Neurobiology of pair bonding in fishes; convergence of neural mechanisms across distant vertebrate lineages

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Abstract

Pair bonding has independently evolved numerous times among vertebrates. The governing neural mechanisms of pair bonding have only been studied in depth in the mammalian model species, the prairie vole, *Microtus ochrogaster*. In this species, oxytocin (OT), arginine vasopressin (AVP), dopamine (DA), and opioid (OP) systems play key roles in signaling in the formation and maintenance of pair bonding by targeting specific social and reward-mediating brain regions. By contrast, the neural basis of pair bonding is poorly studied in other vertebrates, and especially those of early origins, limiting our understanding of the evolutionary history of pair bonding regulatory mechanisms. We compared receptor gene expression between pair bonded and solitary individuals across eight socio-functional brain regions. We found that in females, ITR and V1aR receptor expression varied in the lateral septum-like region (the Vv/Vl), while in both sexes D1R, D2R, and MOR expression varied within the mesolimbic reward system, including a striatum-like region (the Vc); mirroring sites of action in *M. ochrogaster*. This study provides novel insights into the neurobiology of teleost pair bonding. It also reveals high convergence in the neurochemical mechanisms governing pair bonding across actinopterygians and sarcopterygians, by repeatedly co-opting and similarly assembling deep neurochemical and neuroanatomical homologies that originated in ancestral osteithes.

Introduction

Pair bonding has independently evolved numerous times across all major vertebrate lineages[1], where it represents a major defining feature of species-specific social structure[2] including that of humans'[3-5]. As such, the neural basis of pair bonding in mammals is particularly well-studied (reviewed by: [6-13]) largely due to its translational implications for the mechanistic underpinnings of human pro-sociality (e.g., "romantic love")[8,14-17], and conversely, for better understanding and treating anti-social psychiatric disorders[7,17,18].

Most of what is known about the neural basis of mammalian pair bonding comes from extensive research on a small rodent, the prairie vole, *Microtus ochrogaster*. In this species, oxytocin (OT), arginine vasopressin (AVP), dopamine (DA), and opioid (OP) neurochemical systems play key interactive roles in signaling the formation and maintenance of pair bonding (reviewed in[8,10,12,19,20]). In females, sociosexual activity triggers OT release, which acts on OT receptors (OTRs) in the striatal nucleus accumbens (NAcc) and the prefrontal cortex (PFC)[21,22]; thereby formulating partner preference. AVP systems also regulate female partner preference formation[23]; however, targeted brain regions remain unknown. In males, both OT and AVP nonapeptide systems also appear to mediate mating-induced partner preference formation[23] (and see[20] reference to Keebaugh et al., unpublished data). This likely involves OT-OTR signaling within the medial PFC (see[20] reference to Keebaugh et al., unpublished data), while it requires both OT-OTR and AVP-V1aR signaling in the NAcc- ventral pallidum (VP) circuitry[20], and AVP-V1aR activity in the lateral septum (LS)[24]. AVP-V1aR also promotes mating-induced selective non-partner aggression (mate-guarding) in males[23,25,26] at least partially by signaling within the anterior hypothalamus[27,28]. These aforementioned nonapeptide behavioral effects may result, at least partially, from their more general roles in regulating individual recognition; which may occur through concurrent OTR and olfactory signaling within the medial amygdala (MeAMY)[20,29]; however, this is yet to be empirically tested. Different dopamine receptor sub-types appear to be involved in pair bond formation and maintenance. In both sexes, NAcc D2R activation promotes pair bond formation[30-32] ; and in turn, pair bond formation subsequently up-regulates NAcc D1R activity, promoting selective aggression towards, and thus inhibiting pair bond formation with, other prospective partners[32,33]. This D1R regulation of selective aggression is mediated by downstream activation of kappa-opioid receptors[33] (see below). The source of DA projections to the NAcc in these pathways is the ventral tegmental area (VTA)[34]. As with dopamine, different opioid receptor sub-types appear to selectively mediate either pair bond formation or maintenance. Specifically, mu-opioid receptors (MORs) within sub-structures of the striatum (ie., the caudate putamen (CP), dorsal striatum, and dorsomedial NAcc shell) regulates pair bond formation[35,36]. Dorsal striatum MORs achieve this through regulating mating, while dorsomedial NAcc shell MORs appear to achieve this through mediating positive hedonics associated with mating[36]. Finally, kappa-opioid receptors (KORs) within the NAcc shell regulate pair bond maintenance[33,36,37] by mediating aversive social motivation[33,37].

Because very little is known about the neurobiology of pair bonding in other species, and especially those of earlier evolutionary origins: reptiles[38], amphibians[39]and fishes[40,41] (but see[42-44]), the evolutionary history of neural circuitry governing vertebrate pair bonding remains poorly understood.

Convergence of evolutionarily labile traits, especially across remotely related lineages has been traditionally thought to be underpinned by entirely different regulatory processes[45]. In the case of vertebrate pair bonding, there have been literally hundreds of independent transitions (e.g., there have been at least 61 among mammals[46], and 13 among coral reef fishes[41]) that have transpired across taxon that are separated by up to 450 million years of independent evolution (i.e., actinopterygians and sarcopterygians)[47]. Hence, it is conceivable that the far-ranging convergence of vertebrate pair bonding may be a product of entirely different regulatory systems being selected upon in order to achieve the same phenotypic outcome[48,49]. This may be especially true for nonapeptide involvement, since these systems are expected to evolve in very species-specific ways, depending upon the evolutionary background of the species[49]. Indeed, while the role of nonapeptides in governing pair bonding

in *M. ochrogaster* is well established, evidence for their involvement in certain other species has been absent. Among eight species of *Peromyscus* mice, there is no association between pairing sociality and V1aR density within the VP, nor within other brain regions examined[50]. In male zebra finches, *Taeniopygia guttata*, AVT V1a-like binding sites within the VP are of low density[51], and central administration of AVP V1R antagonist cocktail does not affect pair bond formation[52]. With regards to the OT-like system, in *Peromyscus* mice, NAcc OTR expression is not associated with species differences in pairing sociality[53]; and pairing finches, *T. guttata,* and sparrows, *Zonotrichia albicollis,* exhibit no detectable expression of OTRs (or binding sites) in the NAcc nor the surrounding striatum[54,55].

Key neuro-chemical components of *M. ochrogaster* pair bonding have ancient evolutionary origins, as they were already established in the last common ancestor of ray- and lobe-finned fishes (ancestral osteichthyes) ~450 MYA, and their structure and functions have since remained highly conserved across vertebrates (**Fig. 1**). Vertebrate nonapeptides all derived from arginine vasotocin (AVT), which originated in jawless fishes (agnathans) ~500 MYA[56]. In early jawed fishes (gnathostomes), the AVT gene duplicated[56], giving rise to two lineages, AVP- and OT-like nonapeptides. In the AVP-like lineage, AVT has remained present in all non-mammalian species; whereas, a single amino acid substitution was made in AVT in most mammals, giving rise to AVP. In the OT-like lineage, the gene duplication event in early jawed fishes gave rise to isotocin (IT), which is found in all extant bony fishes (teleosts). Prior to water-land transition, IT was replaced by mesotocin (MT), which is mostly present in extant lungfish, amphibians, reptiles, and birds[57]. Finally, MT was replaced by OT in most mammals[58-60]. Cartilaginous fishes have evolved at least six OT-like peptides, including the mammalian OT form[61]. Despite these alterations with the nonapeptide family, OT and AVT differ by only one amino acid[62]. Nonape[63]ptides play fundamental roles in regulating social behavior and physiology in all vertebrate taxa[48]. Dopamine and the two major classes of dopamine receptors (D1 and D2Rs) pre-date the origin of chordates, and have since remained highly conserved across the phylum[64,65]. The dopamine system serves a diverse array of behavioral and physiological functions, some of which, including associative reward learning, are shared across different lineages[65,66]. The opioid system, primarily consisting of three endogenous ligands (endorphins, enkephalins, and dynorphins) and their conjugate receptors (mu-, κappa-, and delta-receptors)[67,68], was established before the origin of jawed vertebrates, and is found in all vertebrate lineages where it mediates a variety of functions[67,69-71]. Pain and reward/pleasure affect are two prominent roles in mammals[67,69,72]. Whether these affective functions are shared in other lineages is poorly studied, but available data suggest that both roles exist in birds[73,74], pain/nociception roles exist in amphibians, and both roles exist in teleosts[75-77] (but see[78]).

