Modulation of neural circuits by the neuropeptide, arginine vasotocin, in the central nervous system of zebrafish

by

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List of Abbreviations

avt; arginine vasotocin

avp: arginine vasopressin

avtr: arginine vasotocin receptor

avpr: arginine vasopressin receptor

Dm: Medial zone of dorsal telencephalon

Vd: Dorsal nucleus of ventral telencephalon

Vv: Ventral nucleus of ventral telencephalon

Dp: Posterior zone of dorsal telencephalon

PPa: Parvocellular preoptic nucleus, anterior part

PPp: Parvocellular preoptic nucleus, posterior part

SC: Suprachiasmatic nucleus

SD: Dorsal sac

Had: Dorsal habenula (Ha)

Chab: Habenular commissure

Abstract

The mammalian neuropeptide arginine vasopressin (AVP) has been linked to a wide variety of complex social behaviors in mammalian species. The neuropeptide changes behaviors presumably by modulating neural circuits via V1a receptor signaling. However, due to the extreme complexity of the large mammalian brain and array of complex behaviors it generates, the mechanism by which AVP modulates neural circuits is still poorly understood. To better understand the mechanism, we decided to study the non-mammalian homologue of AVP, arginine vasotocin (AVT) in the brain of zebrafish, a popular vertebrate model species with a simpler brain and simpler behaviors. The major goal for the research was to gain mechanistic insights into how the neuropeptide modulates behaviors. We first cloned the cDNA of two V1a-like receptors, *avtr1a1* and *avtr1a2*, and conducted *in situ* hybridization to examine the location of *avtr* expression in the brain. To our surprise, both receptors are expressed in the CNS during embryogenesis prior to the appearance of any complex social behaviors. Given the anatomical location of the *avtr* expression, we hypothesized that *avt*/*avtr* signaling modulates embryonic sensorimotor behaviors. In fact, the pharmacological increase of *avt* enhanced, and anti-sense morpholino knockdown of *avt*, *avtr1a1*, and *avtr1a2* genes disrupted embryonic sensorimotor behaviors. We also found that chemosensory stimuli that induce locomotor responses activate *avtr*+ neurons in the posterior hindbrain, further supporting the hypothesis that *avtr*+ neurons modulates sensorimotor responses. Moreover, we examined the expression patterns of *avtrs* in the adult zebrafish brain via whole brain *in situ* hybridization to better understand how *avt*/*avtr* signaling modulates adult social behaviors. In fact, both *avtrs* are expressed in brain regions known to modulate social behaviors including aggression and fear responses. We also investigated inter-male aggressive interaction that is repeatedly linked to *avt* signaling across species, and how the aggressive acts get modulated in large social groups. As a whole, the current study gives

mechanistic insights into how the neuropeptide modulates both early and mature vertebrate behaviors.

Chapter I. Introduction

Some animals are rather social and prefer to stay in social settings with many others around. Others are less social and prefer staying alone or in a small group. Such individual differences in sociality are often dismissed simply as difference in personality or personal preference. However, how does a brain of an animal decide how to interact with others in social settings? Is the sociality of an animal determined genetically? Do neural circuits in the brain control sociality? If there are neural circuits regulating sociality in the brain, how can we explain the variability in sociality exhibited by individuals within the same species that share the same brain structures and organizations? Is the variability of sociality a result of differential expression of genes affecting the performance of the circuit? How do environmental context and learning and memory affect sociality of individuals? Recent advances in molecular, genetic techniques have made it possible to answer some of these questions. Increasing evidence in fact indicates that social behaviors of animals are controlled tightly by neural circuits in the brain (Asahina et al., 2014; Falkner et al., 2014; Hong et al., 2014; Lee et al., 2014; Lin et al., 2011; Tayler et al., 2012). The neural circuits consist of neurons that express a unique set of genes to perform specific functions. Incredibly, scientists have succeeded in inducing specific social or asocial behaviors in animals by stimulating genetically defined populations of cells in specific brain regions including the amygdala and ventromedial hypothalamus (Asahina et al., 2014; Hong et al.,

2014; Lin et al., 2011). Subpopulations in specific brain regions control distinct social behaviors, but how upstream brain centers such as the prefrontal cortex (PFC) select a certain behavior over many others likely depends upon the social situation of the animal. Importantly, animals within the same species share the same neural circuits consisting of the same subpopulations of cells. Therefore, variability seen in social behavior among animals within the same species suggests that intrinsic brain mechanisms, neuromodulators, modulate the functions of these behavioral neural circuits (Bargmann, 2012; Marder, 2012; Marder et al., 2014). Unlike neurotransmitters that act locally at synapses, a large amount of neuromodulators can be released from single neurons, and the neuromodulators can diffuse over long distances in the brain. Therefore, large populations of cells in distant neural sites can be targeted as long as the target cells express the receptors for the modulators, slow-acting G-protein coupled receptors (GPCRs) that can alter the synaptic transmission for an extended period of time. This means that cell groups in neural circuits can be targeted by neuromodulators and subjected to long-lasting modulation of the synaptic transmission efficiency, and as a result, the behavioral outputs can be significantly altered. For instance, the nematode *C. elegans* uses two opposing neuromodulators, serotonin and the pigment dispersing factor (PDF) to shift between two behavioral states (e.g. roaming and dwelling) and prolongs one behavioral state once it is initiated (Flavell et al., 2013).

One such modulator of neural circuits is neuropeptides. There are dozens of different neuropeptides expressed by a variety of neurons in the brain. The complexity of neuropeptide ligands is matched by the myriad of neuropeptide receptors to mediate diverse effects on neural circuits by modulating synaptic transmission. As neuropeptides

do not require synapses to cause effects but can diffuse long distances inside the brain, it is extremely challenging to predict what behavioral outputs certain neural circuits generate just from a wiring diagram (Bargmann, 2012). Neuropeptides can also work as neurotransmitters and hormones (Burbach, 2011). To exert their unique functions, neuropeptides must bind their receptor proteins on the membrane surface of the target cells. Binding the receptor causes a cascade of biochemical reactions that leads to changes in behaviors or physiological states of the organism. In fact, neuropeptides play pivotal roles in modulating behaviors across the animal kingdom (Asahina et al., 2014; Beets et al., 2012; Beets et al., 2013; Dolen et al., 2013; Dolen & Malenka, 2014; Flavell et al., 2013; Garrison et al., 2012; Hergarden et al., 2012; Kim et al., 2013; Leinwand & Chalasani, 2014; Taghert & Nitabach, 2012; Woods et al., 2014), and uncovering the underlying mechanisms will be essential for fully understanding how the brain controls behaviors. Importantly, deficits in neuropeptide signaling has been associated with various human disorders including debilitating psychiatric disorders such as schizophrenia and autism (Hammock & Young, 2006), and a deeper understanding of the mechanism by which neuropeptides modulate behaviors may lead to substantial therapeutic benefits.

A neuropeptide called arginine vasopressin (AVP), a hypothalamic nonapeptide (consisting of nine amino acids), has attracted significant attention because it plays important roles in controlling mammalian social behaviors. Mounting evidence indicates that a type of AVP receptor, called V1a receptor, modulates the social behaviors of animals (Hammock & Young, 2006). In fact, the differential expression patterns of V1a in the brain appeared to determine the sociality of the animals. Socially monogamous voles (prairie voles) express more V1a receptor in the ventral pallidum than do promiscuous voles

(montane and meadow voles) (Lim et al., 2004a; Lim et al., 2004b). Amazingly, genetically altering the expression pattern of V1a receptors of mice, which are promiscuous, to the pattern of monogamous voles induced social bonding behaviors in mice (Young et al., 1999). Furthermore, virally mediated expression of the V1a receptor in the ventral pallidum of promiscuous voles, which stimulates normal expression of V1a receptors in monogamous voles, increased social bonding in these voles (Lim et al., 2004c) while virally mediated down-regulation of pallidal V1aR impairs pair bonding behaviors in monogamous voles (Barrett et al., 2013). This clearly demonstrates the critical roles of V1a in determining social behaviors. This change in social behaviors is thought to be mediated by AVP/V1a receptors modulation of the reward circuitry within the ventral pallidum (Hammock & Young, 2006). However, V1a receptors are actually expressed in multiple brain regions, and it is unclear how these various regions may be involved with the modulation of social behaviors. Also unclear is how AVP is delivered to different regions of the brain from AVP synthesizing neurons in the hypothalamus. Moreover, recent studies suggest that V1aR is also expressed in the spinal cord of neonatal rodents (Liu et al., 2003; Tribollet et al, 1991), and application of AVP onto isolated spinal cord preparation modulates motor central pattern generator functions (Barriere et al., 2005; Pearson et al., 2003). AVP application also appears to directly excite hypoglossal and facial motoneurons (Reymond-Marron et al., 2006) and spinal motoneurons of neonatal rats (Oz et al., 2001). This suggests that AVP may modulate central pattern generators (CPGs) in the spinal cord at least in the neonatal rodent CNS. However, it is unclear how AVP is delivered from the hypothalamus to these distant sites in the CNS (e.g. the brainstem and the spinal cord) although the known direct projection from hypothalamic AVP neurons into the spine may

carry the peptide. Besides, autoradiography binding assay suggests that AVP binding sites are ubiquitous in the spinal cord of neonatal rats (Liu et al., 2003), but the specificity of the autoradiography may be questionable as the entire central gray matter were shown to bind AVP, and the identity of the receptor subtypes was not clear. It is thus still unclear how AVP signaling modulates neural circuits that regulate locomotion or social behaviors.

The studies investigating roles of V1a receptor in social behaviors in the rodent brain, however, do provide some key insights into how neuropeptide signaling can modulate behaviors. i) A single neuropeptide can have multiple types of receptors, each of which can be expressed in multiple brain regions. For instance, AVP binds to AVPR1a, AVPR1b, & AVPR2, and a receptor for oxytocin, a neuropeptide similar to AVP, to a lesser degree (reviewed by Holmes et al., 2003; Raggenbass, 2008). AVPR1a is expressed widely in the brain, AVPR1b is expressed in the pituitary, and AVPR2 is expressed in the periphery including the kidney. ii) as a consequence each receptor subtype can serve a distinct behavioral function depending on where they are expressed and how they affect targeted cell's functions. iii) Manipulations (e.g. genetic, molecular, or pharmacological) of neuropeptide release by the hypothalamic neurons, a major source of the peptide, may affect all receptor subtypes. Therefore, to understand the roles of each receptor subtype, it is important to investigate one receptor type at a time. All these points were considered in designing experiments for the current project.

