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Expression Patterns of Neuroligin-3 and Tyrosine Hydroxylase across the Brain in Mate Choice Contexts in Female Swordtails

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Key Words

Mate choice · Swordtail · Brain · Synaptic plasticity · Amygdala · Hippocampus · Neuroligin · Tyrosine hydroxylase

Abstract

Choosing mates is a commonly shared behavior across many organisms, with important fitness consequences. Variations in female preferences can be due in part to differences in neural and cellular activity during mate selection. Initial studies have begun to identify putative brain regions involved in mate preference, yet the understanding of the neural processes regulating these behaviors is still nascent. In this study, we characterized the expression of a gene involved in synaptogenesis and plasticity *(neuroligin-3)* and one that codes for the rate-limiting enzyme in dopamine biosynthesis (*tyrosine hydroxylase*; TH1) in the female *Xiphophorus nigrensis* (northern swordtail) brain as related to mate preference behavior. We exposed females to a range of different mate choice contexts including two large courting males (LL), two small coercive males (SS), and a context that paired a large courting male with a small coercive male (LS). *Neuroligin-3* expression in a mate preference context (LS) showed significant correlations with female preference in two telencephalic areas (Dm and Dl), a hypothalamic nucleus (HV), and two regions associated with sexual and social behavior (POA and Vv). We did not observe any context- or behavior-specific changes in *tyrosine hydroxylase* mRNA expression concomitant with female preference in any of the brain regions examined. Analysis of TH and *neuroligin-3* expression across different brain regions showed that expression patterns varied with the male social environment only for *neuroligin-3*, where the density of correlated expression between brain regions was positively associated with mate choice contexts that involved a greater number of courting male phenotypes (LS and LL). This study identified regions showing presumed high levels of synaptic plasticity using *neuroligin-3*, implicating and supporting their roles in female mate preference, but we did not detect any relationship between *tyrosine hydroxylase* and mate preference with 30 min of stimulus presentation in *X. nigrensis*. These data suggest that information about potential mates is processed in select forebrain regions and the entire brain shows different degrees of correlated expression depending on the mate preference context.

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Introduction

Mate preferences are learned in a variety of species [Verzijden et al., 2012]. Perceptual differences in a variety of sensory modalities can alter neural activity patterns in the brain, which can facilitate learning or habituation of the signal [Choi et al., 2011; Town and McCabe, 2011; Yu et al., 2013]. Previous studies haves examined which central brain regions process sensory stimuli towards mate decisions in songbirds and frogs [Gentner et al., 2001; Hoke et al., 2004], and which brain regions [Desjardins et al., 2010; Wong et al., 2012] and dynamic gene cascades [Cummings et al., 2008; Lynch et al., 2012] are involved when female fish discriminate between potential mates. In order for females to remember and learn the location and qualities of previously sampled males, the underlying neural mechanisms should be plastic and subject to modification through reinforcement [Zupanc and Lamprecht, 2000; Pfaus et al., 2001]. Observing learned attractive stimuli or motivation to interact with such stimuli increases dopamine release [Wise, 2004; Salamone and Correa, 2012]. To this end, we evaluated the expression of genes associated with synaptic plasticity *(neuroligin-3)* and the dopaminergic signaling pathway (*tyrosine hydroxylase*, the rate-limiting enzyme for dopamine biosynthesis) across teleost brain regions in the social decision-making network (SDMN) [O'Connell and Hofmann, 2011] during female mate preference.

One mechanism of modulating neural plasticity involves the regulation of neuroligins (cell adhesion proteins which are critical in synaptogenesis and reinforce synaptic connections [Nam and Chen, 2005; Varoqueaux et al., 2006; Gutierrez et al., 2009; Soler-Llavina et al., 2011; Wright and Washbourne, 2011]). Neuroligins are functionally connected to social behavior in mammals [Wright and Washbourne, 2011] and their mRNA expression patterns are related to sensory perception in invertebrates [Biswas et al., 2010; Hunter et al., 2010]. While there are many potential markers for synaptic plasticity, we have demonstrated that whole-brain *neuroligin-3* expression is associated with female mate preference in the northern swordtail fish *(Xiphophorus nigrensis)* [Cummings et al., 2008; Lynch et al., 2012; Ramsey et al., 2012]. Hence, this gene can serve as a proxy for synaptic plasticity that is relevant to female mate choice.

Swordtail fish belong to a larger family of internal fertilizing and live-bearing fish, Poecillidae, which are a useful system for studying the evolution of female mate choice [Houde, 1997; Ryan and Rosenthal, 2001]. In *X. nigrensis*, females prefer larger size class males over the small size class males that use forced copulation tactics [Ryan et al., 1990; Cummings and Mollaghan, 2006], and the strength of this preference changes with female age [Wong et al., 2011]. Experience-dependent changes in female mate preference have also been demonstrated in other poeciliid species [Morris et al., 2006; Rios-Cardenas et al., 2007; Verzijden and Rosenthal, 2011], suggesting that learning is a common component of mate choice across poeciliids. Swordtail females only need visual information to display these preferences [Ryan and Rosenthal, 2001], and swordtail preferences in the laboratory lead to actual copulation in both the lab and the wild [Ryan et al., 1990; Walling et al., 2010]. Among large males, females prefer those with visible UV ornamentation [Cummings et al., 2003]. Female preference is not predicted by the reproductive state but is weakly and negatively correlated with a proxy for circulating estradiol levels [Ramsey et al., 2011]. We have identified genes associated with female mate preference (e.g. *neuroserpin* and *neuroligin-3*) in the whole brain through microarray and quantitative real-time PCR analyses that show predictive patterns of expression with variation in *X. nigrensis* female preference across 30-min behavioral trials [Cummings et al., 2008; Lynch et al., 2012; Ramsey et al., 2012]. Subsequent experiments localizing the expression of *neuroserpin* have identified possible brain regions involved in the processing of female mate choices [Wong et al., 2012]. Given these characteristics, *X. nigrensis* represents a promising system to evaluate the neural and molecular mechanisms underlying female mate preference without the confounds of reproductive state and physical contact [Cummings, 2012].

The localization of the specific brain regions that underlie female mate choice behavior in teleosts is only beginning to be understood [Desjardins et al., 2010; Wong et al., 2012]. Identifying areas that show a greater molecular activity of synaptic plasticity will give insight into the specific processes that occur in relevant brain regions underlying mate choice. A variety of social behaviors have been implicated in brain regions composing the SDMN [O'Connell and Hofmann, 2012]. This network of brain regions (SDMN) consists of areas involved in the processing of social information and the reinforcement of positive experiences (e.g. the mesolimbic reward system). In this study, we exposed female *X. nigrensis* to different sets of mates and social stimuli (e.g. ornamented courting male phenotypes and non-ornamented coercive male phenotypes or other females) to identify the brain regions associated with mate assessment. To identify important regions in mate preference, we related the expression of *neuroli-*

gin-3 (synaptic plasticity marker) to variations in female mate preference. Similarly we assessed whether changes in the transcript abundance of the rate-limiting enzyme in dopamine biosynthesis *(tyrosine hydroxylase)* are simultaneously associated with variations in female *X. nigrensis* mate preference. We focused on a subset of brain regions in the SDMN implicated in learning and reward [O'Connell and Hofmann, 2012; Wong et al., 2012]. We predicted that *neuroligin-3* expression would be context dependent and significantly correlated with female preference in a putative mate choice network, as has been shown for another synaptic plasticity gene, *neuroserpin* [Wong et al., 2012]. Furthermore, we explored whether the initial stages of dopaminergic signaling pathways, specifically the rate-limiting enzyme for dopamine biosynthesis (TH), exhibit any context-dependent expression patterns associated with behavior over short time scales (30 min).

