Neuropharmacology 58 (2010) 126–134

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Behavioral effects of hindbrain vasotocin in goldfish are seasonally variable but not sexually dimorphic

James C. Walton*, Brandon Waxman, Kristen Hoffbuhr, Meaghan Kennedy, Ellen Beth, Jennifer Scangos, Richmond R. Thompson

Psychology Department and Neuroscience Program, Bowdoin College, Brunswick, ME 04011, USA

ARTICLE INFO

Article history:
Received 18 May 2009
Received in revised form
2 July 2009
Accepted 2 July 2009

Keywords:
Vasopressin
Vasotocin
V_{1A}
Receptor
Social behavior
Teleost

ABSTRACT

We have previously demonstrated that centrally administered vasotocin (VT) inhibits social approach toward same-sex conspecifics in male and female goldfish, and that this behavioral effect is dependent upon VT projections to the hindbrain. We now show that there are no sex differences in sensitivity to the behavioral effects of VT, though differences do exist in responsiveness across seasons in both sexes. A central dose of 1 μg, but not 200 ng, inhibited social approach in goldfish in non-reproductive condition, whereas a dose as low as 40 ng inhibited social approach in fish in full reproductive condition. In males and females in full reproductive condition, social approach behavior was facilitated by central administration of 500 ng of a V_{1A} specific antagonist. In addition, the behavioral effects of exogenously administered central VT were blocked by central administration of 1 μg of a V_{1A} antagonist. These results demonstrate that the propensity to approach a conspecific, a simple behavior underlying many social interactions, is controlled by a V_{1A}-like receptor, and that VT's behavioral effects depend on reproductive context. Quantitative real-time PCR showed that the seasonal changes in behavioral responsiveness to VT are associated with changes in the expression of a V_{1A}-like receptor in the hindbrain, but not the mid- or forebrain, indicating that the seasonal regulation of social approach behavior likely depends on the local modulation of the expression of this receptor within a primitive peptide circuit in this species.

1. Introduction

Vasotocin (VT) and its mammalian homologue, vasopressin (VP), influence a variety of social behaviors in vertebrate animals, particularly in reproductive contexts (reviewed in Goodson and Bass, 2001; Rose and Moore, 2002). A major focus of research has therefore been to elucidate the molecular mechanisms and neural circuitry that underlie peptide influences, which are often sexually dimorphic, on sexual and aggressive behaviors associated with reproduction. However, few studies have tried to determine if and how these peptides influence simple approach behaviors that typically precede such interactions and that may play an important role in determining how social organisms are, in and out of reproductive contexts.

In goldfish, VT inhibits the tendency to approach conspecifics (Thompson and Walton, 2004). This inhibition is mediated by one of the most pronounced VT projections in goldfish brains, the projection from VT cells in the preoptic area to the hindbrain (Thompson and Walton, 2009), as determined by experiments showing that VT infusions into the 4th ventricle, near the hindbrain, inhibit the behavior more potently than do infusions into the 3rd ventricle (Thompson et al., 2008b). The VT fibers in the hindbrain appear to induce this effect through a peripheral feedback mechanism initiated by interactions with substance P cells in the dorsal motor vagus (DMV), which the VT fibers encapsulate. These substance P cells project to the periphery, and VT no longer inhibits social approach if tachykinin receptors that mediate substance P's peripheral effects are blocked, suggesting that the activation of those cells by VT induces a change in the physiological state of the animal that stimulates ascending pathways that ultimately inhibit social approach responses.

Although our previous studies thus indicate that a simple social behavior is mediated by VT and elucidate the neural circuitry associated with that effect, we know little about the social contexts associated with VT's behavioral effects. No sex differences in VT producing cells or projection patterns have been identified in goldfish brains (Parhar et al., 2001; Thompson and Walton, 2009), and we have observed that high doses of VT can similarly inhibit social approach in males and females (Thompson et al., 2008b), which suggests that VT may have similar functions in males and

* Corresponding author at: 1835 Neil Ave., Psychology Building, The Ohio State University, Columbus, Ohio 43210, USA. Tel.: +1 614 688 4674; fax: +1 614 688 4733.
E-mail address: walton.315@osu.edu (J.C. Walton).

