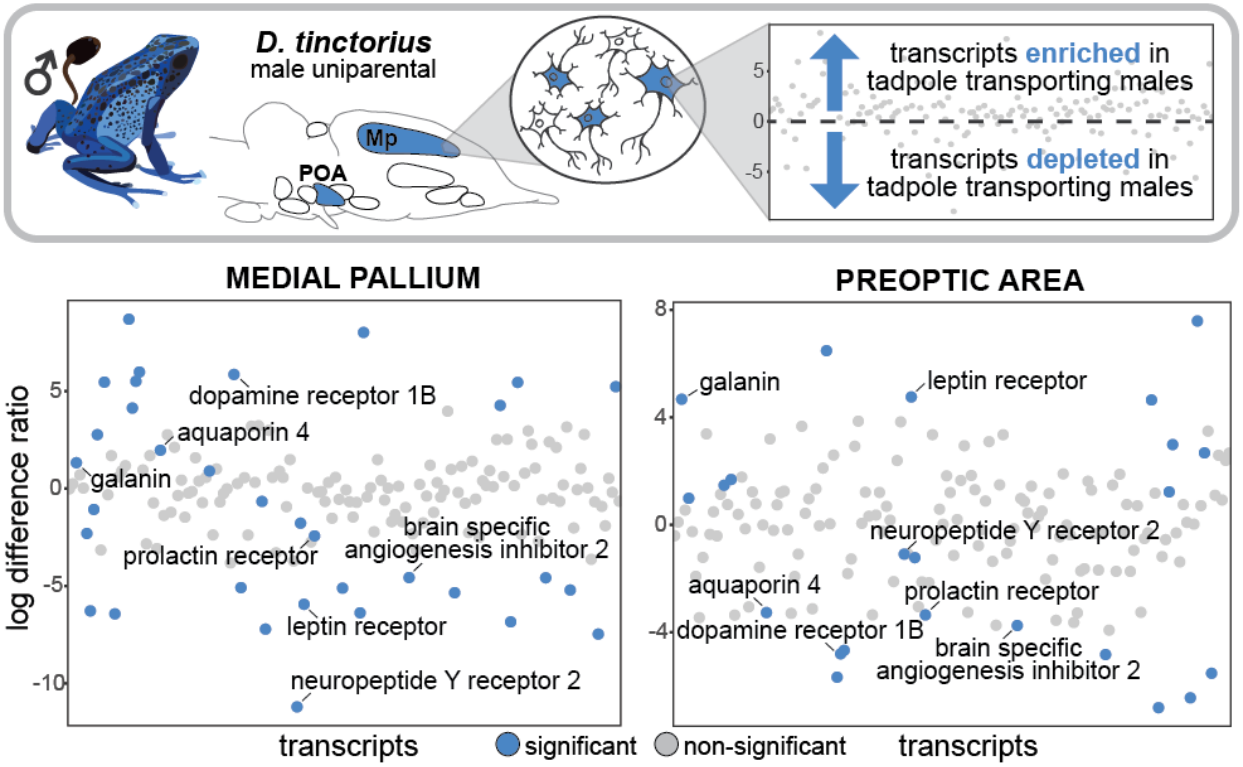


Figure 2. Gene expression in behaviorally relevant neurons. We identified significant expression differences in neurons active during parental care in the preoptic area (POA) and medial pallium (Mp) of tadpole transporting versus non-parental *D. tinctorius* males. We found some unique and some shared transcripts differentially expressed across brain regions (i.e. distribution of blue dots between plots). Those transcripts with significant expression enrichment or expression depletion in tadpole transporting males as compared to control males are highlighted in blue, and the seven transcripts overlapping between brain regions are labeled. The same candidate transcripts are plotted in the same order along the x-axis for both brain regions.

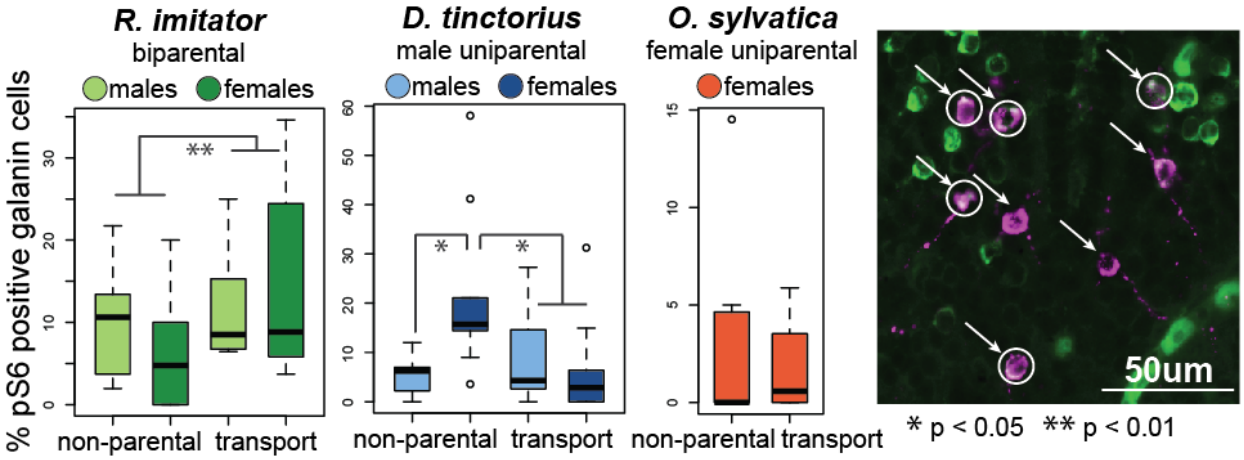


Figure 3. Preoptic area galanin neuron activity. Parental *R. imitator* had a greater proportion of active galanin neurons, as did the female partners of non-parental *D. tinctorius*. Representative micrograph: magenta = galanin positive neurons (arrows), green = pS6 positive neurons, white = co-localization indicating active galanin neurons (circles).