Despite insights into how AVP regulates social behavior, a mechanistic understanding of how AVP modulates neural circuits is lacking. This goal is formidable due to the tremendous complexity of larger mammalian brains. One strategy to get at the underlying mechanisms would be to analyze how AVP or its non-mammalian homolog,

arginine vasotocin (AVT), regulates neural circuits in a vertebrate model organism with a simpler brain and simpler behaviors that can be subjected to molecular and genetic manipulations. We decided to use one such model, zebrafish (*Danio rerio*) that exhibit just these desired attributes. Unlike other popular small model organisms like fruit flies (*Drosophila melanogaster)* and roundworms (*C. elegans)*, zebrafish is a vertebrate organism and thus has a vertebrate brain that contains all the basic regions and neuronal types of the mammal brain. Importantly, both AVT and its receptor genes are expressed in the CNS of embryonic zebrafish at the time when the organism is capable of a limited number of simple behaviors as described below. The simplicity of the zebrafish CNS allows for the identification of specific behavioral roles of the neuropeptide signaling.

AVT, like AVP, is linked to complex social behaviors across non-mammalian vertebrates including teleosts (Filby et al., 2010; Gobrogge et al., 2009; Haller, 2013; Huffman et al., 2014; Larson et al., 2006; Lema et al., 2015; Yokoi et al., 2015). AVT alters behaviors presumably by modulating the underlying neural circuits; however, the circuit identity nor the mechanism by which the circuits are modulated by AVT are largely unknown. In teleost species including zebrafish, AVT is thought to play roles in modulating aggression (Backstrom & Winberg, 2009; Filby et al., 2010; Larson et al., 2006; Lema et al., 2015; Santangelo & Bass, 2006; Yokoi et al., 2015). However, past studies focused on the correlation between AVT expression in brain regions and aggression without addressing what neural circuits are modulated and whether differences in AVT expression lead to changes in aggressive behaviors. For instance, differential expressions of AVT, both on mRNA and protein levels, were reported in aggressive versus non-aggressive zebrafish in the hypothalamus that contains the preoptic nucleus, the sole source of AVT in zebrafish

brains (Filby et al., 2010; Larson et al., 2006). However, the result is purely correlational and it is unclear if the differential AVT expression causes difference in aggression or is simply a consequence of aggressive interactions. To test causation, researchers have injected AVT agonists/antagonists to test if aggressive behaviors change. However, the injection is commonly done peripherally, for instance via intraperitoneal injection (Filby et al., 2010), and this raises serious questions regarding how much exposure the brain has to such AVT injections. Furthermore, it is unclear how much the reported effects of peripheral AVT injections are due to the activation of peripheral AVTR subtypes including AVTR2 that modulates peripheral functions such as kidney functions. As previously stated mammalian studies indicate that the behavioral effect of AVT is mediated by V1a-like subtype receptor expressed widely in the CNS; however these teleost studies may be simply assaying the peripheral effects of AVT. Thus, the genetic, molecular mechanism by which AVT modulates neural circuitry in the brain is to date poorly understood. The main motivation of the current study was to provide mechanistic insights into the modulation of behaviors by AVT mediated V1a-like receptors. The outcome of the research will hopefully contribute to better understanding of neuropeptidergic modulation of behaviors of vertebrates including humans for eventual therapeutic benefits.

When the current study was initiated, there were no published description of V1a-like AVTRs in zebrafish CNS, and it was not known where in the zebrafish brain AVTRs are expressed and when the AVTR expression starts during the development. However, such information is essential to understand the behavioral roles of AVT signaling, and therefore as a first step I cloned two V1a-like AVT receptors (*avtr*) in the zebrafish CNS. We named the cloned receptors *avtr1a1* and *avtr1a2* because of their homology to mammalian V1a

receptors (Iwasaki et al., 2013). Utilizing the obtained clones, we conducted the expression analysis for *avtr1a1* and *avtr1a2* via *in situ* hybridization. To our surprise, both vasotocin receptors are expressed in multiple nuclei in the embryonic zebrafish CNS as early as 1-day post fertilization (dpf) (Iwasaki et al., 2013). These findings correlate with the embryonic expression of *avt* itself (Eaton et al., 2008) and the expression of *avt* and its receptors precedes the appearance of any type of social behaviors, which start much later. In fact, zebrafish embryos engage predominantly in simple sensory-induced locomotor responses. Therefore, this raises an important question of whether this neuropeptide known best for its effect on adult social behaviors in fact modulates early sensorimotor behaviors. Sensorimotor hypothesis of AVT signaling has been proposed by scientists based on studies on amphibian behaviors (Rose & Moore, 2002). However, it was not tested systematically because of the lack of genetic, molecular manipulation techniques in the amphibian system, so how AVT regulates neural circuits for sensorimotor responses is still unknown.

Chapter II describes the expression pattern of the *avtrs* in the embryonic CNS. Based upon this, we hypothesized that AVT signaling regulates sensorimotor circuits and behavior in zebrafish embryos. Chapter III describes the functional manipulations of AVT signaling using molecular and pharmacological techniques designed to test this hypothesis. In fact, the obtained data led us to formulate a mechanistic model that accounts for how the neuropeptide modulates neural circuits. Having established a role for modulation of sensorimotor behavior by AVT, I turned my attention to how AVT signaling might regulate aggression in adult zebrafish. Chapter IV describes the behavioral analysis of aggression by male zebrafish including social conditions that dramatically repress aggression. The chapter also describes the expression pattern of the *avtrs* in the adult zebrafish brain to

better understand how AVT signaling may regulate aggression in mature zebrafish. As a whole, the outcome of the current research will provide new insights into the mechanism by which the neuropeptide in question modulates behaviors throughout the development.

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Chapter II. Expression of Arginine Vasotocin Receptors in the Developing Zebrafish CNS

1. Introduction

Signaling within the CNS via the nonapeptide, arginine vasopressin (AVP) in mammals and its homolog arginine vasotocin (AVT) in nonmammalian vertebrates, regulates social and reproductive behaviors in a wide variety of species (reviewed in Donaldson and Young, 2008). Dysfunction of signaling by AVP and oxytocin, another nonapeptide implicated in social behaviors, is thought to contribute to psychiatric disorders such as autism, affective disorders, obsessive-compulsive disorder, posttraumatic stress disorder and schizophrenia (reviewed in Heinrichs et al., 2009). Furthermore, AVP/AVT released into the circulation by the posterior pituitary in response to sexual stimulation, stress and dehydration mediates a variety of peripheral effects including antidiuretic activity by the kidney (Leng and Bicknell, 1986; Nishimura and Fan, 2003).

AVP is expressed by neurons of the supraoptic, paraventricular and suprachiasmatic nuclei of the hypothalamus in mammals (Brownstein et al., 1980; Young and Gainer, 2003) and AVT primarily by neurons of the preoptic area in fish (Venkatesh and Brenner, 1995; Acher et al., 1997). Additionally AVP is expressed by the bed nucleus of the stria terminalis and amygdala in the mammalian brain (DeVries and Buijs, 1983; DeVries et al., 1985). There are 3 AVP receptors in mammals with the $AVPR_{1a}$ (V1a) and $AVPR_{1b}$ (V1b) receptors

expressed primarily in the CNS and the $AVPR_2$ (V2) receptor in the periphery (Caldwell et al., 2008). AVT receptors have been identified in a number of teleosts as well (Mahlmann et al., 1994; Conklin et al., 1999; Warne, 2001; An et al., 2008). In the pupfish and perhaps other teleosts there appear to be two V1a receptors and a V2 receptor for AVT (Lema, 2010). Like the mammalian V1a receptor, RT-PCR found that the pupfish V1a receptors are widely expressed throughout the CNS.

AVT is expressed by cells in the ventral hypothalamus and the preoptic area of the diencephalon, and isotocin in the preoptic area (Tessmar-Raible et al., 2007; Eaton et al., 2008; Blechman et al., 2011) during embryogenesis in zebrafish prior to the development of social or reproductive behaviors. During embryogenesis behaviors exhibited by zebrafish embryos are restricted to simple motor responses such as escape swimming evoked by sensory stimulation (Saint-Amant and Drapeau, 1998). The embryonic expression of AVT suggests that it may participate in the development of sensory and/or motor circuits early in development. As a first step in examining the function of AVT signaling in the embryonic CNS, we cloned two V1a type receptors in zebrafish and determined their expression patterns during early stages of development. The expression pattern of the V1a receptors is concordant with the hypothesis that AVT signaling may play a role in early sensory/motor function.

2. Materials & Methods

Fish breeding and maintenance

Zebrafish (*Danio rerio*) were maintained in a breeding facility following the guidelines set forth by the University of Michigan Animal Care and Use protocols (Zhou et al., 2008). The

fish were provided daily with either dry food (Micro pellets, Kyorin, Japan) or brine shrimp (Brine Shrimp Direct, Utah). Embryos were collected and incubated at 28.5^{o}C and staged according to hours post fertilization (hpf; Westerfield, 1995). For *in situ* hybridization of the embryos older than one day post-fertilization, the embryos were kept in 0.2 mM 1-Phenyl-2-thiourea (Sigma, P-7629) to keep them transparent until they were fixed at desired developmental stages. Transgenic lines used in this paper were *otpb.A:GAL4* (Tg(*otpb:GAL4-VP16, myl7:GFP*)^{zc57}) and Tg(*UAS:GFP*) (Fujimoto et al., 2011); *otpb.A:egfp^{caax}* (Tg(*otpb:1EGFP*)^{zc49}) (Xing et al., 2012); and *pitx2c:egfp* (Tg(*pitx2:EGFP*)^{zy8}) (Wolman et al., 2008).

cDNA cloning of zebrafish AVTRs

cDNA cloning for AVTRs was conducted following standard protocols (Zhou et al., 2008). Whole brains were dissected from male adult zebrafish (approximately 14 months post fertilization, N=4) and total RNA was extracted following the manufacture's protocol for TRIzol® Reagent (Invitrogen, #15596-018). The RNA was reverse transcribed with oligo-dT primers (Invitrogen, #58862) following the manufacture's protocol for SuperScript® II Reverse Transcriptase (Invitrogen, #18064-022). For the PCR of *avtr1a1* cDNA, the following primers were used to amplify the coding sequence along with 5'UTR and 3'UTRs; forward primer, 5'-GCTCGCGCCTTTACGCATGA-3', and reverse primer, 5'-ACAGGAGGGTAAATGCTTTTGACT-3'. To PCR *avtr*1a2, the following primers were used; forward primer, 5'-CGCGCGTATTTCCATCAATCAAGC-3', and reverse primer, 5'-AATCGCTGTGCTTTCAGCTGGT-3'. The PCR products were purified and cloned into pGEM

T-easy vector following the manufacture's instructions (Promega, #A137A). The obtained clones were sequenced at the University of Michigan Sequencing Core.