In addition to neurochemicals, brain regions involved in *M. ochrogaster* pair bonding present a high degree of functional homology across vertebrates (prefrontal cortex notwithstanding, because it's homolog is currently unknown)[79-87]. Finally, it has been most recently discovered that protein and gene expression patterns of socially-paramount neurochemicals across key brain regions that regulate reward and social behaviour are strikingly similar across vertebrates[88,89]. This highly conserved social decision making (SDM) neural network, comprised of two sub-circuitries (the mesolimbic dopaminergic system (MDS)[90], and social behaviour network (SBN)[91,92]), were already established in ancestral osteichthyes ~450 MYA[88,89]. Notably, nonapeptide and dopamine systems, as well as brain regions involved in *M. ochrogaster* pair bonding (PFC notwithstanding once more) are constituents of the vertebrate SDM network (**Fig. 1**).

Given that the neurochemical and neuroanatomical components that underpin pair bonding in *M. ochrogaster* originated in early vertebrates and have remained structurally and functionally conserved, it is conceivable that in at least selective cases, they may have been repeatedly co-opted and similarly assembled into a converged regulatory neural network during independent transitions to pair bonding within the sub-phylum. Indeed, nonapeptide and DA systems appear to regulate pair bonding in other species that span several lineages, and appear to do so through targeting similar brain regions. In male and female marmosets, *Callithrix penicillata*, OT promotes while an OTR antagonist reduces affiliation during cohabitation with a prospective partner[93]. Similarly, in male and female tamarins, *Saquinus Oedipus*, urinary OT increases with intra-pair affiliation[94]. In zebra finches*, T. guttata,* both i.c.v. and peripheral OTR antagonist administration impairs pair bonding behaviors, including latency to pair, and pairing stability[95,96]. In male cichlids, *Amatitlania nigrofasciata*, a IT/AVT antagonist cocktail inhibits affiliation with prospective partner and aggression towards non-partners[43]. However, nonapeptides do not appear to be involved in male *A. nigrofasciata* pair bond maintenance[43,44]. Pair bonding pine voles, *M. pinetorum,* exhibit higher NAcc OTR expression[97] and VP V1aR densities[98] than do non-pairing montane voles, *M. montanus*. In five species of zebra finches (f: Estrildidae), LS V1aR density predicts species-typical social group sizes[51]. Similarly, in seven species of butterflyfishes (f: Chaetodontidae) AVT-ir neuron fibre varicosity density within the lateral septum-like region (the ventral and lateral parts of the ventral telencephalon, Vv/Vl) predicts species-typical pairing from non-pairing sociality[42]. Finally, in zebra finches, *T. guttata*, during pair bond formation and in established pairs, DA neurons expressing immediate early gene Fos (a marker of neuron activity) in the VTA is heightened[99,100] and pair bonded birds exhibit higher levels of DA in the ventral medial striatum (the super-structure of the NAcc) than unpaired birds[100]. However, since comprehensive examination of both the functional involvement of nonapeptide, dopaminergic, and opioid systems, and their respective targeting sites is thus far limited to *M. ochrogaster,* it is currently not possible to confidently discern the extent to which pair bonding regulatory neural networks may have converged across vertebrates.

The aim of this study was to determine whether IT, V1a, DA, and MO receptors are involved in pair bonding in a teleost, as well as establish the specific brain region(s) (anatomical substrate(s)) upon which each receptor type operates*. Chaetodon lunutaus*, was selected as a study species because it exhibits both pair bonding and solitary phenotypes among sympatric individuals of otherwise similar ecologies, offering an opportunity for comparative research. Receptor gene expression within eight distinct brain regions (**Table 1**) was contrasted between pair bonded and solitary (control) individuals in order to reveal mechanistic correlates of pair bonding. Importantly, these regions include the putative ancestral homologs of those involved in *M. ochrogaster* pair bonding (**Table 1**).

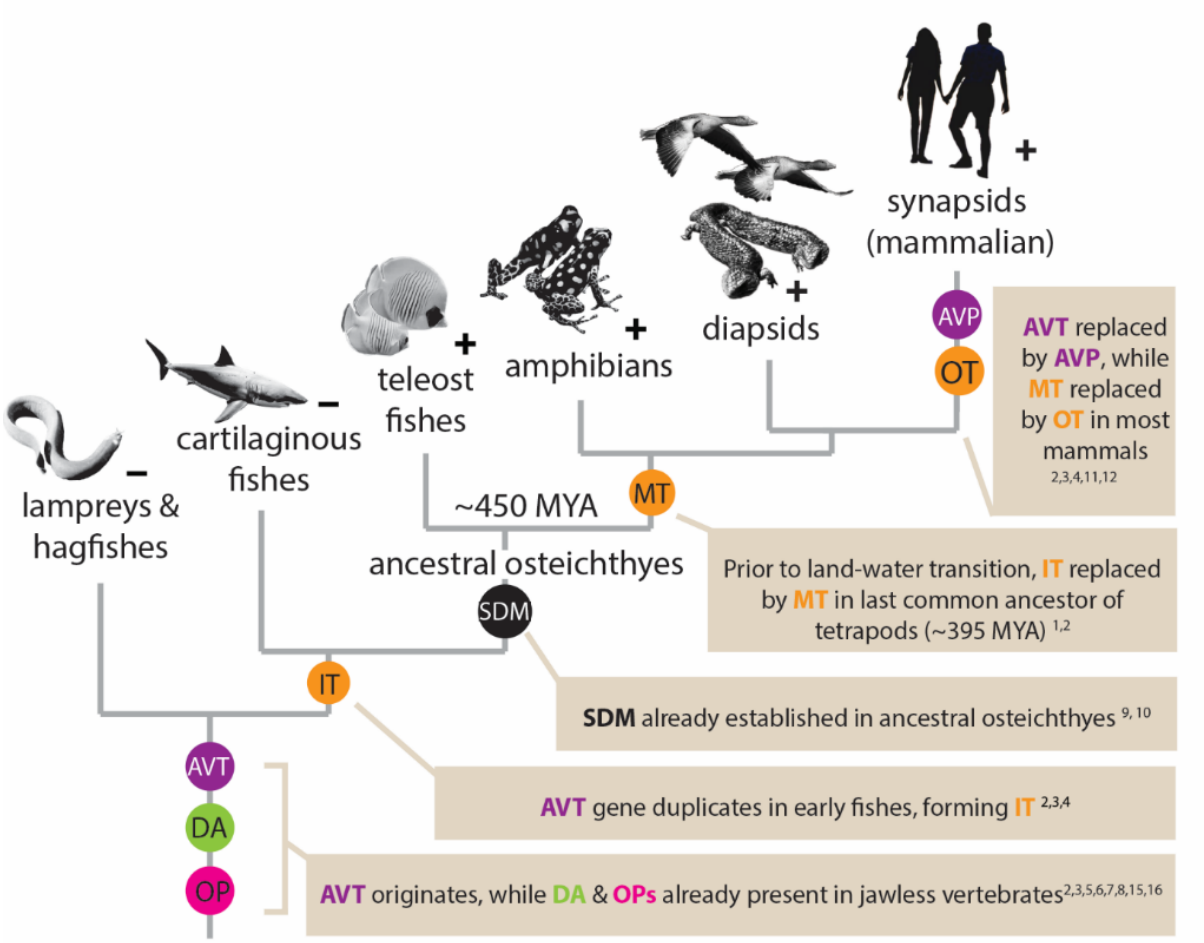

Figure 1. The neurochemical and -anatomical substrates of *M. ochrogaster* pair bonding pre-date the split between ray- and lobe-finned fishes, and have since remained highly conserved. Hence, the convergence of vertebrate pair bonding may have relied on repeatedly co-opting these homologies that were already established in a common ancestor ~ 450 MYA. The distribution of vertebrate pair bonding is indicated by its presence (+) or absence (-) across major groups. The evolutionary origins and history of neurochemical systems (colored circles) and brain regions comprising the SDM (black circle) is shown. *Abbreviations*: AVT = arginine vasotocin; DA = dopamine; OP = opioid; IT = isotocin; SDM =social decision making network; MT = mesotocin; AVP = arginine vasopressin; OT = oxytocin; SDM = social decision making network. *References for neural components*: [1]Shubin, 2008; [2]Goodson, 2008; [3]Archer and Chauvet, 1995; [4]Archer et al., 1995; [5]Callier et al., 2003; [6]Yamamoto and Vernier, 2011; [7]Khan et al., 1999; [8]Le Merrer et al., 2009; [9]O'Connell and Hofmann, 2011, [10]O'Connell and Hofmann, 2012; [11]Moore, 1992; [12]Moore and Lowry, 1998; [15]Dreborg et al., 2008; [16]Sundström et al., 2010.