Materials and Methods

Animals

Our study subjects consisted of 48 sexually mature female *X. nigrensis* that were either wild caught or the progeny of wild caught individuals maintained in seminatural conditions at the Brackenridge Field Laboratories of the University of Texas at Austin (Austin, Tex., USA). When brought to the laboratory for testing, females were housed in mixed-sex tanks for at least 1 month. All experimental females were then sexually isolated for at least 2 weeks. All fish were fed ad libitum daily and kept on a 10:14-hour light:dark cycle. All experimental procedures in this study were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin (protocol No. 07110101).

Behavioral Paradigm

We measured each female's mating preference employing a 2-way choice experimental design using established behavioral measures and natural lighting conditions for this species [Cummings et al., 2003, 2008; Wong et al., 2011, 2012]. The experimental tank was subdivided into three sections. Stimuli fish were placed behind non-perforated UV transparent barriers on each end of the experimental tank unless otherwise noted. The center section of the experimental tank was further subdivided into three 24-cm regions comprised of two association zones adjacent to the barriers and the centermost region termed the neutral zone. Immediately prior to the behavior trials, we measured the excreted estradiol, a proxy for circulating estradiol levels, of each female using a noninvasive waterborne assay to assess associations between estradiol and gene expression (see below). Afterwards, we placed the females in the experimental tank and recorded the behavioral activity for 30 min. During this time, females were allowed to interact with either stimulus by swimming into the adjacent association zones or remain in the neutral zone. Halfway through the trial, we switched the sides of the fish stimuli (the exception being the LL group, see below) in order to avoid confounding side bias with preference.

Females were exposed to one of five conditions: two size-matched large males (LL, n = 10), with one male behind a UV pass filter and the other behind a UV blocking filter (the UV filters, and not the males, were switched half way through the trial); one large and small male (LS, n = 13); two size-matched small males (SS, n = 7); two size-matched females (FF, n = 12), or collection from their home tank (HT, n = 6), a treatment which served as an asocial control group (females are housed in isolation). Home tank females underwent estradiol measurements as in the other groups but were then returned to their home tanks for 30 min prior to sacrifice. The UV pass and blocking filters in the LL trials allowed the females to discriminate between two attractive males based on a feature other than size in this more complex condition. We selected our three male-exposure groups to represent a presumed gradient in mate choice complexity and sensory stimulation, with the LL treatment group representing a relatively complex mate preference environment with high sensory stimulation (two preferred phenotypes varying in the presence or absence of UV ornamentation and presumably having the highest incentive value). The LS treatment group represented a simple mate preference environment with a presumably intermediate level of sensory stimulation and incentive value. As previous studies have shown that a small proportion of females display a preference for small males [Ramsey et al., 2011, 2012; Wong et al., 2011], the SS treatment group represents a presumably minimum mate preference environment with the lowest incentive value and sensory stimulation (two small males that employ forced copulation strategies only). All size-matched stimuli differed by no more than 1 mm in standard length (the length from the tip of the snout to the caudalmost part of the caudal peduncle).

For each female, we measured the number of glides (a proxy for receptivity, which can precede copulatory events with any male [Liley, 1965; Cummings and Mollaghan, 2006]), transits to the center (a proxy for general locomotor activity), and association bias (a common measure of preference in poecillid fish [Ryan and Rosenthal, 2001; Walling et al., 2010; Wong et al., 2011]). As we were interested in preference regardless of stimulus, we defined association bias as the amount of time spent with stimulus *a* (where the time spent with stimulus *a* was greater than the time spent with stimulus *b*) divided by the total amount of time spent in the association zones of both stimuli. Arbitrarily assigning the preferred stimulus by size (e.g. larger vs. smaller) or UV ornamentation (e.g. UV pass vs. block) does not change the behavioral results (data not shown).

Estradiol Measurements

To account for a potential relationship between estradiol levels and *tyrosine hydroxylase* and *neuroligin-3* expression, we quantified a proxy for circulating estradiol levels. We measured estradiol levels for all females using a noninvasive waterborne assay following an established protocol for teleosts [Scott et al., 2008; Kidd et al., 2010] and has been validated in our focal species [Ramsey et al., 2011]. Briefly, females were placed in 150 ml of reservoir water (treated tap water used for home and experimental tanks) for 1 h prior to the behavior trials. Estradiol was extracted from the water using C18 solid-phase extraction columns (Sep-Pak Plus C18 cartridge, 55–105 lm; Waters Corporation, Milford, Mass., USA) and measured on a Correlate-EIA 17β-estradiol enzyme immunoassay kit (Assay Designs) according to the manufacturer's protocol.

Hormone samples were run in duplicate on three 96-well EIA assay plates; the interassay CV was 6.5% and the intra-assay CV was 1.9%.

Cloning Tyrosine Hydroxylase and Neuroligin-3

While there are two tyrosine hydroxylase genes in teleosts (TH1 and TH2 [Yamamoto et al., 2010]), we cloned TH1 (referred to here as TH) because it is more widely expressed across the brain [Filippi et al., 2010; Yamamoto et al., 2010]. Further, TH2 encodes for tryptophan hydroxylase [Ren et al., 2013]. To create a *tyrosine hydroxylase* (TH) probe for in situ hybridization (see below), we first cloned a fragment of the gene in *X. nigrensis* using methodology described elsewhere [Cummings et al., 2008]. Briefly, after isolating total RNA from whole-brain homogenates, we synthesized cDNA according to the manufacturer's protocol (SuperScript First-Strand Synthesis; Invitrogen). Using CODEHOP (http://blocks.fhcrc.org/codehop.html), we generated the degenerate primers 5′-TGGATCTTCAGGGGGTTGTCNARNACYTC-3′ and 5′-GGCAGTCCCTGATCGAGGAYGCNMGNAA-3′ to amplify a 1,268-bp TH fragment. The reaction conditions were 1 denaturing cycle (94°C for 2 min) followed by 30 amplification cycles (94°C, denaturing for 30 s; 61°C, annealing for 90 s, and 72°C, elongation for 1 min) and a final 10-min elongation cycle (72°C). After amplification, we ligated the fragment into the pCR4-TOPO vector and transformed into One Shot chemically competent cells according to the manufacturer's protocol (TOPO TA Cloning Kit for Sequencing with One Shot TOP10 Chemically Competent *Escherichia coli*; Invitrogen). We subsequently verified the sequence identity (gene accession No. HM107109.1). We used our previously cloned *neuroligin-3* (gene accession No. DQ835282) template from a previous study [Cummings et al., 2008].

