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Oxytocin/Vasopressin-Related Peptides Have an Ancient Role in Reproductive Behavior

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Abstract

Many biological functions are conserved, but the extent to which conservation applies to integrative behaviors is unknown. Vasopressin and oxytocin neuropeptides are strongly implicated in mammalian reproductive and social behaviors, yet rodent loss-of-function mutants have relatively subtle behavioral defects. Here we identify an oxytocin/vasopressin-like signaling system in *Caenorhabditis elegans*, consisting of a peptide and two receptors that are expressed in sexually dimorphic patterns. Males lacking the peptide or its receptors perform poorly in reproductive behaviors, including mate search, mate recognition, and mating, but other sensorimotor behaviors are intact. Quantitative analysis indicates that mating motor patterns are fragmented and inefficient in mutants, suggesting that oxytocin/vasopressin peptides increase the coherence of mating behaviors. These results indicate that conserved molecules coordinate diverse behavioral motifs in reproductive behavior.

Animal behaviors such as mating, feeding, or foraging typically involve combinations of simpler actions and motor patterns that develop over different time scales. In the case of reproductive behavior, neuropeptide signaling through oxytocin and vasopressin peptides may provide a global organizing role. The mammalian hypothalamic neuropeptide oxytocin, released during birth, stimulates maternal behaviors as well as uterine contractions and lactation, and the related peptide vasopressin is linked to male-typical behaviors in rodents and fish, in addition to its role in fluid homeostasis (1). Similarly, administration of the oxytocin/vasopressin-related peptide conopressin to leeches stimulates reproductive behaviors and mating-related neuronal activity (2). A more general role for oxytocin in social behaviors is suggested by the social amnesia of oxytocin mutant mice (3) and by altered social decision-making in humans after oxytocin administration (4). However, mammalian oxytocin and vasopressin mutants have subtle behavioral defects relative to the potency of the administered peptides (5). To define the role of these neuropeptides in endogenous reproductive behaviors, we here address their function through genetic analysis of an oxytocin/vasopressin-related neuropeptide in the nematode *Caenorhabditis elegans*.

Through homology searches, we identified a gene in *C. elegans* with similarity to mammalian vasopressin and oxytocin (*ntc-1*; Fig. 1, A and B, and fig. S1). To determine whether this gene generates an authentic neuropeptide, we isolated total neuropeptides from

Supplementary Materials

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Materials and Methods

Figs. S1 to S14

Table S1

References (18–36)

wild-type animals and characterized them by tandem mass spectrometry (6), identifying a peptide matching the mass predicted for an 11-amino acid cyclized peptide with an amidated C terminus (Fig. 1C and fig. S2). Fragmentation of this peptide by liquid chromatography–tandem mass spectrometry confirmed the existence of a *C. elegans* neuropeptide, nematocin. The *C. elegans* genome also predicts two genes encoding G protein–coupled receptors related to vertebrate oxytocin and vasopressin receptors, the nematocin receptor (*ntr*) genes (Fig. 1A and figs. S3 and S4). To determine whether these encode nematocin receptors, we expressed *ntr-1* and *ntr-2* cDNAs in HEK293T cells with the promiscuous G protein G₁₅ and administered a synthetic cyclized peptide corresponding to nematocin. Cells transfected with *ntr-1* responded to nematocin with calcium transients and a nanomolar median effective concentration (EC₅₀) (Fig. 1, D and E, and fig. S1). Cells transfected with *ntr-2* did not mobilize calcium in response to nematocin, but cells cotransfected with both receptors responded to nematocin with decreases in intracellular cyclic adenosine monophosphate (cAMP), suggesting coupling to a signaling pathway that antagonizes adenylyl cyclase (Fig. 1F and fig. S1) (7). Although heterologous expression may not capture all native functions, these results suggest that NTR-1 and NTR-2 can contribute to nematocin-mediated signaling alone (NTR-1) or as heterodimers (NTR-1/NTR-2).