Table 1. Teleost brain regions examined in current study, and their putative mammalian homologs.

Teleost brain region	Putative mammalian homolog***
1. Medial part of the dorsal telencephalon (Dm)	Basolateral amygdala (blAMY)[1,2,3]
2. Dorsal part of the ventral telencephalon (Vd)	Nucleus accumbens(NAcc)[3, 11] and striatum (Str/CP)[3,12,13,14]
3. Lateral part of the dorsal telencephalon (Dl)	Hippocampus (HIP)[1,2,3]
4. Ventral and lateral parts of the ventral telencephalon (Vv/vl)	Septum, lateral septum (LS)[3,2,4,5,6]
5. Supracommissural nucleus of the ventral telencephalon (Vs)	Extended amygdala (medial amygdala/bed nucleus of stria terminalis (meAMY/BNST))[3,5]
6. Central nucleus of the ventral telencephalon (Vc)	Striatum (Str)[3,8]/ caudate putamen (CP)**
7. Preoptic area and (POA/PVN)	Preoptic area (POA)[3,7]
8. Periventricular nucleus of posterior tuberculum (TPp)	Ventral tegmental area (VTA)[3,9,10*]/ substantianigra pars compacta (SNc)[15]

References: [1]Portavella et al., 2002;[2]Northcutt, 2006;[3]O'Connell and Hofmann, 2011; [4]Wullimann and Muller; 2004; [5]Northcutt, 1995; [6]Bradford, 1995; [7]Moore and Lowry, 1998, [8]Wullimann and Rink, 2002; [9]Rink and Wullimann, 2001; [10]Luo et al., 2008; [11]O'Connell et al., 2011; [12]Sharma et al., 1989; [13]Batten et al., 1990; [14]Weld and Maler, 1992; [15]Fallon and Moore, 1978. *Notes*: *The teleost TPp has been suggested to be at least functionally equivalent (Rink and Wullimann, 2001) if not homologous (Lou et al., 2008) to the VTA/substantianigra pars compacta (SNc) (Fallon and Moore, 1978). **In most mammals, the caudate putamen is a sub-structure of the striatum (O'Connell and Hofmann, 2011). ***While a tentative consensus for putative partial homologies between mammals and teleost brain regions has emerged, homologies should still be considered debatable (O'Connell et al. 2011; Goodson and Kingsbury, 2013). ****Brain regions involved in *M. ochrogaster* pair bonding that were not examined in the current study include the PFC and the VP (because their ancestral homologs are unknown (O'Connell and Hofmann, 2011)), and the anterior hypothalamus (teleost homologue = vTn) (O'Connell and Hofmann, 2011).

Methods

Animal collection and sexing

This study was conducted at Lizard Island, located in northern section of the Great Barrier Reef (GBR), Australia (14°40'08"S; 145°27'34"E). To compare receptor gene expression within brain regions between pair bonded and solitary individuals, we first collected solitary and paired individuals of *C. lunulatus* from fringing reefs around Lizard Island. The social system of individuals was recorded following 5-min observations prior to collecting fishes by spearing through the dorsal musculature. Individual fishes were immediately placed in an ice-water slurry for 5 min after which the brain was dissected (within 10 minutes of capture), embedded in optimal cutting compound (OCT), and frozen in liquid nitrogen for transportation to the laboratory where they were then transferred to -80 ˚C freezer until sectioning. In order to sex individuals, gonads were removed and fixed in formaldehyde-

acetic acid-calcium chloride (FACC) for at least one week. Thereafter, gonads were dehydrated in a graded alcohol series, cleared in xylene, embedded in paraplast, sectioned transversely (7 µm thick), and stained with hematoxylin and eosin. Sections were examined under a compound microscope (400 X magnification) for the presence of sperm (male) or oocytes (female)[101].

Brain region extraction and measuring gene expression

Frozen brains were transversely sectioned on a cryostat at 110µm, thaw mounted onto Superfrost Plus slides (Fisher Scientific) and stored at -80˚C prior to brain region extraction (approx. one week). Brain regions, identified using a butterflyfish brain atlas[102,103], were manually extracted at -30˚C using a hand-held micro-punching device (50mm diameter; Stoelting, model # 57401) (**Fig. 2**), incubated in RNAlater® at 4˚C over night, and then stored at -20˚C for up to one week. Brain region punching regime was standardized across individuals (see **Supplementary Table 1** for regime). Tissue punches were immediately transferred into a lysis buffer and homogenized by passing through a 21-gauge needle 15 times. Following, RNA was extracted using an E.Z.N.A® HP Total RNA kit (Omega, model # R6812-02) according to the manufacturer's instructions, and stored at -80˚C prior to cDNA synthesis. RNA was reverse transcribed into cDNA using Superscript III reverse transcriptase (Life Technologies) and gene-specific primers (see **Supplementary Table 2** for primer sequences). Residual primers and salts from reverse transcription were removed using an E.Z.N.A ® Tissue DNA purification kit (Omega, product # D3396-02), and cDNA was stored at -20˚C for up to 10 days prior to qPCR.

The whole brain transcriptome of *C. lunulatus* was sequenced in order to use as a reference for designing species specific cloning primers for each gene of interest. One *C. lunulatus* brain was taken out of RNAlater, rinsed in 1X phosphate buffered saline (PBS) and placed immediately in Trizol (Life Technologies, Grand Island, NY) where RNA was extracted according to manufacturer instructions. Poly-adenylated RNA was isolated from each sample using the NEXTflex PolyA Bead kit (Bioo Scientific, Austin, TX, USA). Lack of contaminating ribosomal RNA was confirmed using the Agilent 2100 Bioanalyzer. A strand specific library was prepared using the dUTP NEXTflex RNAseq kit (Bioo Scientific), which includes a magnetic bead-based size selection of roughly 350 bp. The library was pooled in equimolar amounts with sample from an unrelated study after library quantification using both quantitative PCR with the KAPA Library Quantification Kit (KAPA Biosystems, Wilmington, MA, USA) and the fluorometric Qubit dsDNA high sensitivity assay kit (Life Technologies), both according to manufacturer instructions. Libraries were sequenced on an Illumina HiSeq 2000 to obtain paired-end 100bp reads. We first corrected errors in the Illumina reads using Rcorrector (parameters: run_rcorrector.pl -k 31) and then applied quality and adaptor trimming using Trim Galore! (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/; parameters: trim_galore --paired --phred33 --length 36 -q 5 --stringency 5 --illumina -e 0.1).

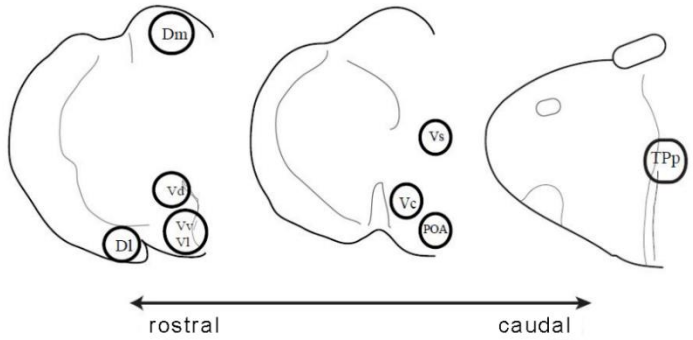

Figure 2. Transverse sections of oval butterflyfish brain are shown with circles identifying brain region micro-punches extracted for gene expression analysis.

After filtering and trimming, a total of 64,795,096 paired reads remained for de novo assembly. We created a *C. lunulatus* de novo transcriptome assembly using Trinity (parameters: --seqType fq --SS_lib_type RF). The raw Trinity assembly produced 376,338 contigs (N50: 1148 bp).

