SUPPLEMENTAL MATERIAL

Vasoactive intestinal peptide as a mediator of the effects of a supergene on social behavior

Brent M. Horton[1, 2], Christina M. Michael[1], Mackenzie R. Prichard[2], and Donna L. Maney[2]
[1] Department of Biology, Millersville University, Millersville, PA
[2] Department of Psychology, Emory University, Atlanta, GA

Correspondence to: dmaney@emory.edu

Proceedings of the Royal Society B
DOI: 10.1098/rspb.2020.0196

Methods

Behavioral Observations and Tissue Collection

We studied free-living white-throated sparrows during the 2010 and 2011 breeding seasons in the Hemlock Stream Forest near Argyle, Maine, USA. We characterized behavior and collected tissues from birds during two phases of breeding, hereafter referred to as the 'early breeding' and 'nestling' stages. The early breeding stage, which followed territory establishment and pair formation, and included birds in the pre-laying, nest-building, egg-laying, and early incubation phases, coincides with the highest levels of territorial aggression in white-throated sparrows (Horton et al., 2012; 2014a), and because only females build nests and incubate (Falls & Kopachena, 2010), males are not yet engaged in parental behavior at this time. During early breeding, we quantified song rate in response to simulated territorial intrusions according to the methods described in Horton et al. (2014a). Singing in this context is a component of territorial aggression, and in this study population, song rates in response to simulated territorial intrusions are higher in WS birds than TS birds in both sexes (Horton et al., 2014a). During the nestling stage, and on different territories than those studied during early breeding, we quantified nestling provisioning rates (number of feeding trips per hour) by parental adults and spontaneous territorial song rates by parental males when nestlings were 5 and 6 days old according to the methods described in Horton et al. (2014a); young typically fledge at 9-10 days old (Falls & Kopachena, 2010). In this study population, parental WS males provision nestlings at lower rates but sing spontaneously at higher rates than do parental TS males; WS and TS females provision young at similar rates (Horton et al 2014a) and rarely sing spontaneously during the nestling stage. Our sample during both stages consisted entirely of opposite morph pairs, the typical pair type for this species (Falls & Kopachena, 2010).

We captured focal birds on the day following their last behavioral observation either by luring them into mist nets with conspecific song or by placing mist nets near the nest. Blood samples were collected and analyzed for gonadal steroid concentrations according to the methods described in Horton et al. (2014a); capture and blood sampling times did not vary according to morph in either sex or stage (Grogan et al., 2019). Immediately after capture and blood sampling, whole brains were extracted and rapidly frozen on powdered dry ice in the field, and shipped frozen to Emory University (Atlanta, GA, USA) where they were stored at -80°C until cryosectioning.

Cryosectioning and In Situ Hybridization

Brains were cut coronally into seven replicate sets of 20µm sections. Three sets were used for previously published studies on gene expression (Grogan et al., 2019; Horton et al., 2014b). In this study, we labeled VIP mRNA expression in another set using *in situ* hybridization with an ^{35}S-labeled riboprobe; our *in situ* hybridization protocol is described by Leung et al. (2011). Briefly, frozen brains were sectioned onto Superfrost™ Plus microscope slides (Thermo Fisher Scientific). After sections were defrosted, delipidated, acetylated, and dehydrated, 100 mL hybridization buffer containing 1 X 10^6 cpm of riboprobe was applied to each slide. Slides were then coverslipped and incubated overnight in a mineral oil bath at a hybridization temperature of 55.5°C. The next day, the slides were washed, treated with RNase, washed again at 49.7°C, and dehydrated. Dry slides were placed into film cassettes against Kodak

BioMax maximum-resolution film and protected from light for 3 days. The film was then developed in a Konica SRX101-A developer and scanned at 1600 dpi using an Epson V700 digital scanner. The slides were then Nissl-stained in toluidine blue and scanned at the same resolution.

Tissue from each sex and breeding stage was run separately; that is, we performed separate runs of *in situ* hybridization on tissue from early breeding males, early breeding females, nestling stage males, and nestling stage females. With the exception of the early breeding females, each group was further divided into two separate runs with year and morph balanced across them. Thus, the tissue was processed in a total of seven runs.

The ^{35}S-labeled riboprobe used to label VIP mRNA was prepared according to the protocol described by Leung et al. (2011). Briefly, we amplified a 340bp sequence (Table S1) that spanned exons 4-6 (GenBank reference sequence ID: NW_005081596.1) and cloned it into pCRII vector using a TOPO TA Cloning kit (Invitrogen). The sequence of the insert, which was confirmed by Retrogen (San Diego, CA), contained no ZAL2/2^{m} polymorphisms. Antisense and sense riboprobes were generated from linearized plasmids using a T7/SP6 Riboprobe *in vitro* Transcription Systems kit (Promega) and ^{35}S-UTP (PerkinElmer). The resulting riboprobes were purified using Illustra ProbeQuant G-50 Micro Columns (GE Healthcare) and diluted in hybridization buffer to a concentration of 10^{7} cpm/mL.