Sequence analysis

Amino acid alignments for human V1a (NP_000697), zebrafish $AVTR_{1a1}$ and $AVTR_{1a2}$ (2. 1.1) were constructed with ClustalW (BiologyWorkbench, SCSD; http://seqtool.sdsc.edu). Default settings of the textshade option (Biology Workbench) were adopted except that amino acid identity (dark gray) and similarity (light gray) were color-coded. The amino acids were numbered corresponding to that for zebrafish $AVTR_{1a1}$. The transmembrane domains for zebrafish $AVTR_{1a1}$ (black bars) were determined using MEMSAT3 (http://bioinf.cs.ucl.ac.uk/psipred). The white box indicates the DUF1856 domains (unknown function) conserved in the C-termini of various AVP receptors (conserved domain search; http://www.ncbi.nlm.nih.gov/cdd). The intracellular loops (ICL) and extracellular loops (ECL) are also indicated. Phylogenetic tree was constructed based on the alignment of amino acid sequences for vasopressin/vasotocin receptors across vertebrate species with the MEGA 4.0 software (neighbor-joining method with pairwise deletion and bootstrap values from 1000 replicates) as described by Lema (2010). GenBank accession numbers are provided in the parentheses.

Whole-mount in situ hybridization & immunolabeling

To synthesize RNA probes for *in situ* hybridization for *avtr* genes, partial coding sequences (685 bp for *avtr1a1* & 658 bp for *avtr1a2*) were cloned into pGEM T-Easy vectors using the following primers; forward primer (*avtr1a1*), 5'-CTTGGGAATGTTCGCGTCCACTTA-3',

reverse primer (*avtr1a1*), 5'-TTAGGCTGTTTCCCATGCTGGAAC-3', forward primer (*avtr1a2*), 5'- GGACTTTCTGTGCAGGATCGTCAA-3', and reverse primer (*avtr1a2*), 5'-TCCGCTGAACACCATGTAGATCCA-3'. The partial clones were sequenced at the University of Michigan Sequencing Core and confirmed to be identical to the complete AVTR cDNA clones described above (cDNA cloning of zebrafish AVTRs). The partial cDNA clones were used to synthesize, *in vitro*, digoxigenin (DIG)-labeled antisense probes with DIG RNA labeling mix (Roche) following the manufacture's instructions for mMESSAGE mMACHINE Kit (Ambion, Life Technologies). The sense probe for *avtr1a1* was used as a negative control and found to generate no significant labeling (not shown). The standard protocol was followed to conduct *in situ* hybridization (Zhou et al., 2008). Anti-DIG-AP Fab fragments (Roche) was used to carry out color reactions using NBT/BCIP as a substrate (Roche). For fluorescent double-labeling, Fast Red (Roche) was used as a substrate for *in situ* hybridization and immunolabeling was carried out with primary antibodies [anti-acetylated α tubulin (1:1000, Sigma-Aldrich), anti-GFP (1:1000, Torrey Pines Biolabs), anti-Neurofilament-M/RMO44 (1:500, Invitrogen, Life Technologies)] and Alexa488-anti-mouse/rabbit IgG (1:500, Invitrogen, Life Technologies). The labeled embryos were mounted in Vectashield mounting medium (Vector Laboratories) and fluorescent images were acquired with a Leica SP5 laser scanning confocal microscope.

For double *in situ* hybridization, dinitrophenol (DNP)-labeled anti-sense riboprobes for *avtr1a1* were synthesized with DNP-11-UTP (PerkinElmer), and DIG-labeled riboprobes for *avtr1a2* were synthesized using DIG RNA labeling mix (Roche). Prior to *in situ* hybridization, the embryos were treated with 2% hydrogen peroxide, and dextran sulfate was added to the hybridization reaction as described by Lauter et al. (2011). The alkaline

phosphatase-mediated color reaction was first carried out for *avtr1a2* using anti-DIG-AP Fab fragments (Roche) with NBT/BCIP (Roche) as a substrate. After anti-DIG-AP was inactivated by 0.1 M glycine-HCL (pH 2.2), the color reaction for *avtr1a1* was conducted using anti-DNP-AP (Vector Laboratories) with INT/BCIP (Roche) as a substrate. The labeled embryos were mounted in 70 % glycerol to obtain bright-field microscope images.

3. Results

3.1. Zebrafish contain two AVT receptors homologous to $AVPR_{1a}$

We cloned two AVT receptors by RT-PCR from adult zebrafish brain tissue, $AVTR_{1a1}$ and $AVTR_{1a2}$, that were 60% and 62% identical at the amino acid level with human V1a receptor and 54% and 52% identical with human V1b receptor, respectively (blastp, www.ncbi.nlm.nih.gov) (Fig. 2.1A). A phylogenetic analysis of zebrafish $AVTR_{1a1}$ and $AVTR_{1a2}$ was consistent with the assignment of the zebrafish receptors as V1a type (Fig. 2.1B). These findings are consistent with previous findings of a duplicated V1a receptor in teleosts (Lema, 2010).

3.2. avtr1a1 and avtr1a2 are expressed primarily in the CNS in early stage zebrafish

By 25 hours postfertilization (hpf) *avtr1a1* and *avtr1a2* are expressed by a cluster of cells in the forebrain and by a small number of discrete cells in the hindbrain (Fig. 2.2A). The earliest expression seen via *in situ* hybridization was *avtr1a1* in the forebrain and hindbrain at 22 hpf (not shown). Examination of *avtr1a1* expressing cells in embryos in which all axons are labeled with anti-acetylated α-tubulin found that the forebrain cells are in apparent contact with the postoptic commissure (POC) and/or tract of the postoptic

commissure (TPOC) (Fig. 2.2B; Chitnis and Kuwada, 1990), which is consistent with these cells projecting axons into the POC/TPOC. These *avtr1a1+* neurons as the putative *avtr1a1+* epiphyseal and nucleus of the posterior commissure neurons (see below) may also express HNK-1 since the pattern of early neurons/axons labeled with anti-HNK-1 and anti-acetylated α-tubulin are similar (Wilson et al., 1990). Additionally there are irregularly spaced, occasional dorsal cells in the spinal cord of unknown identity that express *avtr1a1* (Fig. 2.2C).

By 48 hpf *avtr1a1* is expressed by a cluster of ventral forebrain cells (I), forebrain cells near the dorsal midline (II), dorsal cells located at the forebrain/tectum boundary (III) ventral cells near the forebrain/tegmentum border (IV), and two longitudinal stripes of cells in the midbrain/anterior hindbrain region (V and VI) when viewed from a dorsal perspective as well as the posterior hindbrain cells (Fig. 2.3A-C). Lateral views show that in the ventral forebrain cluster I is located anterior and ventral to cluster IV (Fig. 2.3D). Examination of cluster I *avtr1a1*+ cells in 48 hpf embryos with all axons labeled with anti-acetylated α–tubulin showed that these neurons appear to project axons into the TPOC (Fig. 2.4A) suggesting that these are the POC/TPOC forebrain neurons observed earlier (Fig. 2.2B). Examination of forebrain *avtr1a1*+ cells in *otpb.A:GAL4; UAS:gfp* embryos that express GFP in preoptic neurons (Fujimoto et al., 2011) showed that cluster I *avtr1a1*+ cells are adjacent to the preoptic neurons at 48 hpf (Fig. 2.4B). Furthermore at 72 hpf *avtr1a1* is expressed in the region immediately posterior to the preoptic region, either ventral or dorsal to the preoptic neurons (not shown). Thus preoptic neurons do not express *avtr1a1* during the embryonic stages examined.

The dorsal forebrain cells (II) are found at the lateral base of the epiphysis, which can be seen with DIC optics in 48 hpf embryos (Fig. 2.3A inset). These cells are likely neurons since they appear to extend axons in the dorsal-ventral diencephalic tract (DVDT) (Fig. 2.5A; Chitnis and Kuwada, 1990; Wilson et al., 1990). The cluster of neurons on the left side of the brain appears to be larger than that on the right side (Fig. 2.3A inset) in accord with the left-right asymmetry exhibited by the pineal complex in embryonic zebrafish (Concha et. al., 2000). The forebrain/tectum boundary cells (III) could be neurons of the nucleus of the posterior commissure (nucPC) since they appear to extend axons in the PC (Fig. 2.5B; Chitnis and Kuwada, 1990). The *avtr1a1* expressing cells near the midbrain/hindbrain boundary (V) at approximately 48 hpf could be neurons of the nucleus of the Medial Longitudinal Fasciculus (nucMLF) since these neurons reside in this region. nucMLF neurons express *pitx2c* and are labeled by EGFP in *pitx2c:egfp* zebrafish (Wolman et al., 2008). However, *in situ* hybridization for *avtr1a1* in these transgenic embryos showed that the *avtr1a1* expressing cells were in the same region as the nucMLF neurons but did not express *pitx2c:egfp* suggesting they were not nucMLF neurons (Fig. 2.5C). It is noteworthy that the medial longitudinal stripe of cells (V) are also located in a position similar to that of the raphe serotonergic neurons (Lillesaar et. al., 2007).

Given that many neurons in the hindbrain are characterized by their stereotyped locations, morphology and molecular properties, we also examined the *avtr1a1*+ hindbrain cells in order to determine their identities. The caudal most *avtr*+ cells are located in the region of the T reticular neurons that project into the contralateral MLF and can be labeled with MAb RMO44 (Skromne et al., 2007), which recognizes a specific neurofilament expressed by these neurons. Double-labeling with RMO44 and *in situ* hybridization for

avtr1a1 showed that the caudal *avtr1a1* positive neurons are not T reticular neurons (Fig. 2.5D). Additionally, the double-labeled embryos demonstrated that the M cell which projects posteriorly into the contralateral MLF and are also labeled by RMO44 do not express *avtr1a1*.

At 48 hpf *avtr1a2* appears to be expressed by fewer cells in the midbrain/anterior hindbrain region compared with *avtr1a1* while the forebrain expression of the receptors is similar to that for *avtr1a1* (Fig. 2.6A,D). Since *avtr1a1* and *avtr1a2* are expressed in similar patterns in the brain, it is possible that they may be co-expressed in neurons. However, double *in situ* hybridization of 48 hpf embryos for both receptor genes found that the two receptors appeared generally not to be co-expressed by neurons (Fig. 2.6B,C,E,F).

avtr1a1 is expressed by cells outside of the CNS with expression seen in the endothelial cells of the developing vasculature in the trunk at 24 hpf (Fig. 2.2C); the pharyngeal arches; a midline structure ventral to the brain between the eyes (Fig. 2.3D); and cells in the space between the lens and the retina by 48 hpf (Fig. 2.3E).