Tissue Processing

We cryosectioned female brains at a 16-μm thickness into 4 serial series. All series were postfixed in cold 4% paraformaldehyde/PBS solution, washed in PBS, and acetylated in 0.25% acetic anhydride/triethanolamine. Subsequently, slides were washed in 2× standard saline citrate, dehydrated in increasing ethanol series, and stored at –80°C. All slides in a series were processed simultaneously. Using a digoxigenin (DIG)-labeled riboprobe (see below), one series was used to localize and quantify *neuroligin-3* expression and an adjacent series was used to localize and quantify *tyrosine hydroxylase* expression. The other series were used in a different experiment [Wong et al., 2012].

Probe Synthesis

DIG-labeled *neuroligin-3* and TH riboprobes were generated using a 1:3 ratio of UTP and DIG-UTP (Roche) following a modified manufacturer's protocol (Megascript T7/T3, Ambion). A 393-bp TH DIG probe template (gene accession No. HM107109.1) was subcloned into a pCR4-TOPO vector (Invitrogen) using the primer pair 5′-TTTGAGGAGGAGGACGGAAAAG-3′ and 5′-TCTTCTCTGTCTGTAGGCAGGGTC-3′. The 345-bp *neuroligin-3* probe template was subcloned using the primer pair 5′-CCAGATGACATCCCTCTGATGACC-3′ and 5′-GTGCTG TATGGACTCATGTTGGAG-3′ on the *X. nigrensis neuroligin-3* transcript (gene accession No. DQ835282). After probe synthesis, we removed the unincorporated nucleotides via column filtration according to the manufacturer's protocol (Megaclear, Ambion) and checked the probe quality through gel electrophoresis.

In situ Hybridization

We used an established DIG in situ hybridization protocol [Wong et al., 2012]. Briefly, we first prehybridized the slides for 6 h at 60°C in a hybridization chamber. The slides were then hybridized overnight (16 h) at 65°C with fresh prehybridization solution containing 0.25 ng of antisense riboprobe per slide in hybridization chambers. Following hybridization, the slides were washed to remove nonspecific probe binding and then incubated overnight at 4°C with Anti-Digoxigenin-AP antibody (Roche). After antibody incubation, we blocked endogenous alkaline phosphatase activity and used NBT/BCIP stock solution (Roche) for colorimetric detection. After stopping the colorimetric reactions (3 h for *neuroligin-3* and 20 h for TH), we progressively dehydrated sections in ethanol washes and finally coverslipped the slides with Permount adhesive (Fisher). Within each series, all individuals that were compared in the analyses for each gene were processed in a single in situ hybridization to avoid any potential colorimetric development differences across individuals due to batch effects [for technical details, please see Wong et al., 2012]. DIG-labeled *neuroligin-3* and *tyrosine hydroxylase* sense riboprobes showed no expression.

Gene Expression Quantification

Using an *X. helleri* brain atlas for reference and terminology [Anken and Rahmann, 1994], we identified and quantified DIG-labeled *neuroligin-3* riboprobe expression in 9 brain regions. Those regions are (teleost nuclei: abbreviation, putative tetrapod homolog) [Bruce and Bradford, 2009; Forlano and Bass, 2011; O'Connell and Hofmann, 2011]: cerebellum (Cb, cerebellum), area dorsolateralis telencephali (Dl, pallial hippocampus), area dorsomedialis telencephali (Dm, basolateral amygdala), central gray (GC, periaqueductal gray), hypothalamus ventralis (HV, ventral hypothalamus), nucleus preopticus (POA, preoptic nucleus), nucleus tuberis anterioris (TA, ventromedial hypothalamus), ventralis supracommissuralis telencephali (Vs, medial amygdala), and area ventroventralis telencephali (Vv, lateral septum). For *tyrosine hydroxylase*, we identified the expression in the following 8 brain regions previously demonstrated to contain TH in other teleosts [Parafati et al., 2009; Filippi et al., 2010; Yamamoto et al., 2010; O'Connell and Hofmann, 2011]: olfactory bulb (OB, olfactory bulb), area ventroventralis telencephalic (Vv, partial homologue to the nucleus accumbens), area centroventralis telencephalic (Vc, striatum), nucleus preopticus (POA, preoptic nucleus), nucleus pretectalis periventricularis pars ventralis (PPv, pretectal nucleus), nucleus periventricularis tuberculum posterioris (TPp, partial homologue to the ventral tegmental area), nucleus tuberis posterioris (PTN), and isthmus nucleus (is, locus coeruleus). The isthmus nucleus contains a dense population of noradrenergic neurons and was only included for completeness [Kaslin and Panula, 2001; Filippi et al., 2010; Yamamoto et al., 2010]. While TH is the rate-limiting step in catecholamine synthesis [Levitt et al., 1965], none of the brain regions examined express a marker for noradrenalin (except the isthmus nucleus) but they do contain dopamine immunoreactive cell bodies in other teleosts [Filippi et al., 2010; Yamamoto et al., 2011]. Hence, it is likely TH expression in all areas measured, and not noradrenalin, leads to dopamine synthesis. After tissue processing, the final sample sizes were: LL, n = 10; LS, n = 10; SS, n = 5; FF, n = 9, and HT, n = 5.

DIG Quantification. For both genes, we quantified expression by measuring the optical density (OD) of the DIG-labeled probes,

which has been validated as a semiquantitative measure of gene expression in the focal species [Wong et al., 2012]. For each slide, we normalized the mean intensity of all measures to the background (the mean intensity of slide not containing the tissue), producing a value for the fractional transmittance of the brain region in each section. Fractional transmittance was mathematically converted to OD using the equation OD = 2 – log(fractional transmittance), which was derived specifically for the imaging setup (Nikon Eclipse 80i) in our laboratory using neutral density filters of 0, 8, and 32. Using NIS Elements image analysis software (Nikon), we measured the OD of *neuroligin-3* and *tyrosine hydroxylase* expression across individuals based on a standardized portion of each brain region (ranging from 1,737 to 29,152 μm^2 depending on the size of the brain region of interest).