Quantification of Gene Expression

The distribution of VIP mRNA in our material was similar to that previously reported in other birds (Kingsbury et al., 2015; Kuenzel et al., 1997). Label was seen, for example, in the ventromedial hypothalamus, median eminence, and medial preoptic area. Because our hypothesis pertained specifically to the VIP cell populations in the anterior hypothalamus and the infundibular region, we restricted our analysis to these two regions.

We used ImageJ to measure the average gray value for VIP mRNA signal within each region of interest bilaterally in two to four consecutive sections, which were ~120 µm apart since multiple sets of sections were cut from each brain, and only one set was used to measure VIP expression. Regions of interest were located using corresponding Nissl-stained slides and visible landmarks as a guide (see Supplemental Fig. S1, below). VIP signal in the anterior hypothalamus was measured in two sections where the anterior commissure was visible by placing a 400 µm diameter circle over a distinctive cluster of VIP mRNA-expressing neurons in the anterior hypothalamus (Fig. S1; see also Goodson et al., 2012). VIP signal in the infundibular region was measured in four sections by tracing the margins of the ventral portion of the tuberal hypothalamus known to contain a dense population of VIP mRNA-expressing neurons. The traced region, which excluded the median eminence, was similar to that referred to as the infundibular nuclear complex (Chaiseha & El Halawani, 1990), infundibular complex (Surbhi et al., 2015), and nucleus infundibulum (Zhao et al., 2018) in other studies of VIP expression in birds. Gray values for VIP signal in each section were corrected for background by subtracting the gray value of a nearby region with no discernable VIP signal. For each region of interest, average corrected gray values across hemispheres and sections for each individual were used in analyses.

Supplemental Table and Figure:

Table S1. Sequence of primers used to amplify the VIP riboprobe, and the sequence of the resulting VIP mRNA riboprobe used for *in situ* hybridization.

	Size:	Sequence:
Forward Primer:	22 bp	TGCTGTCTTCACCGACAACTAC
Reverse Primer:	28 bp	CGTAAGAAACAAATATATCACAACTTTC
Riboprobe:	340 bp	TGCTGTCTTCACCGACAACTACAGCCGCTTTCGAAAGCAGATGGCTGTGAAGAAATA CTTAAACTCAGTTTTAACTGGAAAAAGAAGCCAGGAAGAGCTAAATCCTGCTAAACT TCGAGATGAAGCAGAACTTCTTGAACCATCCTTTTCAGAAAACTACGATTCTGTAGAT GAGCTGCTGAGCCACCTCCCACTGGACCTCTGAAGGACACCTGGTAAAGTCTATGAC GAGAACAAGCTATTTTTGAGTTCCACATAGTATTTCAAAGAGATGACTTTAGTCATCA AACCAGAACAAATATGTTGTGAAGTGAAAGTTGTGATATATTTGTTTCTTACG