3.3 The morphology and location of hindbrain avtr1a+ neurons are consistent with a role in sensory/motor responses

We determined the axonal trajectories of *avtr1a1*+ neurons by combined *in situ* hybridization and immunolabeling with anti-acetylated α–tubulin. We found that the hindbrain *avtr1a1*+ neurons project axons towards and into the ipsilateral MLF (Fig. 2.7A). The fact that these *avtr1a1*+ neurons project into the MLF indicates that these neurons may participate in regulating motor responses since many neurons with axons within the MLF are known to have a motor function in teleosts including zebrafish (Zottoli, 1977; Nissanov

et al., 1990; Gahtan et al., 2002). A group of cells known as IC neurons are located in the caudal hindbrain and project axons in the ipsilateral MLF (Mendelson, 1986; Metcalfe et al., 1986). IC neurons are rhythmically active during spontaneous coiling of the body (Saint-Amant and Drapeau, 2001). At present there are no markers for these neurons so it is not possible to readily identify whether IC neurons are *avtr*+.

Many neurons that participate in motor responses in zebrafish receive sensory input (Gahtan et al., 2002; Kohashi and Oda, 2008). In the zebrafish, hindbrain sensory axons extend along the lateral longitudinal fasciculus (LLF). Axons in the LLF appear to course over the cell bodies of the *avtr1a1*-expressing neurons as seen in confocal stack images and in single focal planes (Fig. 2.7B), so potentially could make synapses with the *avtr1a1*+ neurons.

The finding that the axons of *avtr1a1*+ neurons project into the MLF and the proximity of their cell bodies to the RB sensory axons of the LLF are consistent with a role for *avtr1a1*+ neurons in motor responses of embryos to sensory stimulation. Further physiological and morphological analysis of *avtr1a1*+ neurons will be required to establish this hypothesis.

3.4 Preoptic neurons project axons in the vicinity of avtr1a1-positive neurons

Since *avt*, *avtr1a1,* and *avtr1a2* are expressed early in zebrafish, we wondered if the *avt*+ neurons innervate the receptor-expressing neurons in embryos. Unfortunately anti-AVT appears not to label AVT-expressing neurons until 5 dpf (Eaton et al., 2008), so we examined transgenic embryos in which *avt*+ neurons expressed GFP. Preoptic neurons express AVT and isotocin, an ortholog of mammalian oxytocin, and this requires expression

of the homeobox transcription factor, *orthopedia-b*, in preoptic neurons (Blechman et al., 2007; Ryu et al., 2007; Eaton et al., 2008; Lohr et al., 2009). Preoptic neurons can be visualized in the *otpb.A:egfp* and *otpb.A:GAL4; UAS:gfp* transgenic zebrafish that express GFP in preoptic neurons (Fujimoto et al., 2011). To examine whether preoptic neurons could innervate *avtr*+ neurons, transgenic *otpb.A:egfp*^{caax} embryos that express membrane-targeted EGFP in preoptic neurons were examined in combination with *in situ* hybridization for *avtr1a1*. In *otpb.A:egfp*^{caax} embryos EGFP-labeled axons from preoptic neurons projected posteriorly. In confocal stack images the posteriorly-projecting axons extend in the medial longitudinal catecholaminergic tract (MLCT; Kastenhuber et al., 2010) in close proximity to *avtr1a1* expressing neurons in the anterior and caudal hindbrain (Fig. 2.8). This could be seen in single focal planes as well (not shown). Thus preoptic neurons including those presumably expressing *avt* could be presynaptic to the *avtr*+ neurons. However, since preoptic neurons include those that express isotocin as well as vasotocin, physiological and anatomical examination of confirmed *avt* expressing neurons and *avtr*+ neurons will be required to establish synaptic interactions.

4. Discussion

Although AVP/AVT signaling within the brain has been widely studied as an important factor for reproductive and social behaviors (reviewed in Stoop, 2012), relatively few investigations have examined the development of AVP/AVT signaling during CNS development. Indeed AVP and oxytocin binding sites are found in the mammalian neonatal spinal cord (Liu et al., 2003; Stoop, 2012). Furthermore, it is likely that these neonatal spinal neurons receive input from AVP expressing axons: AVP is expressed by

paraventricular (PVN) and supraoptic hypothalamic neurons in rat embryos (Lipari et al., 2001), AVP expressing PVN cells innervate the spinal cord (Sawchenko and Swanson, 1982; Motawei et al., 1999; Hallbeck et al., 2001), and PVN axons project to the neonatal spinal cord (Leong et al., 1984; Kudo et al., 1993; Lakke, 1997). Our study demonstrates that AVT receptors are expressed in the CNS early in zebrafish development together with AVT (Eaton et al., 2008). This suggests that AVT signaling may regulate some aspect of CNS development (e.g. Boer et al., 1982a & b; Hammock and Levitt, 2012) and/or control early behaviors.

The expression of AVT and its receptors correlate with the earliest behaviors of zebrafish embryos. These are spontaneous coiling of the body and motor responses to sensory stimulation, touch-induced fast escape response and swimming (Saint-Amant and Drapeau, 1998). Thus it is possible that AVT signaling may play a role in regulating these early motor behaviors. Several findings from our study are consistent with this hypothesis. First, AVT receptor expressing neurons in the hindbrain project axons into the MLF as do many other neurons known to regulate motor behaviors in zebrafish. Second, the morphological relationship between sensory axons of the LLF and the AVTR expressing hindbrain neurons suggests that sensory neurons could synaptically activate *avtr*+ neurons. Third, *otpb* expressing preoptic neurons, which include AVT expressing neurons, project axons into the hindbrain with the axons in close proximity to *avtr*+ neurons. Since isotocin, the fish homolog to oxytocin, is also expressed during embryogenesis (Eaton et al., 2007), it is possible that both AVT and isotocin may regulate motor behaviors in zebrafish embryos.

Our hypothesis assumes that the interaction between the *avt+* preoptic neurons and the hindbrain *avtr+* neurons is a synaptic one based upon release of neuropeptide from axon terminals. AVP and oxytocin, as are other neuropeptides, are found in large dense core vesicles in neurons and can be released in a Ca^{2+} dependent fashion directly from the cell bodies and dendrites (Brownstein et al., 1980; Ludwig and Leng, 2006). Recently, however, physiologically relevant synaptic release of oxytocin from axon terminals was elegantly demonstrated in the amygdala (Knobloch et al., 2012). In the case of zebrafish embryos the cell bodies of *avt*+ preoptic neurons are in the diencephalon while the *avtr*+ neurons in question are located in the hindbrain. The distance between the *avt*+ preoptic cell bodies and *avtr*+ hindbrain neurons and the physical proximity of preoptic axons and the hindbrain neurons is consistent with a synaptic release from axon terminals of preoptic neurons. However it is also possible that AVT could act in a humoral fashion via release into the early vascular system and/or ventricles.

We hypothesize that binding of AVT by the hindbrain *avtr*+ neurons increases the excitability of these neurons and thus regulates the responsiveness of the receptor expressing neurons to sensory stimulation in zebrafish (Fig. 2.9) much like the sensorimotor processing role proposed for AVP/AVT (Rose and Moore, 2002). In fact AVP and oxytocin depolarize motor neurons, interneurons and preganglionic neurons in the rodent spinal cord (Suzue et al., 1981; Kolaj and Renaud, 1998; Oz et al., 2001; Liu et al., 2003). Application of AVP onto facial motor neurons and oxytocin onto vagal motor neurons leads to a TTX insensitive, voltage-dependent persistent I_{Na} (Raggenbass et al., 1991) while in neurons in the lumbar spinal cord AVP suppresses resting conductance to K^+ and enhances conductance to cations (Ogier et al., 2006). Furthermore, AVT induces an

inward current in oocytes expressing flounder *avtr* (Warne, 2001). Finally AVP can induce rhythmic activity in the spinal cord by itself or in combination with serotonin suggesting that AVP can activate central pattern generators (Pearson et al., 2003). Interestingly, the invertebrate homologs of AVP and oxytocin, conopressin, hirudotocin and annetocin, induced activation of a central pattern generator for reproductive behaviors in the Medicinal leech (Wagenaar et al., 2010) suggesting that regulation of motor behaviors might be an evolutionarily ancient function for these neuropeptides.

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Figure Legends

Fig. 2.1. Zebrafish $AVTR_{1a1}$ and $AVTR_{1a2}$. (A) Amino acid alignment for human V1a (NP_000697), zebrafish $AVTR_{1a1}$, and zebrafish $AVTR_{1a2}$. There is a high degree of amino acid conservation between human V1a and zebrafish AVTRs. The dark gray boxes indicate amino acid identity and light gray boxes similarity. The amino acid number corresponds to that for zebrafish $AVTR_{1a1}$. The black bars mark the transmembrane domains (TMs) for zebrafish $AVTR_{1a1}$. The white box indicates the DUF1856 domains conserved in the C-termini of various AVP receptors. The locations of the intracellular loops (ICL) and extracellular loops (ECL) are also indicated. (B) The phylogeny of AVP/AVT receptors.

Fig. 2.2. Vasotocin receptors (*avtr1a1* and *avtr1a2*) are expressed in discrete populations of cells in the CNS of 24/28 hpf embryos. (A) Dorsal views (anterior up) of *in situ* hybridizations of 25 hpf embryos showing that both *avtr1a1* and *avtr1a2* are expressed in the forebrain (A1, A3) and the posterior hindbrain (A2, A4). Arrows denote position of the posterior border of the otocyst. Scale:100 μm. (B) Combined *in situ* hybridization (red) and anti-acetylated α tubulin labeling of axons (green) showing *avtr1a1* expressing neurons in the forebrain appear to project axons into the postoptic commissure (white arrow) and tract of the postoptic commissure (28 hpf, lateral view with anterior right and dorsal up). Yellow arrow denotes the anterior commissure. Scale: 50 μm. (C) Lateral view of trunk of 24 hpf embryo showing *avtr1a1* expressing neuron in the dorsal spinal cord (arrow) and apparent endothelial cells forming blood vessels (arrowhead). Scale: 100 μm.

Fig. 2.3. More neurons express *avtr1a1* at 48 hpf. (A) View of the brain focused dorsally showing *avtr1a1*+ cells in the base of the epiphysis (II) and the forebrain/tectum boundary (III). Inset shows a higher magnification view of the cluster II *avtr1a1*+ cells with asterisk denoting the epiphysis and dotted line the midline. Scale: 100 μm for (A-C). (B) Same view focused more ventrally than (A) showing the *avtr1a1*+ cells at the forebrain/tegmentum boundary (IV) and two rough stripes of cells that cross the midbrain/hindbrain boundary (V and VI). (C) Same view focused even more ventrally showing the anterior forebrain *avtr1a1*+ cells (I). (D) Lateral view showing the *avtr1a1*+ cells in the anterior forebrain (I), near the dorsal midline of the forebrain (II), at the forebrain/tectum boundary (III), at the forebrain/tegmentum boundary (IV) and the lateral stripe near the midbrain/hindbrain boundary (VI). Outside the brain the pharyngeal arches (arrowhead) and a midline structure in between the eyes (arrow) also express *avtr1a1*. Scale: 100 μm. (E) View of eye showing *avtr1a1*+ cells (arrowhead) between the lens and the retina. Scale: 100μm.