Brain section images were captured at a magnification of ×4 with a Nikon 12-bit 2-megapixel monochrome camera (DS-2MBWc). For each brain region, we used Nikon NIS Elements 2.3 to measure a standardized rectangular box (the area of the box is given after each brain region; see below) within the borders of each brain region and measured the mean intensity of *neuroligin-3* or TH expression within the box. Unless otherwise stated, the measuring box was always placed in the middle of the brain region on the dorsal-ventral plane. We measured the mean intensity in both hemispheres if available and then averaged the values for the section. For all brain, regions we then proceeded to measure the mean intensity for all sections of the individual in which we could identify the desired brain region and then calculated the average for that individual. Depending on the size of the brain region, the number of sections averaged per individual ranged from 2 to 8 consecutive sections. Consecutive sections were 48 μm apart. For *neuroligin-3* specifically, we measured: Dl (19,906 μm^2) and Dm (19,906 μm^2) for sections from the rostralmost section until the disappearance of the anterior commissure; Vv (5,877 μm^2) and Vs (5,877 μm^2) for sections only containing the telencephalic ventricle; POA (2,843 μm^2) for sections with an anterior commissure present until the appearance of the optic tectum; HV (1,737 μm^2) from the pituitary until the lateral extension of the lateral hypothalamus; TA (5,339 μm^2) for 3 sections preceding the lateral extension of the dorsal hypothalamus, and Cb (29,152 μm^2) and CG (7,298 μm^2) for all sections observed (see Gene Expression Quantification for brain region abbreviations). For *tyrosine hydroxylase*, we measured: OB (17,983 μm^2) for all sections observed; Vv (4,599 μm^2) for all sections only containing the telencephalic ventricle and one section preceding the appearance of the ventricle; Vc (4,599 μm^2) from the appearance of the telencephalic ventricle until the disappearance of the anterior commissure; POA (4,134 μm^2, the box was placed in the most medial and ventral part of the region) for sections with an anterior commissure present until the appearance of the optic tectum; PPv (2,716 μm^2) and TPp (2,716 μm^2) for all sections containing the posterior commissure, PTN (6,564 μm^2) for all sections observed until the disappearance of the torus longitudinalis, and is (1,486 μm^2) for all sections observed. All image capturing and data collection was performed by a single person (R.Y.W.) who was blind to the treatments.

Statistics

All statistics were performed using SPSS (version 18) and network statistics were conducted using Ucinet [Borgatti et al., 2002]. To examine group-wide behavioral and gene expression differences between individuals expressing high (>median) versus low (<median) behavioral patterns within each treatment group, we used a t test. Due to the small sample size (n = 5), we did not perform any statistical tests involving high and low behavioral patterns in the SS group. To account for multiple hypothesis testing, we used a Benjamini-Hochberg correction [Benjamini et al., 2001]. To assess relationships between individual variations in preference, gene expression, or estradiol levels, we used Pearson's correlation when the data was normal and Spearman's correlation when the data was nonnormal (glides and transits).

We characterized *neuroligin-3* and TH network expression patterns by looking at pairwise correlations of gene expression between brain regions in specific social contexts. To analyze network patterns, we converted all Benjamini-Hochberg-corrected significant correlations of *neuroligin-3* and TH expression between brain regions into binary values in an association matrix. Significant relationships had a value of 1 while nonsignificant values were designated 0. We compared the densities [Bullmore and Sporns, 2009] of networks across conditions using UCINET [Borgatti et al., 2002] as in the study by Wong et al. [2012]. For each mate preference group (LL, LS, and SS), we defined unique correlations as those that were not also significant in the FF or HT groups.

Results

Behavior

As reported elsewhere [Wong et al., 2012], females exposed to the LS treatment condition showed a clear preference for the large male by spending a significantly greater amount of time in the association zone next to the large male relative to the small male ($t = 8.4$, $p = 1.1 \times 10^{-7}$; online suppl. fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000360071). Females did not differ in terms of the time spent next to the larger stimulus in the other test groups [two females, (FF, $t = 0.84$, $p = 0.4$) two small males (SS, $t = 0.12$, $p = 0.9$) (online suppl. fig. 1) or the large male with visible UV ornamentation (LL, $t = 0.75$, $p = 0.45$) (online suppl. fig. 1)]. There were no significant differences in association bias ($F = 1.7$, $p = 0.18$; fig. 1), the number of glides ($\chi^2 = 1.36$, $p = 0.71$) or transits ($\chi^2 = 4.69$, $p = 0.19$) across treatment groups. There were no significant correlations between association bias and glides in any treatment group (online suppl. table 1). For the LS females only, there was a significant correlation between transits and estradiol levels (online suppl. table 1), which is consistent with previous findings in this species [Ramsey et al., 2011].

Neuroligin-3 Expression

Of all the treatment groups tested, only the females exposed to the LS environment showed a differential expression in *neuroligin-3* OD that was matched by differences in association bias (fig. 2; table 1). Specifically, the

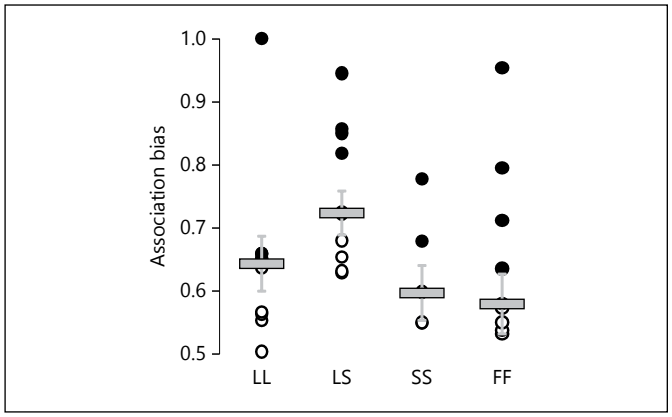


Fig. 1. Association bias towards the preferred stimulus in each treatment group. Bars represent the median with standard error. Black and white circles represent high- (>median) and low (<median)-preference females, respectively.

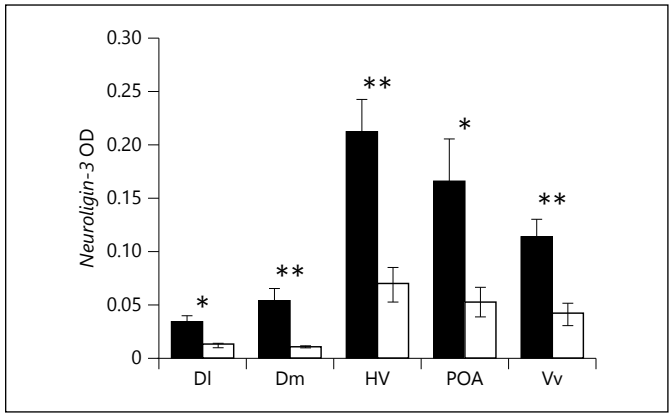


Fig. 2. *Neuroligin-3* expression (mean ± SE) in females exposed to a large vs. a small male (LS) context. Differences in *neuroligin-3* expression between groups of high-association-bias (>median) females (n = 5, black) and low-association-bias (<median) females (n = 5, white) for LS females. * $p < 0.05$, ** $p < 0.01$.