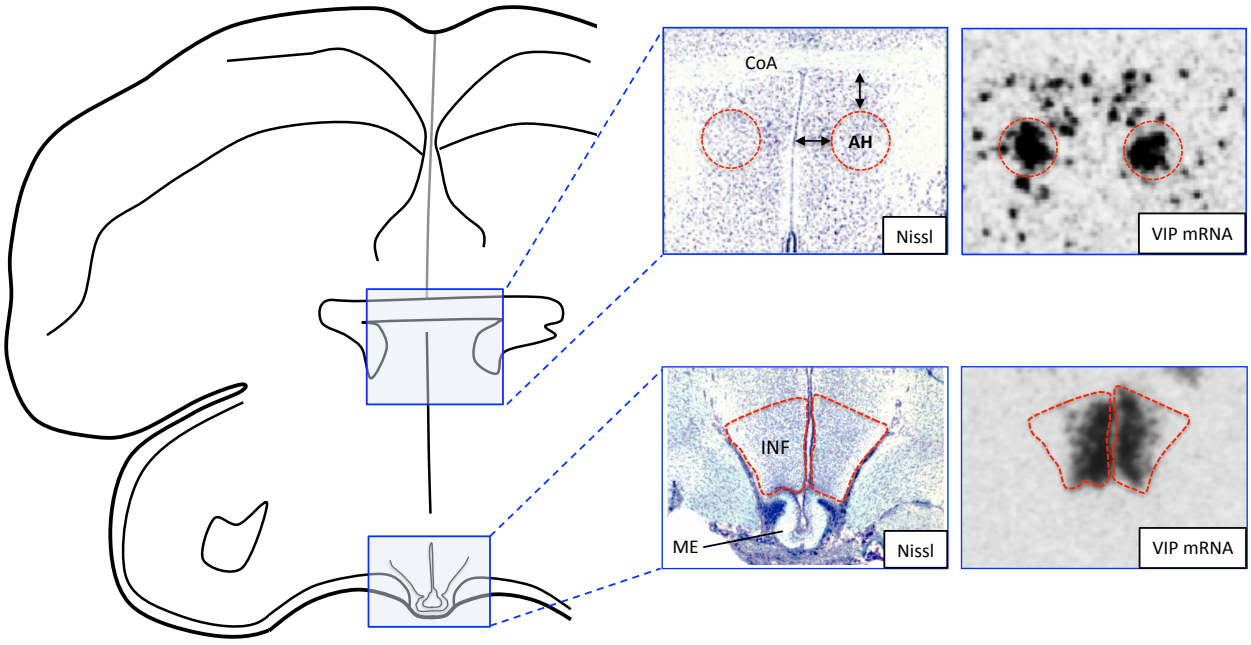

Figure S1. Measuring VIP mRNA expression in the anterior hypothalamus (AH) and infundibular region (INF). For AH, a circle (400 µm diameter) was placed with its dorsal edge 250 µm ventral to the anterior commissure (CoA) and its medial edge 250 µm lateral to the midline (i.e., immediately lateral to the paraventricular nucleus); arrowed lines in the top insert are 250 µm. The cluster of VIP expression captured by the circle is typical of VIP mRNA expression in the AH (see Goodson et al., 2012). For INF, the margins of the ventral portion of the tuberal hypothalamus containing a dense population of VIP mRNA-expressing neurons was traced; as shown, traces excluded the median eminence (ME). The brain drawing represents a coronal section approximately 1.4 mm anterior to a zero point defined by Stokes et al. (1974).

References

Chaiseha, Y., El Halawani, M.E. (1999). Expression of vasoactive intestinal peptide/peptide histidine isoleucine in several hypothalamic areas during the turkey reproductive cycle: Relationship to prolactin secretion. Neuroendocrinology. 70, 402–412.

Falls J. B., Kopachena J. G. (2010). White-throated sparrow (*Zonotrichia albicollis*). The Birds of North America, ed. Poole, A. (Cornell Laboratory of Ornithology, Ithaca, NY).

Goodson, J. L., Wilson, L. C., Schrock, S. E. (2012). To flock or fight: Neurochemical signatures of divergent life histories in sparrows. Proceedings of the National Academy of Sciences. 109, 10685-10692.

Grogan, K. E., Horton, B. M., Hu, Y., Maney, D. L. (2019). A chromosomal inversion predicts the expression of sex steroid-related genes in a species with alternative behavioral phenotypes. Molecular & Cellular Endocrinology. 495, 110517.

Horton, B. M., Hauber, M. E., Maney, D. L. (2012). Morph matters: Aggression bias in a polymorphic sparrow. PLoS ONE, 7, e48705.

Horton, B. M., Moore, I. T., Maney, D. L. (2014a). New insights into the hormonal and behavioural correlates of polymorphism in white-throated sparrows, *Zonotrichia albicollis*. Animal Behavior. 93, 207-219.

Horton, B. M., Hudson, W. H., Ortlund, E. A., Shirk, S., Thomas, J. W., Young, E. R., . . . Maney, D. L. (2014b). Estrogen receptor α polymorphism in a species with alternative behavioral phenotypes. Proceedings of the National Academy of Sciences. 111, 1443-1448.

Kuenzel, W. J., McCune, S. K., Talbot, R. T., Sharp, P. J., Hill, J. M. (1997). Sites of gene expression for vasoactive intestinal polypeptide throughout the brain of the chick (*Gallus domesticus*). Journal of Comparative Neurology. 381, 101-118.

Leung, C. H., Abebe, D. F., Earp, S. E., Goode, C. T., Grozhik, A. V., Mididoddi, P., Maney, D. L. (2011). Neural distribution of vasotocin receptor mRNA in two species of songbird. Endocrinology. 152, 4865-4881.

Stokes, T. M., Leonard, C. M., Nottebohm, F. (1974). The telencephalon, diencephalon, and mesencephalon of the canary, *Serinus canaria*, in stereotaxic coordinates. Journal of Comparative Neurology. 156, 337-374.

Surbhi, Rastogi, A., Rani, S., Kumar, V. (2015). Seasonal plasticity in the peptide neuronal systems: Potential roles of gonadotrophin-releasing hormone, gonadotrophin-inhibiting hormone, neuropeptide Y and vasoactive intestinal peptide in the regulation of the reproductive axis in subtropical Indian weaver birds. Journal of Neuroendocrinology. 27, 357–369.

Zhao, H., Jiang, J., Wang, G., Le, C., Wingfield, J. C. (2018). Daily, circadian and seasonal changes of rhodopsin-like encephalic photoreceptor and its involvement in mediating photoperiodic responses of Gambel's white-crowned Sparrow, *Zonotrichia leucophrys gambelii*. Brain Research, 1687, 104-116.