Fig. 2.4. At 48 hpf the *avtr1a1*+ cells in the anterior forebrain (I) are the POC/TPOC neurons and not preoptic neurons. (A) Dorsal perspective (anterior up) of an embryo labeled with *avtr1a1* riboprobe (red) and anti-acetylated α tubulin labeled axons (green) showing that the forebrain *avtr1a1*+ cells appear to extend axons in the TPOC (arrow). Scale: 50 μm. (B) A ventral perspective of an *otpb.A:GAL4; UAS:gfp* embryo labeled with *avtr1a1* riboprobe showing that the anterior forebrain *avtr1a1*+ neurons (red) are located just medial to the preoptic neurons (green, yellow arrow). Blue arrow denotes the olfactory neurons that express GFP in the transgenic embryos. Scale: 100 μm.

Fig. 2.5. The epiphyseal and nucPC neurons but not the nucMLF nor T reticular neurons express *avtr1a1* at approximately 48 hpf. (A) Lateral view (anterior left, dorsal up) of a z-stack of confocal images of a embryo double labeled for *avtr1a1* and anti-acetylated α tubulin (axons) showing that the cluster II *avtr1a1*+ cells at the lateral base of the epiphysis (red, asterisk) project axons into the DVDT (green, arrowhead) and the *avtr1a1*+ cluster III cells (red, star) are adjacent to the PC (arrow). The *avtr1a1*+ cells seen ventrally are the neurons in the ventral forebrain and near the forebrain/tegmentum boundary (I and IV). Scale: 50 um. (B) A single focal plane seen in a lateral view showing that the cluster III *avtr1a1*+ cells (red) appear to extend axons in the PC (green, arrows). Scale: 50 μm. (C) Ventral perspective of *pitx2c:egfp* embryos labeled with a *avtr1a1* riboprobe showing that the nucMLF neurons (green, bracket) do not express *avtr1a1* (red). Arrowhead denotes the MLF; asterisk denotes the forebrain *avtr1a1*+ neurons. Scale: 100 μm. (D) Dorsal perspective (anterior left) of the hindbrain of embryo double labeled for *avtr1a1* riboprobe (red, bracket) and MAb RMO44 (green) showing that the posterior hindbrain *avtr1a1*+ cells are not the T reticular neurons. The arrowhead indicates *avtr1a1+* cells in the otocyst. Scale: 100 μm.

Fig. 2.6. *avtr1a2* is expressed in a pattern similar to *avtr1a1* but they are not coexpressed by neurons. Dorsal perspective showing that *avtr1a2* is expressed in the brain (arrow and arrowhead) (A) and hindbrain (D) at 48 hpf. Double *in situ* hybridizations for *avtr1a1* and *avtr1a2* shows that brain neurons generally do not coexpress *avtr1a1* and *avtr1a2* (B and C) nor do posterior hindbrain neurons (E and F) at 48 hpf. B and C are the same field of view from the same embryo as are E and F. Scale for A-D: 100 μm and for E-F: 50 μm.

Fig. 2.7. *avtr1a1* expressing neurons in the caudal hindbrain project axons into the ipsilateral MLF and are in close proximity with the LLF at 28 hpf. (A) Lateral view (anterior left) showing caudal hindbrain, *avtr1a1*+ neurons (red) project axons (labeled with anti-acetylated α tubulin) into the ipsilateral MLF (arrow). Scale: 20 μm. (B) Lateral view showing that the LLF (green, bracket) courses over the *avtr1a1*+ neurons (red). Asterisk denotes a RB sensory neuron. Inset: single confocal plane showing LLF axons coursing over *avtr1a1*+ hindbrain neurons. Scale: 50 μm.

Fig. 2.8. *avtr1a1*+ neurons are in close proximity to the axons of *otpb* expressing preoptic neurons at 49-51 hpf. (A) Lateral view (anterior left) in an *otpb.A:egfp*^{caax} embryo showing that the anterior hindbrain *avtr1a1*+ neurons (red) are in close proximity to the axons of preoptic neurons in the MLCT (arrow). Scale: 50 μm. (B) Lateral view in an *otpb.A:egfp*^{caax} embryo showing that the *avtr1a1*+ neurons in the caudal hindbrain are in close proximity to the preoptic axons in the MLCT (arrow). Scale: 50 μm.

Fig. 2.9. Model for the regulation of the responsiveness of *avtr*+ neurons in the hindbrain to sensory input in zebrafish embryos. In the model AVT is constitutively released at the synapse onto *avtr*+ neurons to enhance activation of these neurons by sensory stimulation and modulate motor responses mediated by the central pattern generator (CPG). Dashed arrow denotes hypothesized connection between the hindbrain *avtr*+ neurons and the CPG.