Table 1. *Neuroligin-3* OD (mean ± SE) comparisons between females with high (>median) and low (<median) association biases

Brain region	LL			LS			FF		
	high	low	p value	high	low	p value	high	low	p value
Dm	0.028±0.009	0.014±0.003	0.19	0.053±0.012	0.011±0.001	0.009[a]	0.038±0.014	0.027±0.013	0.58
Dl	0.026±0.01	0.012±0.002	0.23	0.033±0.006	0.012±0.002	0.01[a]	0.026±0.009	0.024±0.011	0.87
Cb	0.081±0.025	0.035±0.014	0.16	0.087±0.028	0.043±0.014	0.21	0.06±0.024	0.093±0.036	0.48
GC	0.008±0.001	0.004±0.0004	0.11	0.007±0.001	0.004±0.0008	0.19	0.005±0.0008	0.01±0.003	0.19
POA	0.104±0.056	0.03±0.004	0.22	0.165±0.036	0.052±0.014	0.023[a]	0.142±0.065	0.101±0.029	0.58
TA	0.078±0.036	0.026±0.011	0.21	0.085±0.014	0.047±0.009	0.05	0.09±0.038	0.096±0.028	0.91
VH	0.127±0.06	0.054±0.019	0.28	0.213±0.03	0.069±0.015	0.002[a]	0.174±0.065	0.222±0.084	0.66
Vs	0.106±0.042	0.04±0.012	0.16	0.142±0.04	0.055±0.011	0.07	0.093±0.046	0.061±0.023	0.56
Vv	0.106±0.042	0.034±0.014	0.14	0.114±0.016	0.041±0.01	0.005[a]	0.1±0.049	0.064±0.028	0.55

[a] Significant after correcting for multiple hypotheses.

neuroligin-3 OD was significantly higher in Dl, Dm, POA, Vv, and HV for females showing a high preference relative to those showing a low preference after multiple hypothesis correction (fig. 2; table 1). We did not observe any significant differences in *neuroligin-3* OD between high glide or transit females and low glide or transit females in any other brain region or context after correcting for multiple hypothesis testing (online suppl. table 2). All of the five brain regions that showed within-group differences in preference for LS females also showed a significant and positive correlation between *neuroligin-3* OD and association bias (Dl, $r = 0.65$, $p = 0.04$; Dm, $r = 0.68$, $p = 0.02$; HV, $r = 0.82$, $p = 0.003$; POA, $r = 0.90$, $p = 0.0005$, and Vv, $r = 0.8$, $p = 0.004$; fig. 3) after controlling for false discovery rates. Interestingly, when looking at these specific brain regions in other social treatments, a significant positive correlation between *neuroligin-3* OD and association bias was only observed in females exposed to two small males (SS) in Dl ($r = 0.88$, $p = 0.047$), HV ($r = 0.96$, $p = 0.007$), and Vv ($r = 0.95$, $p = 0.01$). The estradiol levels measured from the water did not correlate with *neuroligin-3* expression in any brain region and context (online suppl. table 1).

Tyrosine Hydroxylase Expression

As expected, tyrosine hydroxylase was expressed in all brain regions examined (fig. 4). When examining the relationship between TH expression and social contexts,

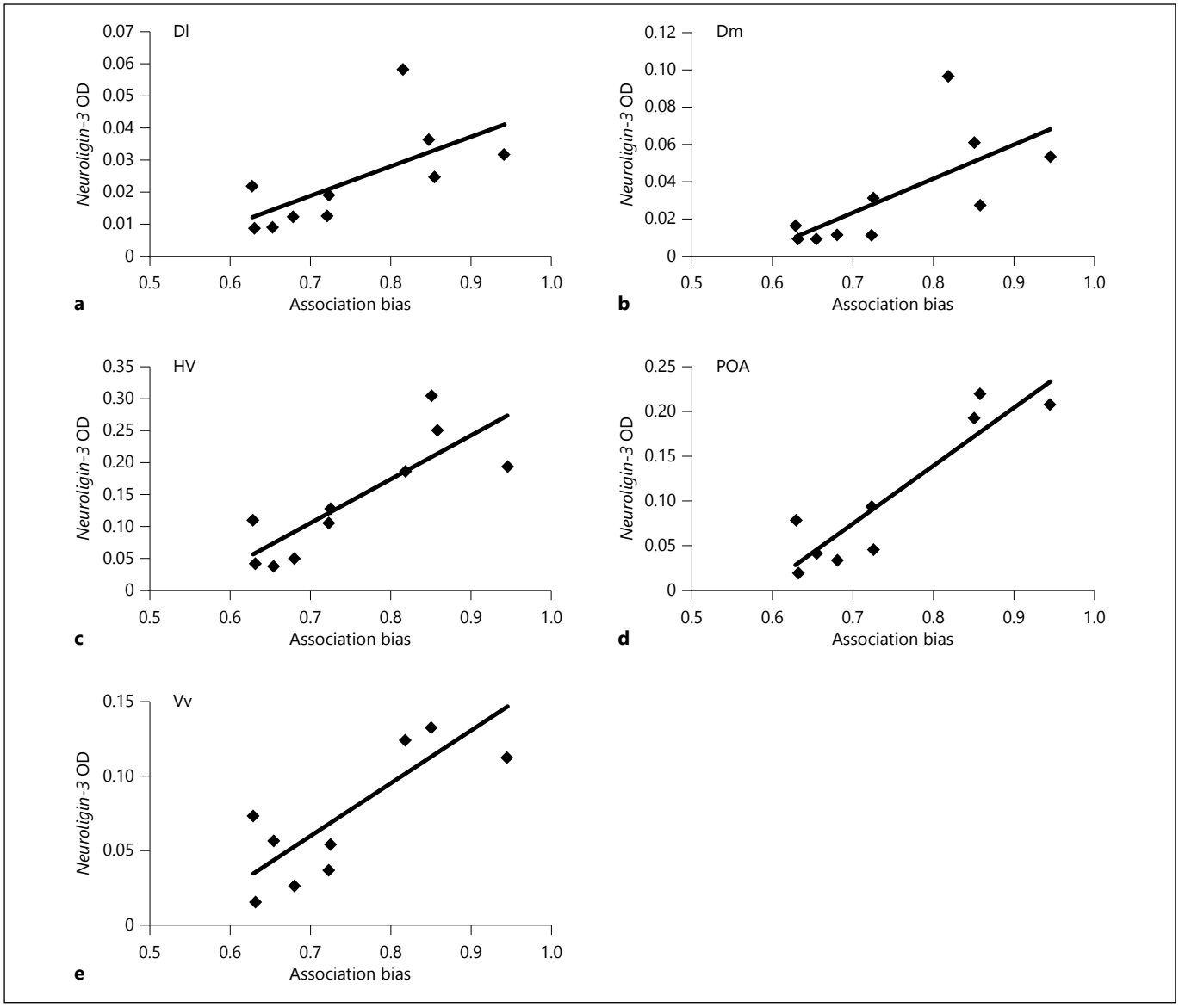
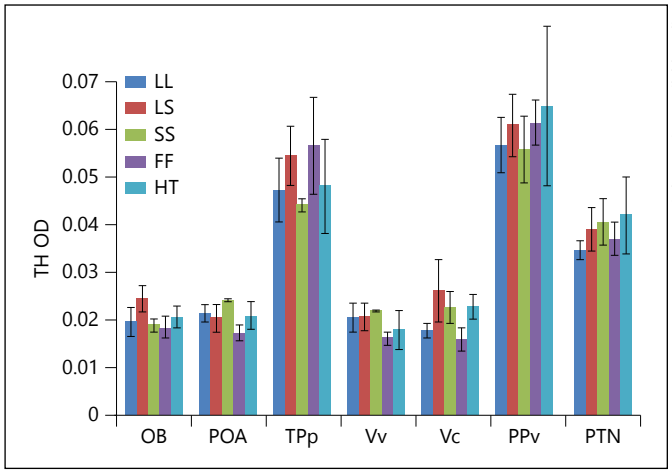