The expression of nematocin and *ntr* genes was examined by conventional and fosmid-recombineered green fluorescent protein (GFP) reporter genes and additionally with antisera generated against synthetic nematocin (Fig. 2, A to H; figs. S5 and S6; and table S1). *C. elegans* has two sexes, self-fertilizing hermaphrodites that can also mate as females, and cross-fertilizing males (8). Both sexes expressed nematocin in the AFD thermosensory neurons, which mediate thermotaxis (9), and in the DVA mechanosensory neuron, which regulates locomotion and posture (10); and males expressed nematocin in male-specific CP motor neurons that control turning behavior during mating (11) (Fig. 2, A and B, and G and H). Both sexes expressed *ntr* receptor reporter genes in partly overlapping sets of head and tail neurons, and males additionally expressed them in male-specific neurons and muscles implicated in mating (Fig. 2, C to F; fig. S6; and table S1): *ntr-1* in hook and tail sensory neurons [HOB, which senses the vulva; and rays 1, 5, 7, and 9, which sense hermaphrodite contact (12)] and in spicule protractor muscles, which act during sperm transfer (13); and *ntr-2* in the male-specific SPC sensory-motor tail neurons that induce spicule penetration and muscle contraction for sperm transfer (13, 14) and in the male-specific oblique muscles that promote prolonged vulval contact (14). The reporter genes rescued all behavioral phenotypes described below, indicating that they are expressed in functionally relevant sites (Fig. 3).

Null mutants for nematocin and the two *ntr* genes were viable and fertile as hermaphrodites, with normal locomotion speed, egg-laying behavior, and numbers of progeny (fig. S7). The males were also viable, but they had reduced mating success: When single males were housed with single mating partners, the number of progeny per male was reduced 2.5-fold in nematocin or *ntr-2* mutants as compared to wild-type males (Fig. 3B). We characterized the male mating defect by quantitative analysis of mating encounters between individual virgin males and hermaphrodite mating partners in a 5-min viewing period, and found that nematocin mutant males were inefficient at multiple mating stages. When placed in a small arena with food and mating partners, a wild-type male typically attempted to mate with the first hermaphrodite that his tail touched, located her vulva after he made one or two turns around her body, and successfully transferred sperm within 5 min (Fig. 3A and C to E, and fig. S8). Nematocin mutant males attempted to mate only after numerous hermaphrodite contacts (Fig. 3D), made more turns around the hermaphrodite before locating the vulva (Fig. 3E), and had difficulty executing turns, maintaining vulval contact, and transferring sperm within the assay period (Fig. 3C and fig. S8). Ethograms showed fragmentation of the

mating sequence and repetition of early mating steps in nematocin mutant males (Fig. 3F and fig. S9). All defects were rescued by transgenes that spanned the nematocin gene (Fig. 3, B to E, and fig. S8).

Null mutations in the receptor genes disrupted partly overlapping aspects of the mating response. *ntr-1* was required for the initial response to hermaphrodite contact (Fig. 3D), matching *ntr-1* expression in male ray neurons that mediate this behavior (12). This defect was partially rescued by *ntr-1* expression in a subset of ray neurons (fig. S10). *ntr-2* had a substantial effect on overall reproductive efficiency (Fig. 3B). Both receptors were required for correct execution of turns and mating success (Fig. 3, C and E).

C. elegans males have a long-term mate search behavior in which they leave a bacterial food source that lacks hermaphrodite mating partners (15). Nematocin mutant males and *ntr* mutant males were partly deficient in male-typical leaving behavior, suggesting a defect in mate search (Fig. 3G). This behavioral change was not due to a general locomotion or sensory defect, because nematocin mutants had normal locomotion parameters on and off of food and normal responses to aversive stimuli, touch, and drugs (figs. S11 and S12).

The broad actions of nematocin on male mating behaviors could result from humoral secretion that activates many targets, precise release at specific neuronal sites, or a mixture of the two. To identify relevant sites of nematocin action without risking misexpression, we focused on rescue with the endogenous nematocin promoter, manipulating its precise expression pattern through Cre-mediated recombination (Fig. 4A). Cre-mediated recombination in all *ntc*-expressing cells caused defects in all male mating behaviors (fig. S13), whereas specific Cre-mediated recombination in the DVA mechanosensory neuron led to defects in initial contact response and vulva location efficiency, but did not affect turning behaviors (Fig. 4, C to F). Laser ablation of the DVA neuron caused the same defect in the hermaphrodite contact response as the DVA nematocin knockout (Fig. 4B). DVA ablation also generated striking male-specific defects in locomotion speed and posture that were not observed in nematocin mutants (fig. S14). DVA has only mild effects on hermaphrodite locomotion and is not obviously sexually dimorphic, so its strong male-specific effects on movement were unexpected.