Using the *C. lunulatus* transcriptome as a reference, species-specific primers were designed to clone target gene sequences. Cloned target sequences were examined to determine whether they contained an exon-exon boundary using *Danio rerio* and *Stegastes partitus* complete genomes as a reference. If target gene sequences did not flank an exon boundary, then a second set of primers were designed to extend the obtained sequence towards the exon(s). Exon-containing sequences of ITR, V1aR, D1R, D2R and MOR genes were then used to design qPCR primers that flanked exon boundaries (18S ribosomal notwithstanding, because it does not contain an exon boundary) (see **Supplimenary Table 2** for primer sequences). Prior to qPCR, primer sets and instrument cycling parameters were empirically optimized on standard curves using several metrics of quality control (i.e., assay amplification R^2 value of at least 0.95, assay slope of approximately -3.3, assay melting curve that only produced a single amplicon peak, no amplicon signal in the no template control (NTC) or nor reverse-transcription control (NRTC)). Quantitative PCR was then performed on each sample using a reaction mixture and qPCR cycling instrument (CFX380) that was recommended by the enzyme manufacturer (see **Supplimenary Table 3** for parameters). Samples were run in technical triplicate on 384 well qPCR plates with standard curves in order to determine assay efficiency from the slope. Since assay efficiency was not the same across individual assays, averaged gene expression (C_t) values were standardized to assay efficiency prior to normalizing to 18S ribosomal RNA following methods of [104]. Not all focal regions of each brain were measured for gene expression due to insufficient tissue available.

Statistical analysis

For each gene within each brain region, a 2-way ANOVA with social system and sex as fixed factors was used to compare gene expression among treatment levels. Prior to analysis, data was natural log transformed +1 to improve normality of residual variance. Statistical analysis was conducted using SPSS software.

Results

Nonapeptide receptors (ITR and V1aR)

Both ITR and V1aR expression within the Vv/vl differed interactively between sex and social system (**Figure 3A, B**; **Supplimentary Table 4**). In females, ITR and V1aR Vv/Vl gene expression was higher in pair bonding than solitary individuals ($F_{1,13}$ = 9.06, p< 0.01; $F_{1,13}$ = 9.18, p = 0.01, respectively); however, in males, there was no difference in either ITR or V1aR Vv/Vl gene expression between social systems ($F_{1,13}$ = .002, p = 1; $F_{1,13}$ = .036, p = 1, respectively). ITR and V1aR Vv/vl gene expression differed significantly between sexes (p < 0.05), with females having higher nonapeptide gene expression than males; and they also differed between social system (p < 0.05), with pair bonded individuals displaying higher nonapeptide gene expression than singletons. Gene expression of both ITR and V1aR did not differ between sex or social system interactively or independently in any other brain region, namely in the Dl, Dm, POA, TPp, Vc, Vc, or Vs (**Supplimentary Table 4**). V1aR gene expression was detected in all brain regions examined (ie., the Dl, Dm, POA, TPp, Vc, Vd, Vv/Vl, and Vs) and ITR gene expression was detected within all brain regions except for the Vd.

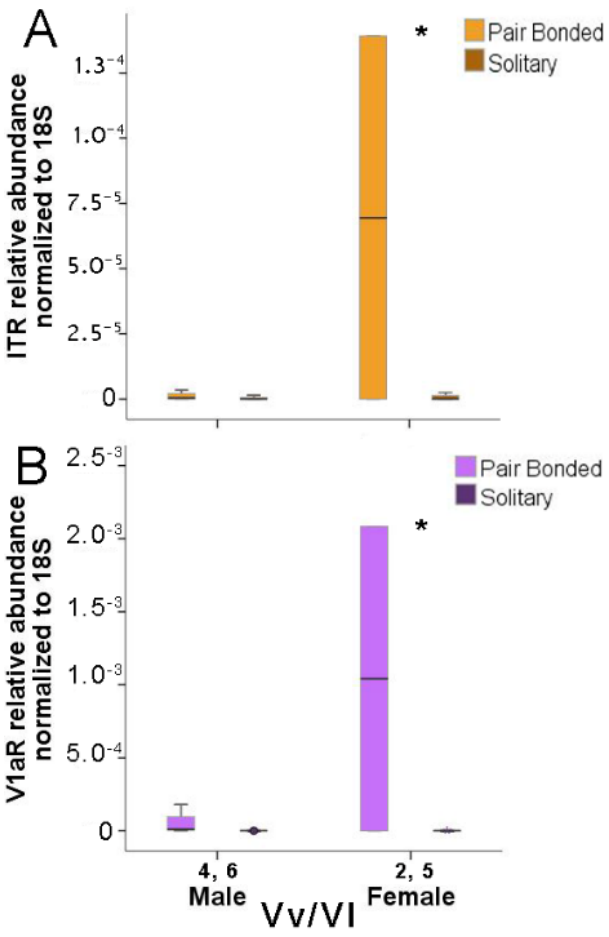

Figure 3. ITR (A) and V1aR (B) gene expression differences between sexes and social systems of *C. lunulatus* within the ventral and lateral parts of the ventral telencephalon (Vv/Vl). Boxes show the first and third quartiles, the black line in box represents median, and whiskers represent minimum and maximum value. Sample sizes are listed below each treatment group. Asterisks indicate statistically significant differences between treatment groups ($P < 0.05$) 2-way ANOVA and HSD Tukey Test.

Dopamine receptors (D1R and D2R)

Gene expression of neither of the dopamine receptor class differed interactively between sex and social system in any brain region (**Supplimentary Table 4**). Furthermore, gene expression of dopamine receptor class did not vary between sexes (**Supplimentary Table 4**). However, in several brain regions, gene expression of both dopamine receptor classes differed between social systems (p <0.05 for each region), with both male and female pair bonded individuals expressing less than their solitary counterparts in these areas: D1R: Dm (trend), Dl, Vs, POA; D2R: Dm, Dl, Vs, POA, Vc, and TPp (**Fig. 4A, B**; **Supplimentary Table 4**). Dopamine receptor class expression did not vary between social systems in other brain regions: D1R: TPp, Vd, Vv/vl; D2R: Vd, Vv/vl (**Supplimentary Table 4**). D1R and D2R gene expression was found in all brain regions examined (ie., the Dl, Dm, POA, TPp, Vc, Vd, Vv/Vl, and Vs).