Figures

Fig. 2.1 Amino acid and phylogenetic analysis of cloned *avtrs*

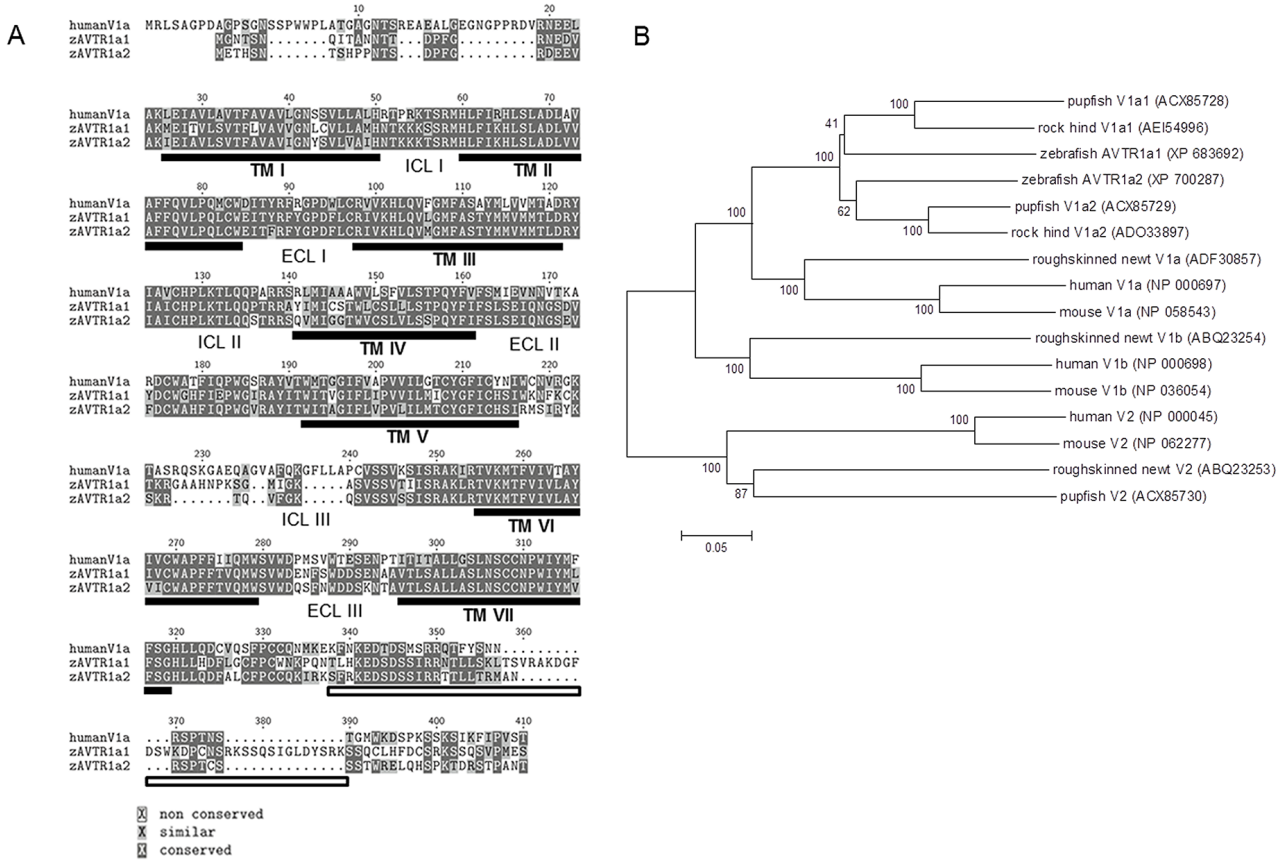


Fig. 2.2. Embryonic expression of *avtrs*

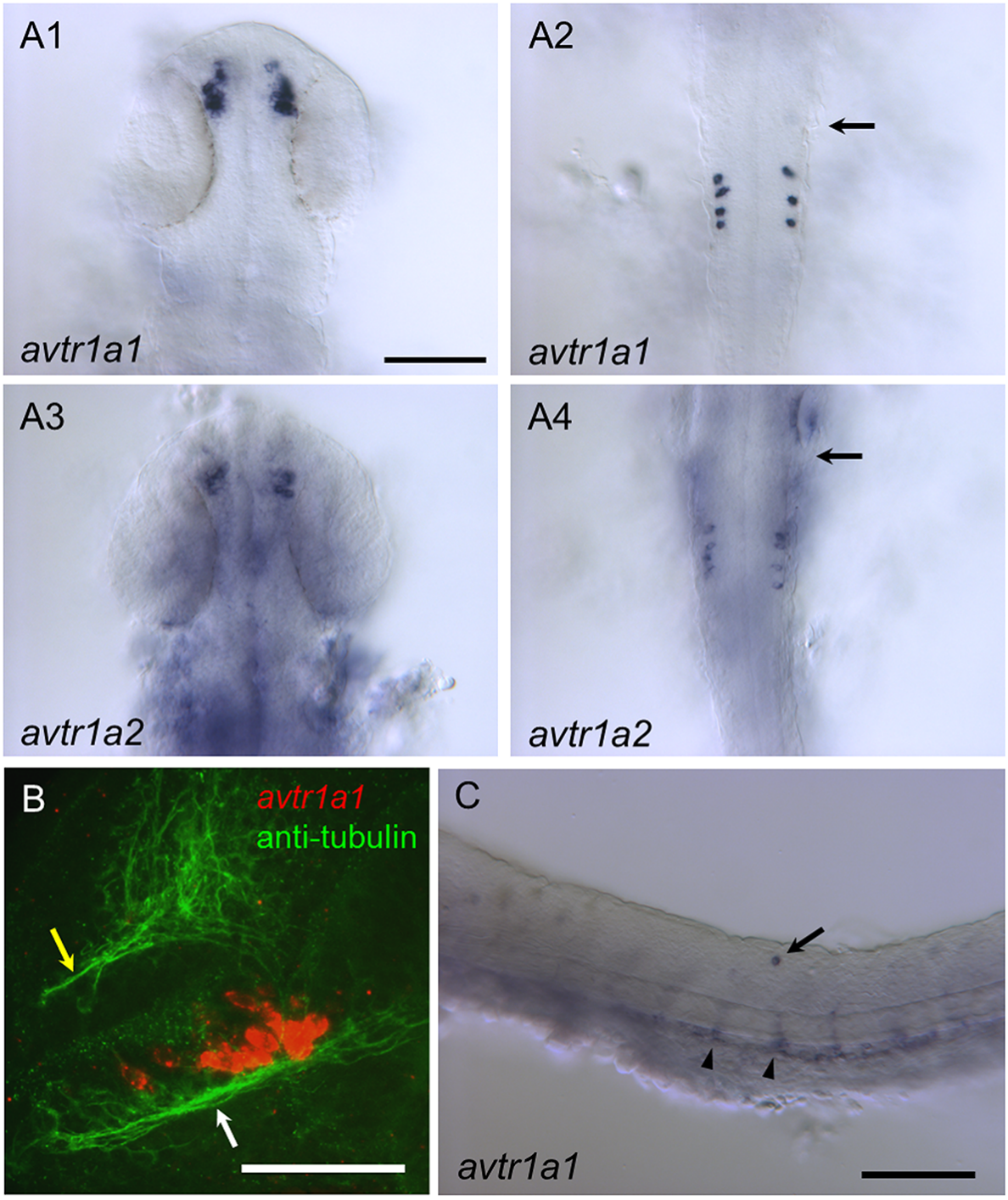


Fig 2.3. *avtr1a1* expression in embryonic brain nuclei

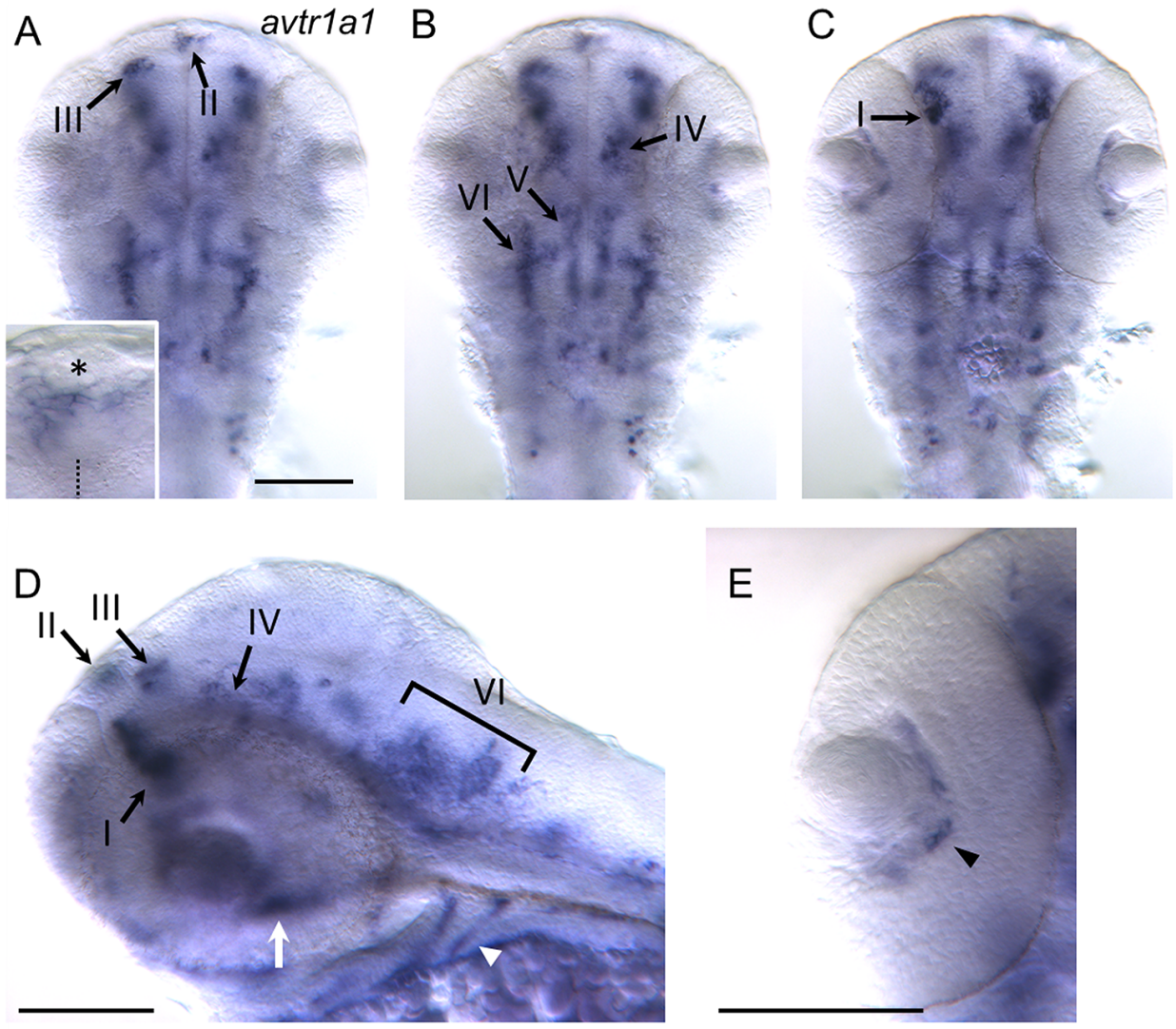


Fig. 2.4. *avtr1a1* expression in the anterior forebrain

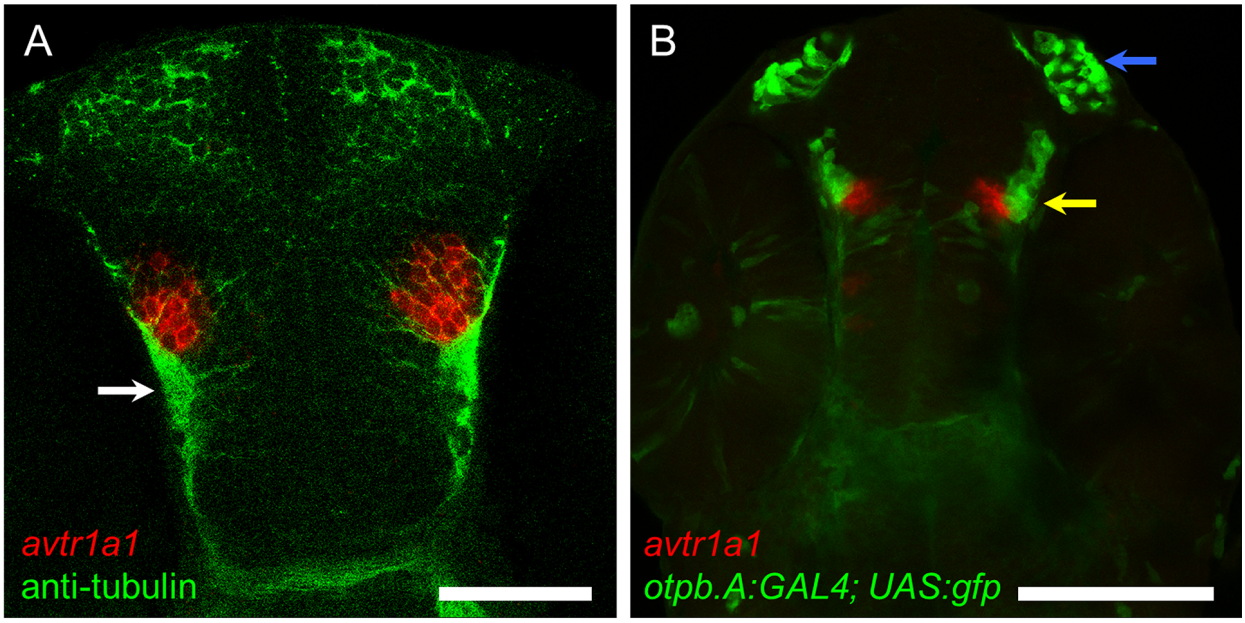


Fig. 2.5. *avtr1a1* expression in epiphyseal and nucPC neurons

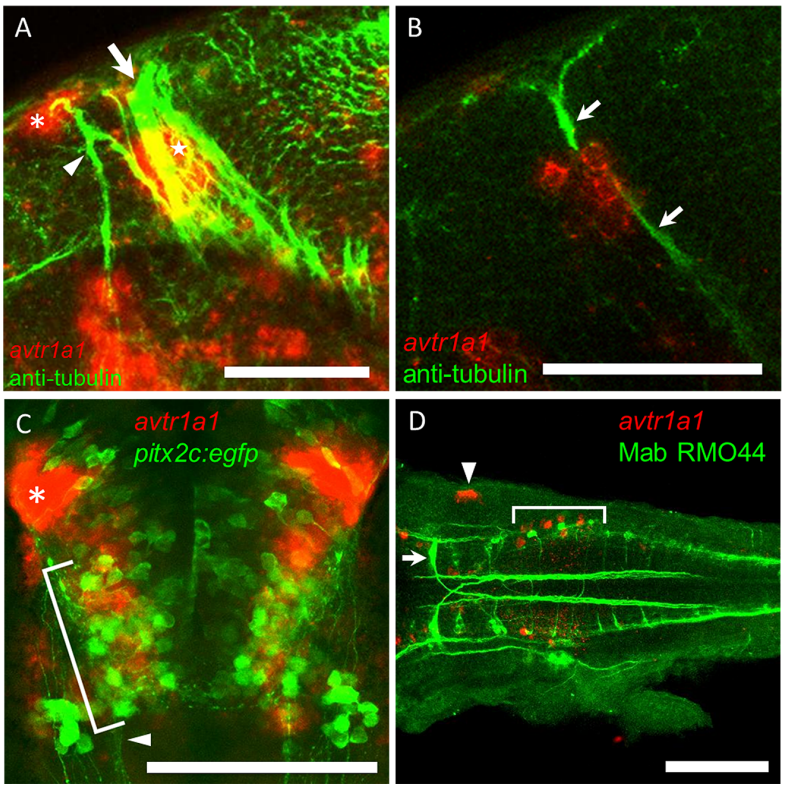


Fig. 2.6. *avtr1a1* and *avtr1a2* are not coexpressed in neurons

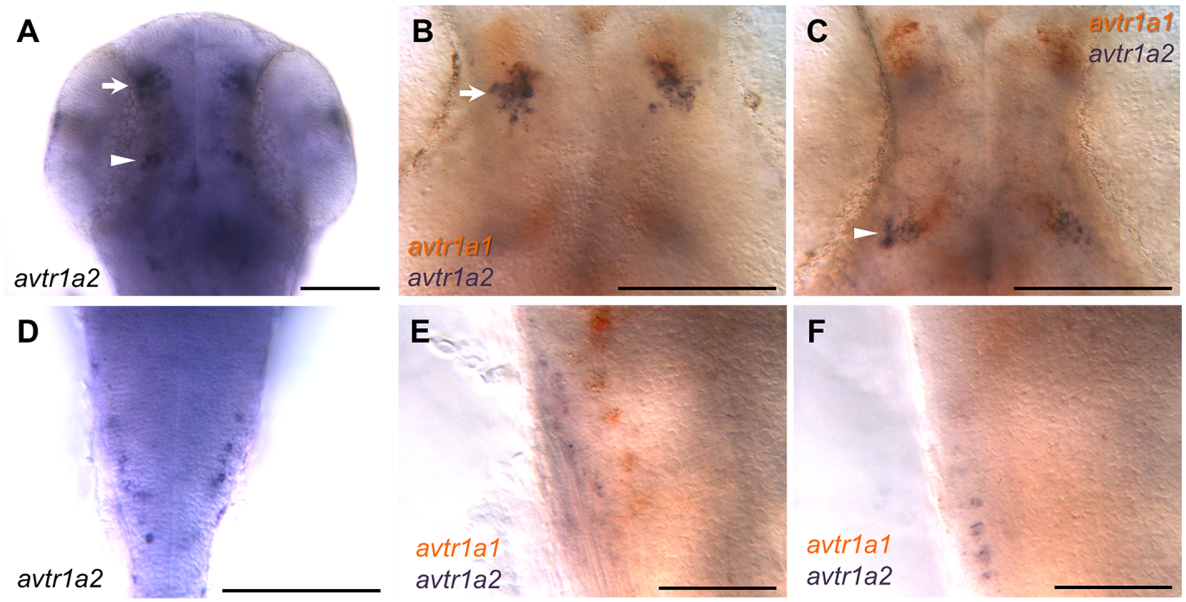


Fig. 2.7. *avtr1a1*+ hindbrain neurons project axons toward MLF

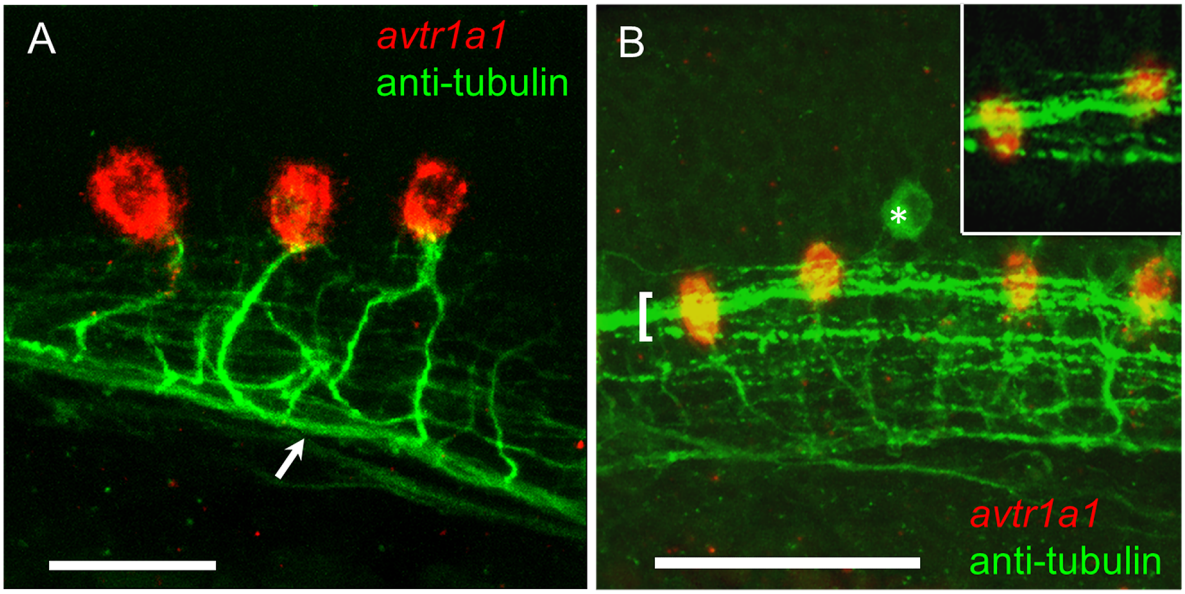