These results indicate that nematocin provides a neuromodulatory input to organize diverse aspects of male mating, increasing the effectiveness by which distributed circuits generate coherent behaviors. DVA is directly pro-prioceptive (10) and also receives synaptic input from the male sensory ray neurons and mechanosensory neurons (16); its ability to release nematocin that activates NTR-1 on ray neurons may provide a feedback signal at the onset of mating. The DVA neuron produces additional transmitters and performs other functions, and nematocin is released from additional sources, so the functions of mating neurons and peptides are partly orthogonal (7, 12, 13). We suggest that nematocin and its receptors prime neurons in a variety of local circuits to generate a neuroethological “appetitive” function in mating. This insight refines the likely functions of oxytocin/ vasopressin-related neuropeptides and suggests that they have ancient roles in reproductive behaviors that are conserved in bilaterian vertebrates, lophotrochozoa, and nematodes. The apparent conservation of oxytocin/vasopressin peptides in reproductive behavior stands in contrast with the diversity of mechanisms for sex determination, sex chromosomes, and dosage compensation (17).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Strains bearing *ntc-1*, *ntr-1*, and *ntr-2* mutations are available through the *C. elegans* National BioResource Project (NBRP), subject to a materials transfer agreement. Sequence accession numbers are as follows: NM_001038459.1 (*ntc-1*), NM_060792 (*ntr-1*), NM_078076 (*ntr-2*). We thank S. Emmons for sharing the male wiring diagram before publication; D. Anderson, S. Emmons, S. Flavell, A. Gordus, W. Kristan, T. Maniar, P. McGrath, L. Voss hall, and Y. Xu for discussions; S. Chalasani for plasmids; S. Mitani and the National Bioresource Project for *ntc-1*, *ntr-1*, and *ntr-2* mutants; and the Rockefeller Proteomics Facility, the Rockefeller Bioimaging Facility, and the Rockefeller High Throughput Screening Facility for technical support. This work was supported by a grant from the G. Harold and Leila Y. Mathers Foundation, by Harvey Karp and Helen Hay Whitney Fellowships to J.L.G., by NIH grant K99GM092859 to J.L.G., and by NIH grant GM07739 to E.Z.M. C.I.B. is an Investigator of the Howard Hughes Medical Institute.

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Fig. 1.

ntc-1 encodes an oxytocin/vasopressin-related peptide that signals through receptors encoded by *ntr-1* and *ntr-2*. **(A)** Gene models with deletions in mutant alleles indicated by horizontal black bars (www.wormbase.org). **(B and C)** Domain structure and amino acid sequence of vasopressin (AVP), oxytocin (OXT), and nematocin (NTC-1) precursors and peptides. **(D and E)** Mean calcium responses and dose-response curves of fura-2/AM-loaded HEK293T cells transfected with *ntr-1* and G 15 and exposed to synthetic NTC-1 peptide. **(F)** Forskolin-stimulated cAMP production in NTC-1-treated HEK293T cells transfected with *ntr-1*, *ntr-2*, and G 15. *** $P < 0.0001$. Error bars in all figures indicate SEM.

Fig. 2. *ntc-1*, *ntr-1*, and *ntr-2* expression patterns. P, gene promoter fragment used. The upper panels show expression in adult hermaphrodite heads; the lower panels show male [(B), (D), and (F)] and hermaphrodite (H) tails. (A to F) Expression of *ntc-1*, *ntr-1*, and *ntr-2* reporter genes (table S1). (G and H) Immunostaining of AFD, NSM, and DVA neurons with antisera to nematocin. White arrows indicate expression in specified neurons. Asterisks mark autofluorescence; scale bars represent 10 μm.

Fig. 3.

Nematocin-deficient males exhibit mating defects. (A) Steps in male mating behavior. The black arrow notes the direction of male movement. (B) Reproductive efficiencies of wild-type (WT, gray bars) and mutant males (black bars) in long-term single-pair mating crosses (no. of cross-progeny/no. of total progeny). (C) Percentage of males that successfully transferred sperm within 5 min of first tail contact with a hermaphrodite. (D) Mean number of hermaphrodites that a male's tail contacted before he initiated mating. (E) Mean total number of turns a male executed around the hermaphrodite's body before locating the vulva. (F) Summary of the behavioral transitions in ethograms of male mating behavior (figs. S8 and S9); the arrow width indicates the probability of behavioral transitions. (G) Male mate search behavior, measured as the rate at which a single male leaves food in the absence of hermaphrodites (15). * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$.

Fig. 4.

Selective inactivation of *ntc-1* in DVA affects individual steps of male mating behavior. (A) Cre/Lox strategy to selectively inactivate *ntc-1* in DVA. (Inset) Tail of larval stage 4 male expressing *mCherry* and not *GFP* in DVA, indicating successful DVA-specific recombination. Scale bar, 10 μ m. (B to F) Behavioral phenotypes of males with *ntc-1* inactivated in DVA [(C) to (F)] or after DVA laser ablation (B). (C) to (E) Behaviors as in Fig. 3. (F) Vulva location ability of males (percentage of positive vulva detections/total vulva encounters). * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$.