0028-3908/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuropharm.2009.07.018

females in this species. However, we do not yet know if both sexes are equally sensitive to VT, if endogenous VT similarly inhibits social approach in both sexes, or whether VT exerts its effects in reproductive contexts. To answer these questions, we performed dose-response and vasopressin receptor antagonist studies in males and females in various reproductive states.

Additionally, we know very little about the receptor mechanisms that mediate VT's behavioral effects. In mammals, the behavioral effects of VP are mediated primarily by the V_{1A} and V_{1B} receptors, though V_{1A}-like receptors appear to mediate most social responses to VT in non-mammalian species (Blanchard et al., 2005; Goodson and Bass, 2001; Wersinger et al., 2002; Young et al., 2001). Although variations in V_{1A} and V_{1A}-like receptor expression patterns in the forebrain are correlated with species-specific and sexually dimorphic behavioral effects of VT and VP across vertebrate groups (reviewed in Goodson and Bass, 2001; Young et al., 2002; Goodson, 2008), nothing is known about if and how hindbrain V_{1A}-like receptors may contribute to behavioral regulation, or if their expression can be affected by factors that influence social behavior. To address the latter question, we sequenced a V_{1A}-like receptor from goldfish and determined if its expression changes in hindbrain circuits involved in the regulation of social approach in goldfish in association with seasonal changes in behavioral sensitivity to VT.

2. Methods

2.1. Subjects

Reproductively mature comet goldfish (*Carassius auratus*; 12–15 cm, 25–50 g) were purchased from commercial hatcheries in March just prior to spawning season. Fish were sorted by sex and held in same-sex group housing in 340 L tanks at 18–20 °C in long photoperiod (14:10 L:D) for a minimum of 3 weeks prior to surgeries and behavioral testing. For animals in non-reproductive winter conditions, fish were received from suppliers and housed as above. Over the following 6 months temperatures and photoperiod were gradually reduced to 15 °C and 10:14 h L:D to mimic natural seasonal cycles. This allowed the use of fish of known sex during a time of year when they cannot be reliably sexed due to lack of secondary sexual characteristics. All subjects were verified for sex and reproductive condition at the conclusion of behavioral testing. All surgical methods, behavioral protocols, and methods of sacrifice were in accordance with guidelines for the use of vertebrate animals established by the Research Oversight Committee (IACUC) at Bowdoin College.

2.2. Surgery

Each fish was removed from its home tank and implanted with a 5-mm single guide cannula (Plastics One, Roanoke VA) extending into the third ventricle, as previously described (Thompson and Walton, 2004). Briefly, fish were anesthetized in 0.1% MS-222 (Sigma–Aldrich, St. Louis, MO) and a hole was drilled through the skull above the juncture of the optic tectum and telencephalon. A micromanipulator was then used to lower and hold the cannula 1.0 mm below the brain surface into the third ventricle. The cavity around the cannula was filled with Gelfoam (Pharmacia, Kalamazoo, MI), and two surgical screws were inserted into the skull. Dental cement (A-M Systems, Carlsbourg, WA) was then applied to cover the surgical site and to anchor the cannula in place. Fish were returned their home tank and allowed to recover for 3 days before behavioral testing. Cannula placement was verified in all fish by an injection of ink after all behavioral testing was complete.

2.3. Behavioral testing

2.3.1. VT dose-response

For each test, fish were placed into the central compartment of a 70 L rectangular tank with two 5 L stimulus compartments on each end, separated by sealed Plexiglas to prevent chemical communication. Time spent within 2.5 cm of each partition during a 15 min baseline was recorded with a video tracking system (Limelight; Coulbourn Instruments, Whitehall, PA, USA). Fish were then captured, infused with 1 μl of the appropriate dose of VT (5, 40, or 200 ng) or vehicle, counterbalanced across days with 24 h between tests, and then placed back into the central test tank. Five minutes later, a stimulus fish was placed in the side compartment behind the partition where the fish spent the least amount of time during the baseline period, and time within 2.5 cm of that partition was again recorded for 15 min. Corrected proximity scores were calculated by subtracting the baseline time in proximity to that partition from the time in proximity during the 15 min while the stimulus fish was present. After the last day of testing, 1 μl India ink was infused through the cannula. Fish were sacrificed 10 min later and the brains removed to evaluate the spread of ink through the ventricular system. Any fish with no ink in the ventricles was excluded from the analysis.