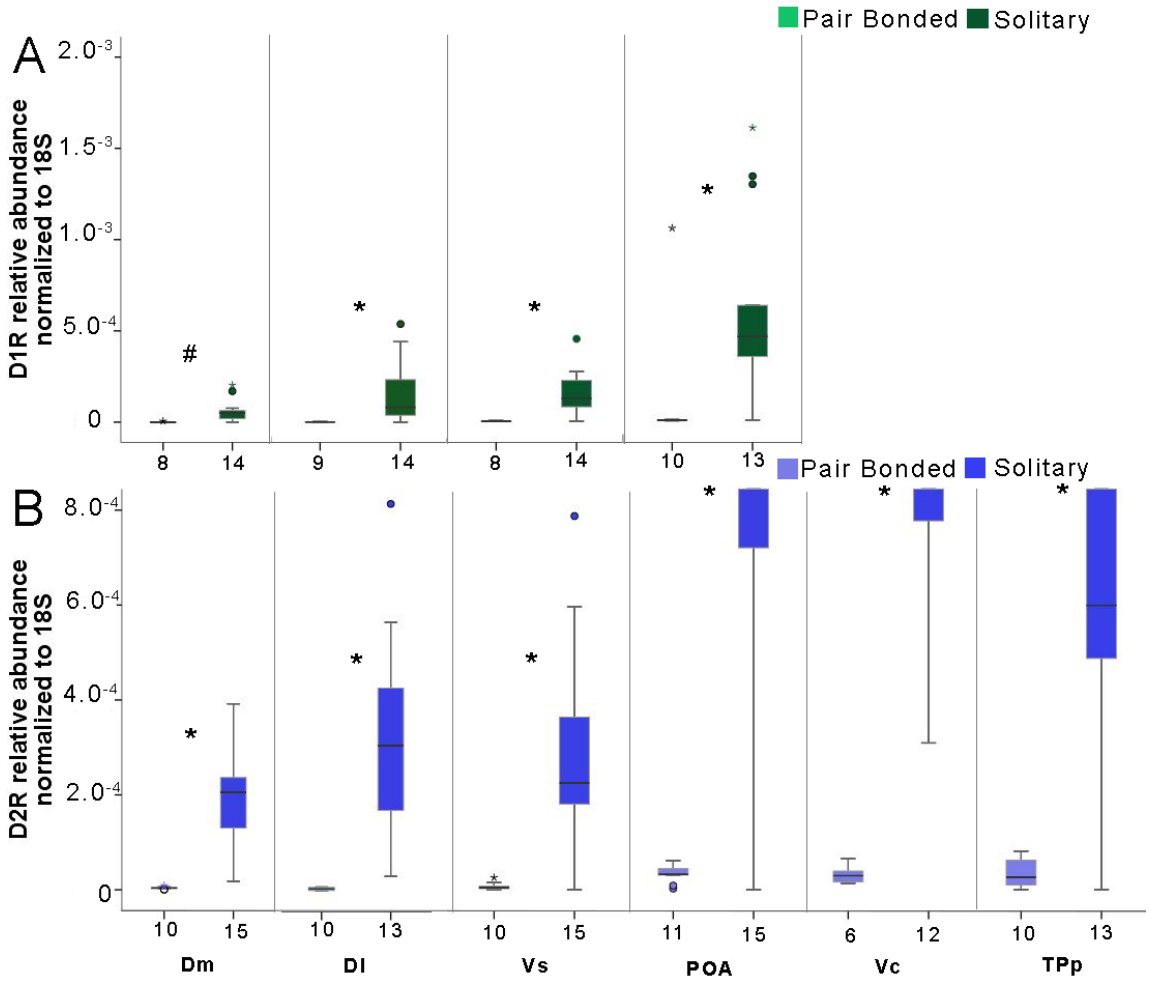

Figure 4. D1R (A) and D2R (B) gene expression differences between social systems of *C. lunulatus* within brain regions. Boxes show the first and third quartiles, the black line in box represents median, and whiskers represent minimum and maximum value. Sample sizes are listed below each treatment group. * = statistically significant ($p < 0.05$), and # = trending (p= 0.053) differences between treatment groups (ANOVA). Abbreviations: Dm, medial part of the dorsal telencephalon; Dl, lateral part of the dorsal telencephalon; Vs, supracommissural nucleus of the ventral telencephalon; POA, preoptic area; Vc, central nucleus of the ventral telencephalon; TPp, periventricular nucleus of posterior tuberculum.

Mu-opioid receptor (MOR)

Gene expression of MOR did not differ interactively between sex and social system in any brain region, nor did it differ independently between sexes in any brain region (**Supplementary Table 4**). However, in several brain regions, MOR gene expression differed between social systems ($p < 0.05$ for each region), with both male and female pair bonded individuals expressing less than their solitary counterparts in the Dl, Vs, POA, and TPp (**Fig. 5A, B**; **Supplementary Table 4**). MOR receptor expression within the Vc trended lower in pair bonded individuals than in solitary counterparts; however, this was to a statistically non-significant extent ($p = 0.059$). MOR receptor expression within the Dm, Vd, and Vv/Vl did not differ between social systems (**Supplementary Table 4**). MOR gene expression was detected in all brain regions examined (ie., the Dl, Dm, POA, TPp, Vc, Vd, Vv/Vl, and Vs).

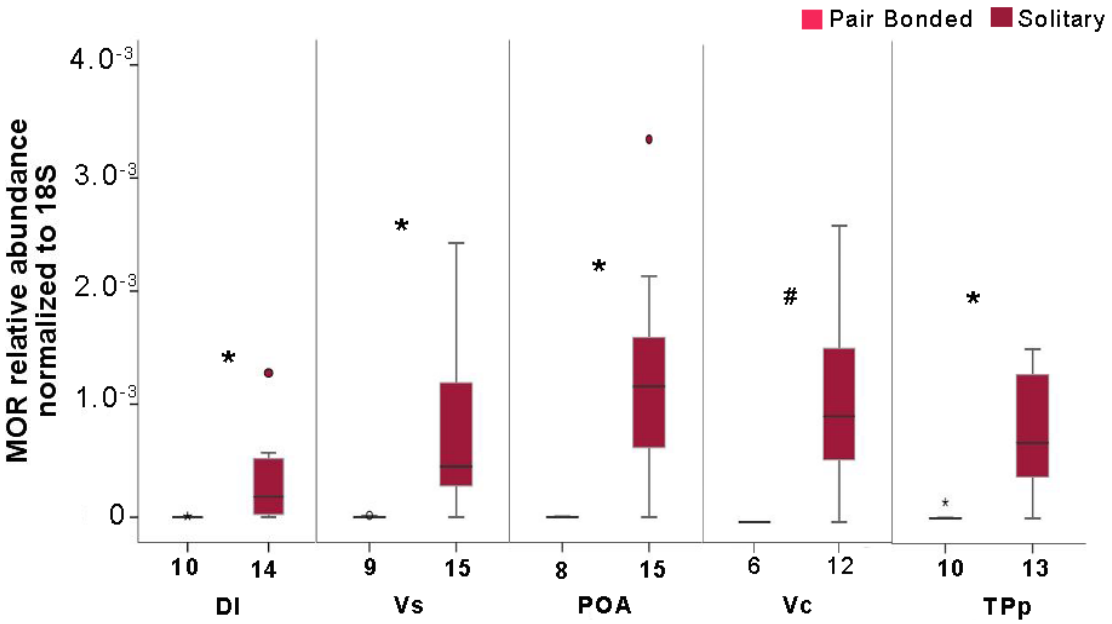

Figure 5. MOR gene expression differences between social systems of *C. lunulatus*. Boxes show the first and third quartiles, the black line in box represents median, and whiskers represent minimum and maximum value. Sample sizes are listed below each treatment group. * = statistically significant ($p < 0.05$), and # = trending ($p = 0.059$) differences between treatment groups (ANOVA). Abbreviations: Dl, lateral part of the dorsal telencephalon; Vs, supracommissural nucleus of the ventral telencephalon; POA, preoptic area; Vc, central nucleus of the ventral telencephalon; TPp, periventricular nucleus of posterior tuberculum.

Discussion

Nonapeptide circuitries of pair bonding in *Chaetodon lunulatus*

Our comparative analyses reveled that in females, paired individuals displayed higher ITR and V1aR receptor expression within the Vv/Vl than solitary individuals, indicating that ITR and V1aR signaling within the Vv/Vl might be important for mediating pair bonding for this sex. However, in males, receptor gene expression did not differ within any brain region between social conditions. Few other studies have explored the involvement of nonapeptides in teleost pair bonding, and they have been mostly on males. In male cichlids, *A. nigrofasciata*, a general nonapeptide receptor antagonist inhibits affiliation with a prospective partner and aggression towards non-partners[43], indicating the involvement both systems in pair bond formation. However, nonapeptide signaling does not appear to be involved in pair bond maintenance in males of this species[43,44]. In established pairs of *Neolamprologus pulcher* (but not of *Telmatochromis temporalis*) cichlids, whole brain gene expression of IT is positively correlated with partner affiliation[105]. Additionally, *N. pulcher* displays higher whole brain gene expression IT than the less affiliative *Telmatochromis temporalis*[105]. Similar to our study, Dewan et al. (2011) found in males of seven species of chaetodontids, Vv/Vl AVT-ir neuron fibre varicosity density predicts species-typical pairing from non-pairing sociality. Taken together, these studies indicate that while nonapeptides play a recurring role in promoting teleost pair bonding, this is species-, gender-, and context-specific.