Fig. 2.8. *avtr1a1*+ hindbrain neurons in close proximity to descending *otpb*+ axons

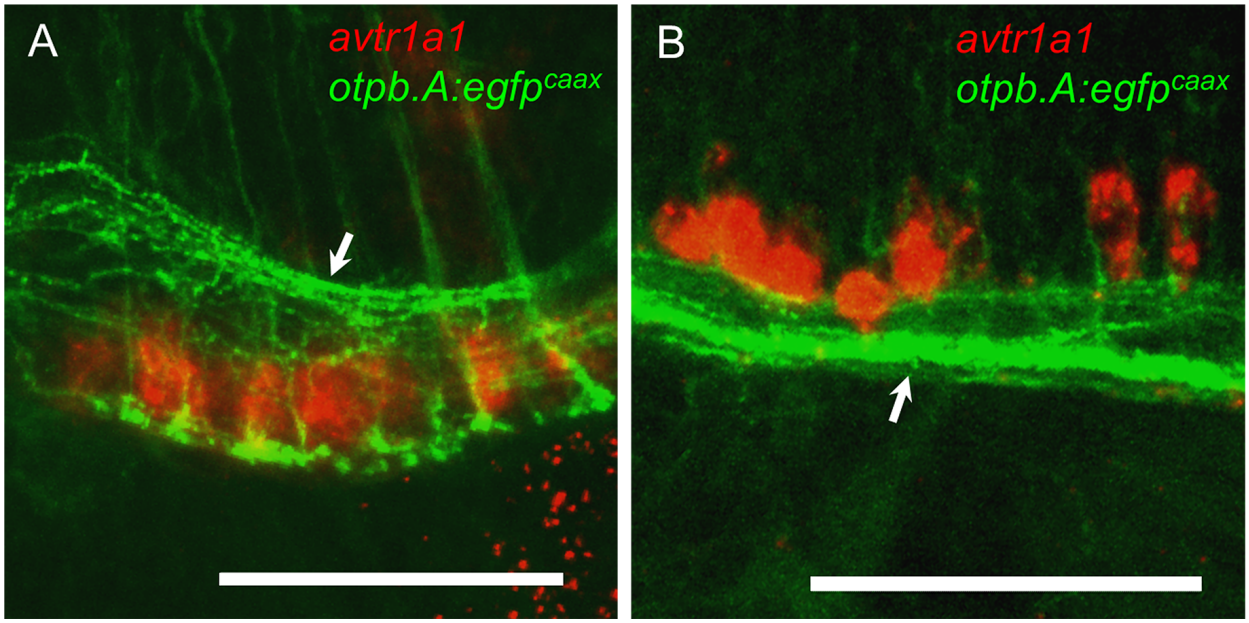
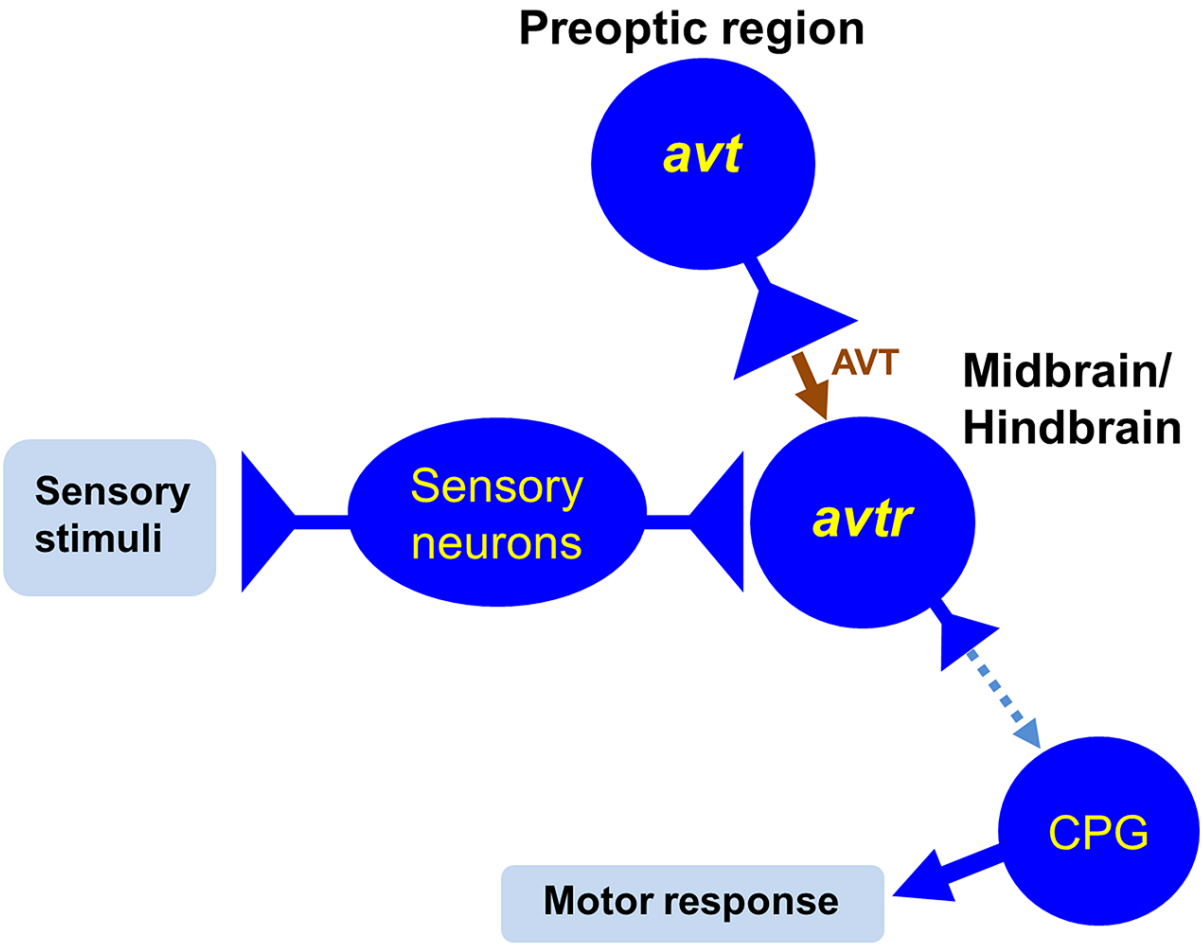


Fig. 2.9. A model proposing a role of *avt/avtr* signaling in sensorimotor behaviors



[1]

[1] Chapter II was published as follows:
Iwasaki K, Taguchi M, Bonkowsky JL, Kuwada JY (2013) Expression of arginine vasotocin receptors in the developing zebrafish CNS. Gene Expr Patterns 13:335–342.
*K. Iwasaki conducted molecular cloning of *avtr1a1* and *avtr1a2*, all RNA probe synthesis, phylogenetic/amino acid analysis of cloned *avtrs*, single/dual *in situ* hybridization, immunolabeling. M. Taguchi assisted in conducting single *in situ* hybridization.