Fig. 3. Individual variation of association bias and *neuroligin-3* expression in LS-exposed females. Significant correlations between individual variation in association bias and *neuroligin-3* expression in: Dl (dorsolateral part of the dorsal telencephalon) (**a**), Dm (dorsomedial part of the dorsal telencephalon) (**b**), HV (ventral hypothalamus) (**c**), POA (preoptic area) (**d**), and Vv (ventral part of the ventral telencephalon) (**e**).

there were no significant differences in TH OD in the any brain region across treatment groups ($F > 0.1$ and $p > 0.1$ for all brain regions; fig. 4). Looking within each treatment group, we also did not observe any differences in TH OD between high-preference females (>median) and low-preference females (<median) in any context (online suppl. tables 3, 4). We also did not observe any significant relationship between TH expression and association time, glides, or locomotor activity after multiple hypothesis correction (online suppl. table 4). Of the two brain regions showing within-group level differences in TH OD for glides and transits in FF females, only the POA showed a significant correlation between glides and TH OD ($r = -0.80$, $p = 0.008$). Furthermore there were no significant correlations between estradiol excreted in the water and association bias, glides, transits, or TH OD in any brain region or context after correcting for multiple hypothesis testing (online suppl. table 1).



Color version available online

Fig. 4. *Tyrosine hydroxylase* expression (mean ± SE) in select brain regions across treatment groups. Blue, red, green, purple, and teal represent LL, LS, SS, FF, and HT, respectively (colors only in online version).

Neuroligin-3 and Tyrosine Hydroxylase Expression Patterns

We examined the density (e.g. the number of significant correlations) of *neuroligin-3* and *tyrosine hydroxylase* expression patterns across brain regions to assess the complexity of the correlation network across the brain for each gene. Focusing on the unique *neuroligin-3* correlations within each male preference group (LL, LS, or SS), we did not find any pairwise correlation that was consistent across all three groups; however, the LS- and LL-exposed females shared many of the same significant correlations (fig. 5). Furthermore, there was a significantly higher density (i.e. the total number of significant correlations between brain regions) for females exposed to LL relative to the LS ($t = 2.27$, $p = 0.013$) and SS ($t = 5.65$, $p = 0.0002$) groups. The *neuroligin-3* network density for females exposed to LS was significantly higher than for those exposed to SS ($t = 3.9$, $p = 0.0002$). In contrast, TH expression showed very little unique correlated expression across brain regions in any context (online suppl. fig. 2). Of note, after correcting for multiple hypotheses testing, we observed only one significant correlation of TH expression and brain regions across all contexts: Vv and OB in the LS environment.

Discussion

In females that display a mate preference, whether innate or learned, theory presumes a comparison among multiple mates to maximize reproductive success. Recent research in many vertebrate taxa indicates that female mate choice decisions vary with experience [for reviews, see Ronald et al., 2012; Verzijden et al., 2012], suggesting that some form of learning or recall is involved in the mate choice process. As such, identifying brain areas of increased synaptic plasticity in a mate choice context can help us identify the neural mechanisms of female mate choice. In this study, we identified five brain regions (Dl, Dm, HV, POA, and Vv) associated with the SDMN [O'Connell and Hofmann, 2012] that showed significant differences in a marker for synaptic plasticity *(neuroligin-3)* between females showing high and low mate preferences. Furthermore, we observed an increase in *neuroligin-3* coexpression patterns across brain regions with increasing complexity and/or increasing signal salience of the mate choice environment. While motivations to act are influenced in part by dopaminergic signaling, we did not find evidence of a relationship between *tyrosine hydroxylase* expression and the strength of female mate preference following our 30-min behavioral assays. In conjunction with findings from previous studies in teleosts [Desjardins et al., 2010; Wong et al., 2012], we are beginning to identify a subnetwork of brain regions within the SDMN that appears to be involved in female mate choice.

Neuroligin-3 and Mate Preference

Within 30 min of stimulus presentation, we saw evidence that specific brain regions exhibit context-dependent differences in *neuroligin-3* expression. In the LS treatment group, high-preference females had a significantly higher *neuroligin-3* expression in Dm, Dl, HV, Vv, and POA relative to females expressing a low preference for a particular male. Furthermore, *neuroligin-3* expression in all of these regions showed a significant positive correlation with preference (fig. 3). These results are consistent with previous analyses of another gene associated with mate preference and synaptic plasticity, i.e. *neuroserpin*, which showed a significant covariation of expression with female preference in four of these five brain regions [Wong et al., 2012]. Two of these brain regions (Dm and Dl) integrate multisensory information [Northcutt, 2008], are part of the putative mesolimbic reward pathway in teleosts, and receive signals from dopaminergic neurons that project from TPp or Vc [Rink and Wullimann, 2001; O'Connell and Hofmann, 2011]. Of note, Dm and Dl are the putative tetrapod homologs for the basolateral amygdala and hippocampus, respectively [Northcutt, 2008; O'Connell and Hofmann, 2011]. Given that both Dm and Dl are sites of neurogenesis in fish [Zupanc et al., 2005], these areas are presumed to be capable