What might be the precise functional role of Vv/Vl IT-ITR and AVT-V1aR signaling in promoting female *C. lunulatus* pair bonding? In teleosts, both AVT and IT mediate a wide

range of behavioral domains that that lack a universal valence and appear to be context specific[106]. AVT regulation of social behavior has been studied extensively, albeit almost exclusively in males, where it has functionally been shown to promote spawning[107-109], mate guarding or other forms of conspecific aggression/avoidance[43,108,110-115], social communication[116], social preference[117], and approach behavior[118]. Few studies have functionally examined IT involvement in teleost social behavior and again, those which have are exclusive to males. Similar to AVT, these studies demonstrate an inconsistent effect of IT on approach and avoidance behaviors, including pair bond formation and maintenance[43,44], social preference[117], and social affiliation[119]. However, since neurochemical effects are often specific to the site(s) of action[120,121] and sites are not confirmed in these studies; it is difficult to know which, if any, of these roles generate insight into the current findings. The rich literature on rodents, however, shows that in pair bonding species, AVP within the lateral septum (LS, the mammalian homolog of Vv/Vl) is involved in both partner affiliation[24] and territoriality[122], perhaps reflecting its broader role in social recognition/memory[123-128]. As with AVP, septal (including lateral septal) OT is essential for social recognition in rodents[128-131]. In pair bonding butterflyfish, both olfactory and visual cues are used for conspecific recognition[132] and are necessary to modulate relationships with partners, territorial intruders[133] and competitors for mates[134]. In teleosts, the ventral telencephalon is the major target of olfactory projections[135-137], but not of optic neurons, nor is it innervated with the optic tectum[138-140], the major brain region in which visual information is integrated and processed in vertebrates[141]. Taken together, we speculate that in *C. lunulatus* females, V1aR and ITR activation within the Vv/Vl might enhance conspecific recognition via olfactory perception[42]. This is certainly an intriguing area of further inquiry.

Convergent evolution with birds and mammals

The teleost, mammalian, and avian lineages share striking similarities in nonapeptide-mediated pair bonding circuitry. We have shown here that, as in other teleosts[42,43], AVT plays an important role in *C. lunulatus* pair bonding, and that its effects are likely exerted within the Vv/Vl through V1aR activation, mirroring the role of AVP in birds[142] and of AVP-V1aR binding within the LS (the mammalian homolog of Vv/Vl) in *M. ochrogaster* voles[24]. Similarly, fMRI studies show that in humans, activation of the septum, which is rich in AVP binding sites[143] is associated with "obsessive love"[144]. We have further discovered that, as in other teleosts, IT is important for *C. lunulatus* pair bonding, paralleling the functional involvement of OT in pair bonding *M. ochrogaster* rodents[21,23,145] and in non-human primates (i.e., marmosets, *Callithrix penicillata*[93]; tamarins, *Saquinus oedipus*[94]).

As with AVP, OT activity is also implicated in human pair bonding: intranasal OT in men within romantic partnerships increases preferred interpersonal distance from non-partner females[146,147], and plasma OT levels predict future success rates in romantic relationships[148]. However, unlike the AVP/AVT system, the site(s) of OT action in humans and *M. ochrogaster* rodents (ie. the prefrontal cortex (PFC), and nucleus accumbens (NAcc) (the mammalian homologue of the Vd)[21,149,150] are different than that of IT in teleosts (i.e., the lateral septum-like area). There are several potential explanations for this. First, since the evolutionary antecedent of the mammalian PFC is unclear[151,152], it couldn't be examined here. Secondly, and somewhat surprisingly, this study found no ITR expression in the NAcc/Vd at all, suggesting that this region is not important for pair bonding and social behavior in general in *C. lunulatus*. Alternatively, this could be an artifact of lack of tissue available for sampling. Given that ITR within the NAcc/Vd is expressed in teleosts[153] and that the NAcc/Vd is

considered a key node in the vertebrate SDM network, we suspect that the latter alternative is more plausible. Technical limitations notwithstanding, we might still expect anatomical targets of ITR/OTR-mediated pair bonding to be distinct between mammals and teleosts, due to differences in pre-existing neural circuitries that would have been available for co-option during their independent evolution. In mammals, a pre-established OT-mediated maternal bonding circuitry, in which the NAcc is a critical site of action[154,155] is thought to have been recruited during the evolution of pair bonding[156-159]. Since parental care did not precede the evolution of pair bonding in butterflyfishes[134], this pre-existing circuitry would have been unavailable for co-option in these organisms.

Dopaminergic circuitries of pair bonding in *Chaetodon lunulatus*

An essential component of pair bonding is the reinforcement of partner affiliation[11], which relies on individuals to perceive their partners as rewarding (i.e., approach eliciting) through heightened "salience"[32,160,161]. In pair bonding prairie voles, conspecific affiliation is not naturally rewarding, so does not facilitate pair bonding independently[11]. However, affiliation coupled with natural reward (specifically mating), is reinforcing and thus promotes pair bonding[162]. Therefore, mammalian pair bond formation is viewed to depend on conditioned reward learning, whereby individuals learn to associate their partner (conditioned stimulus) with mating (natural reward/unconditioned stimulus)[31,163-165]. The associative reward learning involved in this conditioned partner preference (CPP) is dependent upon dopamine acting upon nodes of the mesolimbic reward system--a neural network where the salience of environmental stimuli is evaluated[8,21,161]. Similar to pair bonding prairie voles, *in situ* partner removal experiments on *C. lunulatus* show that widowed males and females initially act antagonistically when approached by opposite sexed conspecifics within their territory. However, persistent "stalking" by the intruder towards the widowed individual while foraging accompanies the development of a new pair bond (Nowicki et al., manuscript submitted). Hence, *C. lunulatus* pair bonding might also rely on the learned association between partner (conditioned stimulus) and food (natural reward/unconditioned stimulus), and this associative learning might also be underpinned by dopamine acting upon reward circuitry. In support of this idea, in teleosts, both DA-D1R and -D2R binding are critical for psychostimulant/food reward learning[76,77,166-169] and a network structured very similarly to the amniote mesolimbic reward system has been identified[88,89]. Importantly, almost all of the brain regions that were associated with DA-mediated pair bonding in this study are nodes of this putative teleost mesolimbic reward system (POA notwithstanding).

Our comparative results revealed that in both male and female *C. lunulatus,* D2R gene expression differed in the TPp and Vc between pair bonded and solitary individuals. This suggests that DA-D2R signaling within these regions may be important for pair bonding in both sexes of this species. The mammalian homologs of these brain regions, namely the ventral tegmental area (VTA) and striatum (STR), comprise the central ascending dopaminergic innervation pathway in the mesolimbic reward system[89,170]. In mammals, this pathway appears to have been co-opted during the evolution of pair bonding in order to mediate partner reward learning[11,30]. In teleosts, the TPp seems to have the densest cluster of DA-synthesizing cell bodies in the brain[170]) and is considered the dopaminergic system ascending to the striatum[80]. Furthermore, DA-synthesizing neurons within the TPp are necessary for conditioned learning of place preference[77,171]. Given the aforementioned homologies and functional similarities, we tentatively hypothesize that DA ascending from the TPp and binding to D2Rs within the striatal Vc might function to mediate partner--

consumatory reward learning in a similar manner to the VTA-NAcc complex in mammals. Yet the hypothesis that the TPp is a major source of DA in *C. lunulatus* pair bonding does not explain why it appears to be a potential target of DA action in this species? Perhaps the TPp is both a source and a site of action in DA-mediation of pair bonding in *C. lunulatus.* This possibility might also apply for mammalian counterparts, because the VTA-mPFC complex within the mesocorticolimbic pathway is reciprocally innervated[172-174] and DA-synthesizing neurons within the VTA display a high density of dendrite D2 receptors[64].

Our comparative results revealed several other potential sites of DA action that are shared by D1R and D2R targeting. This is consistent with the ideas that D1R modulates D2R mediated events[175] and that D1- and D2R subtypes function complementarily to mediate pair bonding behavior[32]. Three of these implicated brain regions, the Dm, Dl, and Vs, belong to the putative teleost mesolimbic reward system, and mediate emotional learning/memory[81], relational/spatial/temporal memory[81] and aggression/spawning[88], respectively. The final brain region implicated, the POA, is a node of the conserved social decision making neural network, where it mediates several social domains across vertebrates, including sexual activity and male aggression[88,176-180]. In voles, in particular, the mPOA appears critical for several pair bonding behaviors, including pair bond formation[181], mating[180], mate guarding, and territorial defense[177,178,182]. mPOA-mediated pair bond formation and mating in particular are believed to be attributed to dopamine[182].