2.3.2. Blocking the effects of exogenous VT

Female goldfish in reproductive condition were surgically implanted with cannula, as described above. On the day of testing, fish were captured in their home tank, infused with either 1 μg of the V_{1A} specific antagonist ([β-Mercapto-β,β-cyclopentamethylenepropionyl[1], O-me-Tyr[2], Arg[8]]-Vasopressin; Manning compound) or vehicle, counterbalanced across days with 48 h between tests, and returned to their home tank for 30 min. Fish were then recaptured and placed in the social approach testing tank, as described above. After a 15 min baseline, fish were removed from the testing tank, rapidly infused with 40 ng of VT, and returned to the tank. Five minutes later they were exposed to a stimulus female, as described above, and behavior was recorded for 15 min.

2.3.3. Blocking the effects of endogenous VT

Male and female goldfish in reproductive condition were surgically implanted with cannula, as described above. On the day of testing, fish were captured from their home tank, infused with either 500 ng of V_{1A} specific antagonist or vehicle, counterbalanced across days with 48 h between tests, and placed in the test tank. After 30 min, baseline behavior was recorded for 15 min. A same-sex stimulus fish was then added to the less preferred side and behavior was recorded for 15 min.

2.4. Gene sequencing

2.4.1. cDNA synthesis

RNA was extracted from seven adult goldfish (3 males and 4 females) in spring breeding conditions and in fall non-breeding conditions. Fish were deeply anesthetized in 0.1% MS-222, rapidly decapitated, and their brains (~0.15 g tissue per brain) were removed intact. Total goldfish RNA was isolated from brain tissue using the Ambion RNAqueous-Midi kit for cellular RNA isolation (Ambion, Austin, TX). For standard and 3′ RACE PCR reactions, cDNA was synthesized using Superscript II according to the manufacturer's instructions (Invitrogen 3'RACE System for Rapid Amplification of cDNA Ends; Invitrogen, Carlsbad, CA). For 5′ RACE reactions, cDNA was synthesized with BD PowerScript Reverse Transcriptase according to the kit protocol (BD SMART RACE cDNA Amplification Kit; Clontech, Mountain View, CA). All cDNA was stored at −80 °C.

2.4.2. PCR amplification of VTR fragments

An initial set of degenerate primers based on highly conserved amino acid sequences for the second transmembrane domain and a region 5′ of the sixth transmembrane domain were used in initial PCR runs to amplify a fragment of the goldfish VTR. A series of gene-specific upstream primers were then designed from that fragment and used in subsequent 3′ RACE reactions with downstream primers (Universal and Abridged Amplification Primers) complimentary to an anchor sequence attached to the Poly-A tail during cDNA synthesis. Similarly, a series of gene-specific downstream primers were designed from the initially sequenced fragments and used in 5′ RACE reactions according to the BD SMART 5′ RACE protocol (Clontech, Mountain View, CA).

2.4.3. Cloning

All PCR products were run on 1% agarose gels and visualized with ethidium bromide. Products were inserted into pCR-II-TOPO vectors and transformed into TOP10 chemically competent *Escherichia coli* according to the TOPO TA Cloning kit protocol (Invitrogen). Bacteria were grown overnight on LB plates containing 50 μg/ml kanamycin and 80 μg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) in dimethyl formamide (DMF). Selected colonies were then grown overnight in Luria broth containing 50 μg/ml kanamycin, and vectors were isolated for sequencing using either the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI) or the QIAprep Spin Miniprep Kit (QIAGEN, QIAGEN Sciences, MD).

2.4.4. Gene sequencing and analysis

All sequencing reactions were performed by the Mount Desert Island Biological Laboratory (Salisbury Cove, ME). Sequence traces were analyzed using either Chromas (Version 2.31, Technelysium Pty Lt) or Finch TV 1.4 chromatogram viewer (Geospiza, www.geospiza.com/finchtv). NCBI BLAST database and ORF Finder (National Center for Biotechnology Information, Bethesda, MD) were used for sequence analysis and sequence translation. Sequence alignments were performed using the alignment software ClustalW. All percent identity calculations were done by JalView.

2.5. qPCR analysis

2.5.1. RNA isolation and cDNA synthesis

Brains were removed from male goldfish in spring, reproductive condition and fall, non-reproductive conditions and immediately frozen and stored at −80 °C. Males in reproductive condition had tubercles, expressed milt, and had enlarged testes; males out of reproductive condition did not display secondary sexual