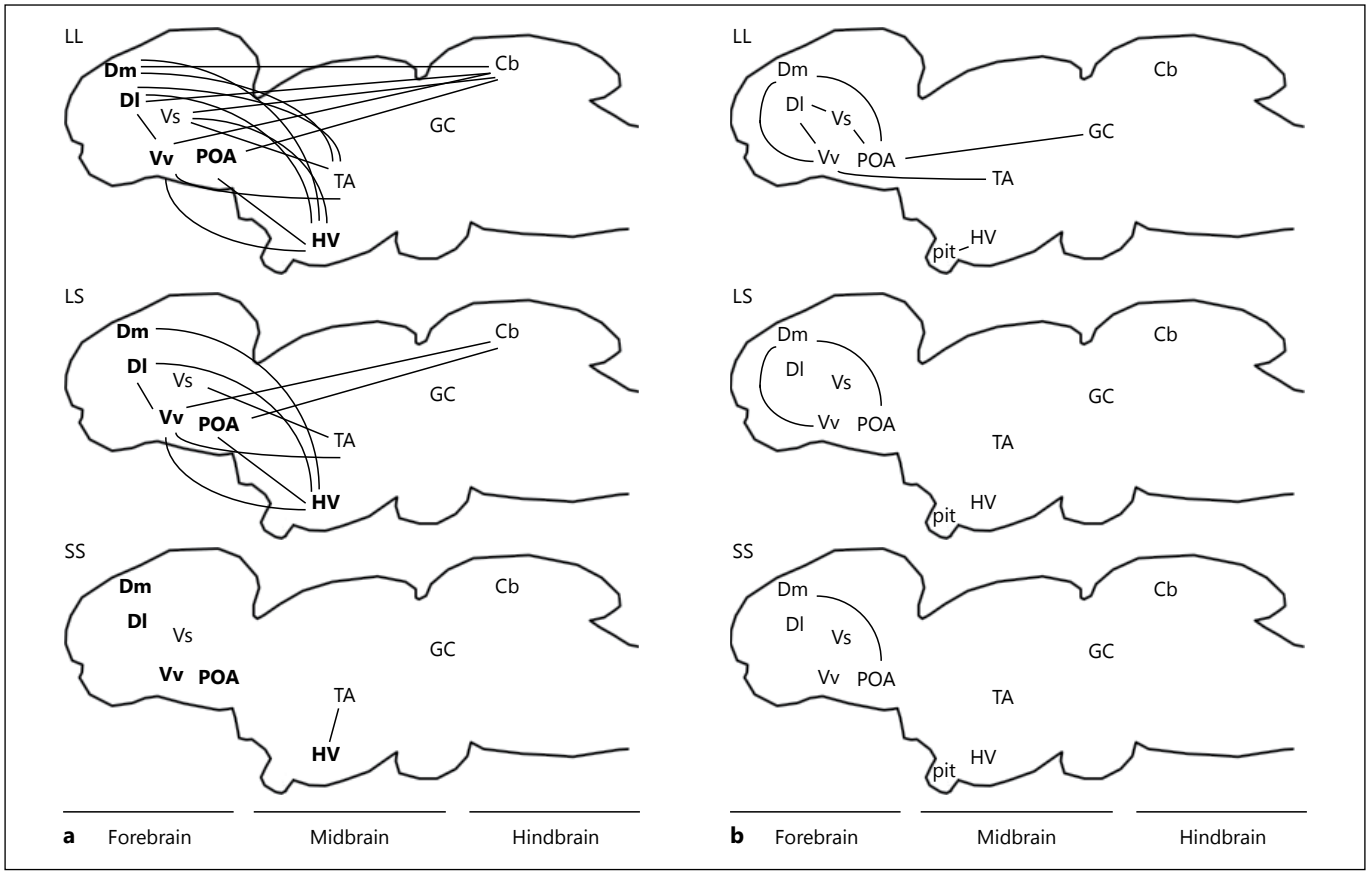


Fig. 5. *Neuroligin-3* expression network (correlation density pattern) by context. **a** Unique significant positive pairwise correlations relative to FF and HT females in *neuroligin-3* expression between brain regions (lines) in LL-, LS-, and SS-exposed females. Brain regions in bold are associated with mate preference as identified in this study. **b** Unique significant positive pairwise correlations relative to FF and HT females in *neuroserpin* expression between brain regions (lines) in LL-, LS-, and SS-exposed females. Modified from Wong et al. [2012].

of easily being remodeled. The relationship between the strength of preference and a marker for synaptic plasticity *(neuroligin-3)* suggests that these areas may be constantly refined for mate preferences. While the current study did not test the learning or memory of mate preferences, it is possible that the differential expression of *neuroligin-3* in Dl (i.e. hippocampus) may reflect such processes and is most salient in the LS condition where discrimination between a courting (L) and coercive (S) male phenotype is required.

The LS group represents males that differ in terms of a number of morphological and behavioral characteristics. Most notably, large males have a larger body size, display courtship behavior, and defend females [Morris et al., 1992; Ryan and Rosenthal, 2001]. On the other hand, small males lack many secondary sexual characteristics and use sneak copulations as a reproductive strategy [Morris et al., 1992; Ryan and Rosenthal, 2001]. Further, females from our study regularly encountered both male phenotypes in their seminatural enclosures that housed more than 200 individuals, where they were courted by the large males and chased by the small males. Studies have demonstrated that females prefer to mate with large males relative to small males, both in a laboratory setting and in the wild [Ryan and Causey, 1989; Wong et al., 2011]. This mate preference appears to be experience dependent (e.g. learned) in this species and in other congeneric species as older females show stronger preferences for the large, courting phenotype [Tudor and Morris, 2009; Verzijden and Rosenthal, 2011; Wong et al., 2011]. *Neuroligin-3* expression levels are linked to the salience and learning/recall of sensory cues in both invertebrates and vertebrates [Tabuchi et al., 2007; Biswas et al., 2010; Hunter et al., 2010]. Of all the male pairings (SS, LS, and

LL), the LS group provided females with the pair of stimuli that differed the most and likely provided the best ability to detect associations of *neuroligin-3* expression and mate assessment. In brain areas implicated in sensory integration, learning, and memory (Dm and Dl), *neuroligin-3* expression may be associated with the female's perception and recall of previous experiences with the male classes.

Three brain regions associated with reproduction (HV, POA, and Vv) also showed an association of *neuroligin-3* expression and mate preference in the LS group. We acknowledge that interpreting the role of synaptic plasticity in these regions can be challenging. While it has been documented that some of these brain regions (e.g. POA) can vary in neuronal properties with social structure, hormone levels, and copulation in males [White and Fernald, 1993; Prince et al., 1998; White et al., 2002; Sakuma, 2008], an analogous situation in females is unlikely in the current study. Female *X. nigrensis* breed year-round [Morris and Ryan, 1992]; we did not detect any relationship between circulating estradiol levels and *neuroligin-3* expression and females were not allowed to physically interact with males (online suppl. table 1). However, we cannot rule out a local neurosteroid release that may have been influencing neuroplasticity in these regions in the context of sexual behavior [Balthazart et al., 2010]. Another interpretation is that we were identifying regions that may be linked to motivational aspects of reproduction. The POA, lateral septum (Vv), and subregions of the hypothalamus (e.g. ventromedial hypothalamus) modulate lordosis displays in rodents (i.e. a measure of the motivation to mate) [Pfaff et al., 1994; Sakuma, 2008]. These areas are also implicated in sexual behavior in a variety of taxa [Newman, 1999; Goodson, 2005; O'Connell and Hofmann, 2011; Sternson, 2013] and they are linked to female mate assessment in cichlids [Desjardins et al., 2010]. Of note, Dm and Dl project to areas involved in receptivity and/or copulation (POA and Vv) or indirectly to HV in the hypothalamus [Northcutt, 2006; O'Connell and Hofmann, 2011]. Hence, our current model of the female mate preference network suggests that mate assessment information is processed in multisensory integration areas (Dm and Dl), possibly being influenced by a learning or memory component (Dl) and then projected to areas more directly involved in receptivity and copulation (HV, POA, and Vv).