Hence, we propose that in *C. lunulatus*, D1 and D2 receptors might act synergistically within the Dm, Dl, Vs, and POA to mediate emotional, spatial/temporal, and sexual/mate-guarding mnemonic events involved in partner reward learning. Of final note, dopamine receptor expression within these brain regions was relatively *lower* in pair bonded fish than in solitary counterparts. This is somewhat contradictory, because since we hypothesize that DA binding promotes partner reward learning, signaling is expected to be relatively *higher* in paired fish. We offer two potential explanations for this. First, reduced DAR gene expression might reflect reduced receptor expression, which might act as a compensatory mechanism for heightened DA release[183]. Secondly, while gene expression often increases with the activity or abundance of protein products, it has also been shown to exhibit an inverse relationship[184], as has been previously shown in pair bonding *M. ochrogaster*[185]. Hence, it is possible that relatively lower DAR gene expression (and MOR gene expression, see below) reflects relatively higher levels of receptor abundance or activation in association with *C. lunulatus* pair bonding.

Convergent evolution with birds and mammals

The teleost, bird, and mammalian lineages share some prominent similarities in dopamine-mediated pair bonding circuitry. Our comparative results suggest that dopamine neurotransmission within the mesolimbic reward network is important for pair bonding in *C. lunulatus*, as appears to be the case in the zebra finch *T. guttata* [99,100,186,187] and in *M. ochrogaster* rodents[30-32]. A notable brain region of this network that appears to be targeted by DA in all three taxa is the striatal Vc/ striatal NAcc[31,32,100,186]. In addition, DA appears to act within the TPp (mammalian and avian VTA) and the POA in both *C. lunulatus* and *T. guttata*[99,186], but whether it targets these regions in mammals remains untested. Finally, our study further implicated that DA-D1R- and -D2R signaling within the Dm, Dl, and Vs might also regulate *C. lunulatus* pair bonding, but their involvement within homologous regions (i.e., the blAMY, HIP, and meAMY/BNST, respectively) remain untested in other taxa. Interestingly, however, a growing body of research implicates that DA targets similar regions of the

mesolimbic reward system to regulate partner affiliation in humans[188,189]. For example, functional magnetic resonance imaging (fMRI) shows that striatal regions, as well as the VTA, AMY, and the HIP, which are rich in dopamine activity[64], are activated differently when participants view images of those with whom they're in an intense romantic or long-term, deeply-loving relationship; than when viewing pictures of other familiar individuals[190-192].

Mu-opioid receptor circuitry of pair bonding in *Chaetodon lunulatus*

Mu-opioid receptor gene expression varied in relation to pairing sociality within the POA and several nodes of the mesolimbic reward system: the striatal Vc, Dl, Vs and TPp (however, in the striatal Vc this was to a statistically non-significant extent (p = 0.059). In teleosts, the POA mediates social and feeding behavior[88]. It is well established in mammals, that MOR plays an essential role in mediating the reinforcing effects of natural rewards (e.g., food, water, sex, social affiliation) and of psychostimulant rewards by eliciting motivational and pleasurable hedonic responses to these stimuli[68,193-205]. Preliminary investigations suggest that opioid and/or MOR action within mesolimbic reward system also mediates reward processing in teleosts[76,77].

In prairie voles in particular, the mu-opioid system plays a critical role in facilitating pair bond formation[35-37]. Its effects are exerted within striatal regions of the brain, including the dorsal striatum, dorsomedial NAcc shell, and caudate putamen[35-37]; where dorsal striatum MORs are believed to facilitate pair bond formation by promoting mating during CPP, and dorsomedial MORs are believed to do so by modulating the positive hedonics of mating during CPP[36]. (See section on dopamine for description of CPP cognitive process.) In teleosts, food is a natural reward whose reinforcing properties are modulated by the opioid system[76]. In *C. lunulatus,* exclusive pair-wise feeding strongly coincides with pair bond formation and maintenance (Nowicki et al., manuscript submitted). Hence, we hypothesize that OP-MOR binding within the POA and nodes of the mesolimbic reward system (i.e., the Vc, Dl, Vs, and TPp) promotes pair bonding in *C. lunulatus* by modulating the positive hedonics of natural consumatory reward during CPP learning. In further support of this idea, our comparative results revealed that the opioid and the dopaminergic systems appear to target several of the same nodes of the mesolimbic reward system (i.e., the Vc, Dl, Vs, and TPp), indicating that they might converge on these regions in order to underpin the learned association between consumatory reward affect and one's partner, respectfully, during the CPP process. Experimental research is needed to empirically test this hypothesis.

Convergent evolution with mammals

To date, potential involvement the opioid-mu-opioid system in pair bonding has only been examined in two species, *C. lunulatus* butterflyfishes (current study), *M. ochrogaster* voles[33,35-37,206]. In both organisms, it appears to play an important role, and effects seem to be exerted by acting upon nodes of the mesolimbic reward network. Specifically, we found comparative evidence that the striatal Vc -MORs are important in *C. lunulatus*, mirroring the role of striatal region (the NAcc and dorsal striatum) MORs in *M. ochrogaster*[36,37]. While several other nodes of the mesolimbic reward system (i.e., the Dl, Vs, TPp) and the POA were implicated in *C. lunulatus* pair bonding, the involvement of their homologs (i.e., the meAMY, LS, VTA and POA, respectively) in other taxa is yet to be explored functionally, so cannot be compared here. Interestingly, however, emerging evidence suggests that OP-MOR activity is important for pair bonding in humans as well, where here too its function is believed to be eliciting motivation and positive hedonics in response to romantic affiliation[207-209]. Moreover,

the implicated brain regions involved share some similarities with those implicated in *C. lunulatus* and established in *M. ochrogaster*. Specifically, fMRI studies suggest that in humans, motivational aspects of partner preference formation are regulated by the dorsal striatum[206], which is rich in MORs[210]. Whereas, the positive hedonics of "romantic love" are associated with the AMY, septal fornix, and VTA[190,192], all of which are also rich in MORs[211,212].

Working model for the neural network of pair bonding in fishes

By synthesizing our current findings with available information on teleost neurochemical synthesis and projection pathways, and functional insight from pair bonding *M. ochrogaster* counterparts, we can now begin to assemble a working model for how isotocin, arginine vasotocin, dopamine, and opioid systems might interplay to comprise a broader neural network of *C. lunulatus* pair bonding (as illustrated in **Fig. 6**). It is important to emphasize from the very onset that the only component of this working neural network model that derived from our findings is the involvement of IT-ITR, AVT-V1aR DA-DR, and OP-MOR signaling within brain regions (olfactory bulb notwithstanding), and that the remainder of this model is purely speculation.

We tentatively hypothesize that pair bonding in *C. lunulatus* relies on conditioned partner preference (CPP), as in the mammalian model, *M. ochrogaster*[11]. Several lines of behavioral evidence support this hypothesis. Field observations reveal that solitary *C. lunulatus* do not find prospective partners naturally rewarding (i.e., they respond to prospective partners by antagonism rather than approach). Only after continued and exclusive cohabitation involving pair-wise foraging, is a pair bond developed (Nowicki et al., manuscript submitted). After development, pair bonds are enduring, and are characterized by selective affiliation and feeding with partner, and selective antagonism towards non-partners (Nowicki et al., manuscript submitted). We propose that during CPP in *C. lunulatus*, individuals form a learned association between natural reward (food, unconditioned stimulus) and their new partner (conditioned stimulus) resulting in the new partner to take on rewarding properties, and thus reinforce selective approach behavior[8]. In our working model for this process, feeding activates the TPp (VTA), concurrently triggering OP-MOR and DA-D2R activity within the mesocorticolimbic reward system, which converges in the Vc (striatum), Dl (HIP), Vs (meAMY, BNST), TPp (VTA), and POA in particular, thereby modulating consumatory reward affect and reward learning/salience of partner-associated cues. In this pathway, the major source of DA projection to at least the Vc (striatum) is most likely the TPp (VTA)[44,64], while OP is most likely to originate from the hypothalamic nucleus lateralis tuberis[213]. Meanwhile, olfactory cues from the partner are transmitted from the olfactory bulb (OB), ultimately reaching the Vv/Vl (LS), where IT and AVT nonapeptide activity converges to promote olfactory learning in females. The source of nonapeptide release originates from cell bodies within the POA[214-216]. After pair bond formation, concordant D1R and D2R activity within the mesocorticolimbic reward system and POA modulates pair bond maintenance by mediating aggression towards non-partner conspecifics[206]. Also involved in this neural network would be higher-order motor circuits that underpin the behavioral outcome of approach and affiliation towards partner, and aversion towards non-partners (not studied nor illustrated here)[217]. This proposed working model is speculative, and requires empirical testing (see below).