Tyrosine Hydroxylase and Mate Preference

Dopamine is widely known to influence decision making, incentive valuation, and reinforcement learning processes [Wise, 2002; Schultz, 2007]. Tyrosine hydroxylase (TH) is the rate-limiting enzyme for dopamine biosynthesis and has been used as a marker for dopaminergic neurons in immunohistochemical studies showing activation of these cells correlating with precopulatory behaviors (e.g. courtship singing in zebra finches) [Goodson et al., 2009]. In this study, we did not observe any relationship between variation in TH gene expression and mate preference behavior in the brain regions examined or across contexts. Considering the limitations of our study (a short exposure time and measurement of mRNA transcripts rather than protein or phosphorylation), the lack of a pattern does not rule out the role of dopaminergic signaling in swordtail preference behavior. We can note, however, that the localized TH gene expression in female swordtails experiencing mate choice contexts is consistent with whole-brain expression patterns in exhibiting no correlational relationship with preference behavior [Ramsey et al., 2012]. Meanwhile, under identical experimental procedures, examination of *neuroligin-3*, and other genes associated with synaptic plasticity, produces significant correlated patterns with preference behavior at the localized (fig. 3) [Wong et al., 2012] and whole-brain [Cummings et al., 2008; Lynch et al., 2012] levels. Furthermore, we cannot rule out the possibility that preference was modulated by changes in dopamine release. We measured mRNA expression level changes within 30 min of stimulus presentation, and dopamine release occurs on a different time scale. Differences in receptor subtype densities/ratios or binding efficiencies underlie variations in other natural behaviors [Graham and Pfaus, 2010; Kleitz-Nelson et al., 2010; Ritters, 2011; Young et al., 2011] and may be an alternate mechanism that allows the reward system to influence mate preference while showing no differences in dopamine production. All of the brain regions showing differential *neuroligin-3* expression (Dm, Dl, HV, POA, and Vv) express dopamine receptors in other teleosts [O'Connell et al., 2011]. Future studies should explore the roles of receptor subtypes in mate assessment.

Gene Expression Dynamics of Mate Preference

Comparisons of individual gene expressions across the entire brain in mate preference contexts revealed that *tyrosine hydroxylase* and *neuroligin-3* have dramatically different responses. TH showed almost no correlated response across brain regions (online suppl. fig. 2), yet the *neuroligin-3* expression complexity increased with increased exposure to courting male phenotypes. Specifically, there are greater numbers of brain regions with correlated *neuroligin-3* expression in the LL and LS environ-

ment compared to SS (fig. 5a). This pattern of differential gene expression complexity mirroring the presumed complexity of the mate choice environment is parallel to that observed with *neuroserpin* expression across these females (fig. 5b) [Wong et al., 2012]. The overlapping expression patterns we observed from these two genes (*neuroligin-3* and *neuroserpin*) suggest that the molecular pathways for the synaptic plasticity response vary by mate preference contexts. That is, if two males are nearly equally attractive (LL), a female's brain may require more cross-talk across the brain than when she is given a simpler choice (LS) or nonattractive options (SS).

Interestingly, there are some major differences in correlation density patterns between *neuroligin-3* (fig. 5a) and *neuroserpin* (fig. 5b). First, there are many more unique connections in the LL and LS contexts in *neuroligin-3* expression patterns relative to *neuroserpin*. Second, the *neuroligin-3* correlation density in LS is significantly higher than that in SS, which was not the case for *neuroserpin*. Differences in social behavior associated with *neuroligin-3* and *neuroserpin*, which can be related to differences in cellular functions, may explain why we observed these correlated expression patterns across the brain within the same females. *Neuroserpin* expression appears to be important for rodents to exhibit exploratory behavior and a decreased neophobia [Madani et al., 2003]. *Neuroligin-3* expression does not seem to influence exploratory behavior but appears to regulate the willingness to be social in rodents [Tabuchi et al., 2007] as well as sensory perception in invertebrates and vertebrates [Radyushkin et al., 2009; Biswas et al., 2010; Hunter et al., 2010]. For instance, *neuroligin-3* knockout mice show abnormalities in social memory that may be linked to an olfactory deficiency [Radyushkin et al., 2009]. As female mate preference requires social interaction and sensory perception, it may not be surprising that *neuroligin-3* expression showed a much higher number of brain regions with correlated expressions relative to *neuroserpin*.

The increasing density of the *neuroligin-3* expression network, concomitant with the complexity of the mate preference environment, may be related to differences in the amount of sensory information or the salience of the male phenotype (e.g. the size of male and ornamentation) and behavioral attributes of the different male phenotypes. In the two-large-male environment (LL), females assessed two courting large males that differed in ornamentation (UV) and individually varied in terms of their display rates to the female. In the LS environment, females were given a simple preference environment with just one large courting male paired with a coercive male, and they were exposed to a coercive-only condition in the SS environment. Increasing sensory and behavioral complexities (LL > LS > SS) may require a more correlated expression of *neuroligin-3* (and *neuroserpin* [Wong et al., 2012]) across the brain to process this information. That is, cellular machinery allowing for the formation of new synapses or changing the strength of the connections between existing synapses may be more active. It is an intriguing possibility that *neuroserpin* and *neuroligin-3* serve complementary roles in facilitating the occurrence of a female encountering a male and subsequently evaluating his attractiveness. Future studies should determine how the amount of colocalization of *neuroserpin* and *neuroligin-3* relates to female mate preference.

In summary, female mate preference is associated with discrete regions of the brain, some of which include associations with sensory integration (Dm), learning and memory (Dl), and reproduction (POA, Vv, and HV). Interestingly, we observed a positive covariation between individual variations of a marker for synaptic plasticity (*neuroligin-3*) and the strength of female mate preference in these brain areas. This suggests that, in *X. nigrensis* and possibly other species, the neural processes associated with mate choice undergo constant refinement and can be modified depending on the context and male availability. Research in the acoustic processing of mate choice cues has found functional shifts in correlated immediate early gene *(egr-1)* expression patterns across the hypothalamus between relevant (conspecific mate calls) and irrelevant (heterospecific mate calls) stimuli [Hoke et al., 2005]. Here we observed that the regulation of *neuroligin-3* expression across brain regions of the SDMN differs depending on the context. There is a shift in regulation patterns across the brain that appears to scale with the complexity or salience of the male social environment. Our study did not identify an association between *tyrosine hydroxylase* – the gene that codes for the rate-limiting step in dopamine biosynthesis – and variations in female mate preference, but at this point we cannot exclude the involvement of dopamine in female mate preference.

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