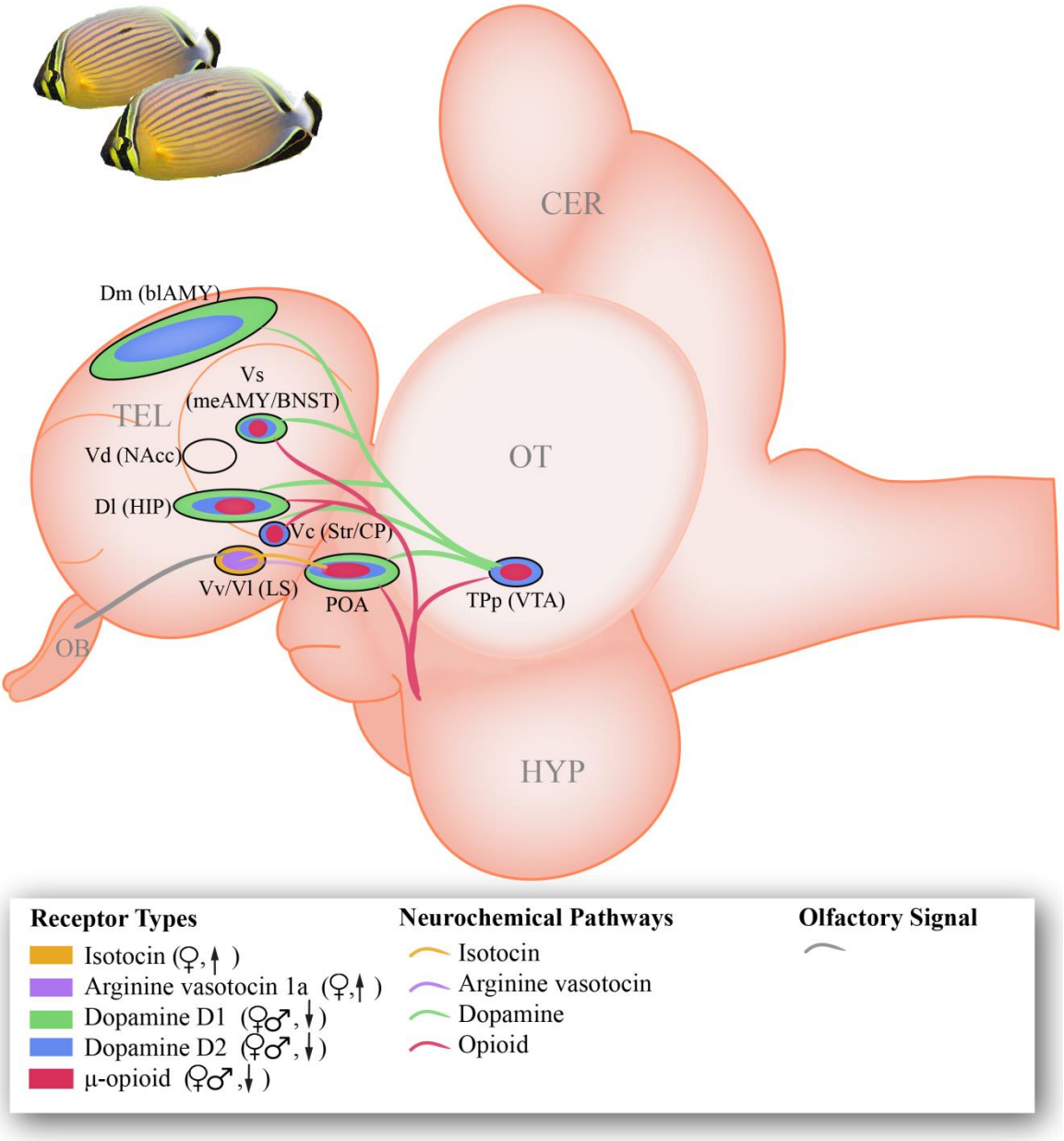

Figure 6. Sagittal view of brain illustrating a working neural network model for pair bonding in *Chaetodon lunulatus*. Colors within brain regions represent putative sites of action for each system based on comparative data on receptor gene expression provided here. Symbols and arrows indicate the sex(es) to which receptor phenotypes apply, and direction of receptor gene expression (up- or down-regulated) within brain regions, respectively. Colored lines represent putative neurochemical projections from predominant sites of synthesis (based on literature) to putative target sites (based on current findings). Illustration made by J.P.N., adapted from Dewan and Tricas, 2014, with permission.

Limitations and future directions

This is one of the first studies to explore the neurobiology of pair bonding in an early vertebrate (i.e., a reptile or an anamniote). While few other studies have researched the involvement of nonapeptides (i.e., teleosts: AVT:[42,43,105,218]; IT:[43,44]); that we are aware of, this is the first to research the involvement of the dopamine and opioid systems, and examine gene expression in specific brain regions. Although we provide support for the involvement of these neurochemicals, their conjugate receptors, and brain regions; our data are only correlative. Furthermore, due to the paucity in neural research on fish pair bonding, we have relied heavily on drawing upon the rich body of literature on the mammalian model, *Microtus ochrogaster,* in order to speculate the cognitive and behavioral functions that these putative neural circuits might subserve. Therefore, it is important to consider that our proposed neural network model of teleost pair bonding is far from conclusive and is certainly incomplete. Nonetheless, we believe it provides a useful foundation from which specific hypotheses related to teleost pair bonding can be tested in the future. A priority should now be to experimentally validate whether the neuroanatomical correlates of pair bonding found here are functionally relevant, and if so, then whether these functions are analogous to those of mammalian counterparts. Furthermore, we advocate exploring the potential involvement of other promising brain regions that are critical to vertebrate social behavior, including the periaqueductal gray/central gray (PAG/CG), ventral tuberal nucleus (vTn) (homologous to the mammalian anterior hypothalamus, (AH)) and anterior tuberal nucleus (aTn) (homologous to the mammalian ventromedial hypothalamus, (VMH))[88,91,92]. Moreover, similar preliminary investigations into the involvement of other likely candidate systems that modulate reward and positive reinforcement behavior (e.g., serotonin[10] and orexin[219]), negative reinforcement behavior (i.e., corticotrophin releasing factor[220,221]), and motor output (e.g., GABAergic and glutamatergic)[217]) are encouraged. Finally, in order to better understand the extent to which neurobiological systems of pair bonding have converged across vertebrate evolution, complementary research needs to be done on multiple species within and across all major taxonomic groups.

Conclusions

In addition to representing an integral part of the human experience[3-5,8], pair bonding has independently evolved in every major vertebrate lineage. It is already clear that this has not occurred through a compete and universal convergence of a single regulatory neural network. However, our study contributes to an emerging pattern that in at least selective cases, even those involving phylogenetically distant taxa with distinct evolutionary histories, this might occur through at least a partially converged neural network. *M. ochrogaster* rodents and *C. lunulatus* teleosts, despite being separated by ~450 million years of independent evolution[47], and despite having opposing parental evolutionary histories (parental vs. non-parental, respectively), appear to share some striking similarities in the neural substrates that underpin pair bonding. We have discovered evidence for the involvement of isotocin, arginine vasotocin, dopamine, and opioid systems in *C. lunulatus* pair bonding, corresponding to their (or their homologs) involvement in *M. ochrogaster* counterparts. Moreover, we have described that in association with pair bonding sociality, nonapeptide receptor expression varies in the lateral septum-like region, while dopamine and opioid receptor expression varies within other regions of the mesolimbic reward network, including the striatum; mirroring sites of action in *M. ochrogaster*. Therefore, we tentatively suggest that the neurobiology of pair bonding between these taxa has at least partially

converged through the repeated co-option of evolutionarily deep molecular and anatomical homologies that were already established in ancestral osteichthyes ~ 450 MYA. In order to determine the extent to which this has occurred across vertebrates, complementary studies across a wider range of lineages (most urgently amphibians and reptiles) are now needed.

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