Chapter III. Signaling by the neuropeptide, vasotocin, in the zebrafish CNS regulates sensorimotor behavior

1. Introduction

In mammals and non-mammalian vertebrates arginine vasopressin (AVP) and arginine vasotocin (AVT), respectively, are released by the posterior pituitary into the vasculature in response to sexual stimulation, stress and dehydration to mediate a variety of peripheral effects including antidiuretic activity by the kidney (Thornton et al., 1986; Nishimura and Fan, 2003; Koshimizu et al., 2012). Social and reproductive behaviors are also regulated in a variety of vertebrates by the action of AVP/AVT within the CNS (reviewed in Donaldson and Young, 2008; Stoop, 2012). Furthermore, aberrant signaling by AVP and oxytocin, another neuropeptide that regulates social behaviors, is thought to be involved in disorders including autism and schizophrenia (reviewed in Heinrichs et al., 2009). Interestingly AVP+ neurons project axons into the spinal cord and AVP binding sites are expressed in the spinal cord of neonatal mammals (Leong et al., 1984; Kudo et al., 1993; Lakke, 1997; Liu et al., 2003; Rood and De Vries, 2011; Stoop, 2012) prior to any reproductive or social behaviors. This suggests that AVP signaling in the spinal cord might regulate sensorimotor functions early in development.

AVP is expressed by hypothalamic, bed nucleus of the stria terminalis and amygdala neurons in mammals (Brownstein et al., 1980; DeVries and Buijs, 1983; DeVries et al., 1985; Young and Gainer, 2003) and AVT primarily by neurons of the preoptic area of the

diencephalon in fish (Venkatesh and Brenner, 1995; Acher et al., 1997). Mammals have 3 AVP receptors with the $AVPR_{1a}$ (V1a) and $AVPR_{1b}$ (V1b) receptors expressed primarily in the CNS and the $AVPR_2$ (V2) receptor in the periphery (Caldwell et al., 2008). AVT receptors have been identified in a number of teleosts as well (Mahlmann et al., 1994; Conklin et al., 1999; Warne, 2001; An et al., 2008) with the pupfish and perhaps other teleosts containing two V1a receptors and a V2 receptor (Lema, 2010).

In zebrafish embryos AVT is expressed by a subset of cells in the preoptic area (Tessmar-Raible et al., 2007; Eaton et al., 2008) that express the transcription factor, *otp* (Herget et al., 2014). Zebrafish have two V1a receptors, $AVTR_{1a1}$ and $AVTR_{1a2}$, that are expressed widely in the CNS (Iwasaki et al., 2013). Both receptor types are expressed in similar regions in the caudal hindbrain. In particular $AVTR_{1a1}$ is expressed by distinct neurons in the caudal hindbrain that project axons into the Medial Longitudinal Fasciculus (MLF). These neurons are in the vicinity of preoptic axons expressing mEGFP in *otp.A:egfp^{caax}* embryos (Fujimoto et al., 2011). Since the MLF contains the axons of reticulospinal interneurons that mediate motor responses to sensory input (Zottoli, 1977; Nisanov et al., 1990; Gahtan et al., 2002), it is possible that the AVTR+ hindbrain neurons may also participate in motor responses such as escape swimming. Furthermore, the axons of Rohon Beard (RB) sensory neurons run in close proximity to the AVTR+ hindbrain neurons (Iwasaki et al., 2013) raising the possibility that the AVTR+ neurons may receive sensory synaptic input. Thus the possibility exists both in mammalian neonates and zebrafish embryos that AVT signaling may play a role in early sensorimotor function. Here we describe experiments that take advantage of the optical clarity and molecular manipulability of zebrafish that test this hypothesis.

2. Materials & Methods

Animals

Zebrafish were maintained in a fish breeding facility following guidelines of the University of Michigan Animal Care and Use protocols (Zhou et al., 2008) and embryos collected and staged according to hours post-fertilization (hpf; Westerfield, 1995). The transgenic fish line used in some experiments is *Isl1(ss):Gal4;UAS:gfp* (Palanca et al., 2013).

in situ hybridization (ISH), immunolabeling & c-fos transcription analysis

For immunohistochemistry (IHC) and *in situ* hybridization (ISH), the embryos were dechorionated and placed in 0.2 mM PTU (1-Phenyl-2-thiourea) at 22 to 23 hours post fertilization (hpf) to prevent the formation of the skin pigment. The embryos were fixed in 4% paraformaldehyde in 1x PBS at a desired developmental stage. Riboprobes against zebrafish *avt*, *avtr1a1* and *avtr1a2* (Iwasaki et al., 2013), *c-fos* (Lau et al., 2011), and *opn4a* (Matos-Cruz et al., 2011) were synthesized *in vitro* to conduct *in situ* hybridization with wholemounted embryos as previously described (Iwasaki et al., 2013). *avt* cDNA was cloned by using a forward primer, 5'-CGAGATGTCAGACTCTCTGCT-3', and a reverse primer, 5'-CATATTTACAGTGATGTGGGGGACAG-3'. The obtained *avt* cDNA sequence was 100% identical to the reference sequence (NM_178293). For ISH, digoxigenin (DIG)-labeled antisense probes were synthesized *in vitro* for the target mRNAs, and NBT/BCIP (Roche) was used as a substrate for the alkaline phosphatase-mediated color reactions. For dual ISH using *c-fos* and *avtrs* or *avt* and *opn4a*, dinitrophenol (DNP)-labeled anti-sense probes were

synthesized *in vitro* for *c-fos* and *avt*, and DIG-labeled antisense probes for *avtrs* and *opn4a* as described before (Iwasaki et al., 2013). The INT/BCIP (Roche) was used as a substrate for the alkaline-phosphatase mediated color reactions for *c-fos* and *avt*, and NBT/BCIP for *avtrs* and *opn4a*.

Double labeling with anti-*avtr1a1* ISH and anti-AVT, anti-acetylated α-tubulin, or anti-GFP was carried out as described by Iwasaki et al. (2013) using the following primary antibodies along with Alexa488-conjugated secondary antibodies (Life Technologies); rabbit anti-AVT (Herget et al., 2014), rabbit anti-GFP (Torrey Pines Biolabs Inc., TP401), or mouse anti-acetylated α-tubulin (Sigma Life Science, T6793). For ISH, fast red (Roche) was used as a substrate for the alkaline phosphatase-mediated color reactions.

Leica SP5 laser scanning confocal microscope was used to image the embryos labeled with fluorescent labels except for the embryos injected with the GFP reporter mRNA plus morpholinos, which were imaged with Nikon eclipse E600 microscope. In Fig. 3.2D and 3.3B, the brightness of ALL images were increased (50%) to ensure visibility of embryos in all conditions. A Leitz, Orthoplan 2 microscope with DIC was used to obtain images for the embryos with non-fluorescent labels. The contrast was increased (set to 20, Scion VisiCapture Image Acquisition application) to image embryos doubly labeled for *avtr* and *c-fos* to ensure that co-labeled cells are reliably distinguished from singly labeled cells.

Injection of synthetic AVT

Synthetic (Arg8)-Vasotocin (BACHEM, H-1785) was injected (4.6 nl) into the forth ventricle of 2 dpf embryos at 0.01, 0.1, or 0.5 mM (in 1x Evan's solution, 0.05% phenol red) using Nanoject II (Drummond Scientific Company) and a stereo microscope (1.6x, CARL Zeiss). The Evan's solution contained (in mM), 134 NaCl, 2.9 KCl, 2.1 $CaCl_2$, 1.2 $MgCl_2$, and

10 HEPES, and pH was adjusted to 7.6 (Bradley et al., 2010). The embryos were anesthetized by 0.01% tricaine (Ethyl 3-aminobenzoate methanesulfonate, Aldrich Chemistry) 3 to 5 min before the injection. The injection was conducted 30 min prior to tail touch-induced swimming assay. Phenol red in the inject solution was used to confirm the placement of the solution into the 4th ventricle. Injected embryos were assayed for touch-induced swimming at 48 hpf using a circular testing dish (14 cm in diameter).

Injection of morpholino oligonucleotides (MO) and morphant rescue

Antisense MOs were designed to block the translation of *avt*, *avtr1a1* and *avtr1a2* (Gene Tools, LLC). The sequences for the translation blocking MOs were; *avt*, 5'-ACAGCAGAGAGTCTGACATCTCGAC-3', *avtr1a1,* 5'-AACGAGCAGTCGTTTTCATGCGTAA-3' and *avtr1a2*, 5'-ACGTGTTACTGTGCGTCTCCATTTT-3'. The MOs were injected (in Evan's solution, 0.05% phenol red) into the yolk of recently fertilized wild-type zebrafish embryos (Nasevicius and Ekker, 2000), and the escape swimming assay was carried out 2 dpf using a square testing dish (22.7 by 22.7 cm). Before behavioral testing, each morphant was examined for the presence of regular heartbeat and gross morphology to ensure that only healthy embryos with no obvious morphological defects were tested in the behavioral assays.

To confirm knockdown by the translation blocking MOs, DNA reporter constructs were made to synthesize *in vitro* (mMESSAGE mMACHINE kit, Ambion, Life Technologies) mRNAs containing each MO target fused 5' to a EGFP coding sequence and the reporter mRNAs (0.58 ng) were co-injected with the MOs (2.3 nl, 100 μM). pEGFP-N1 vector (Clontech) was used as a backbone vector. As a control, standard control MO (STDMO), 5'-

CCTCTTACCTCAGTTACAATTTATA 3' (Gene Tools, LLC), was co-injected with the reporter mRNA with the MOs. Following injections knockdown was assayed by examining GFP expression at 22-28 hpf.

The splice-blocking MO for *avtr1a2* (AGCTTTAGGTCTCACCGTCCCAGTT), targeted the splice junction of *avtr1a2* pre-mRNA between the exon 1 and intron 1. The splice-blocking MO predicts a severely truncated product due to a premature stop codon near the start of intron 1. The knockdown of *avtr1a2* was confirmed by RT-PCR. Total RNA was extracted from pooled MO-injected embryos using TRIzol® reagent (Invitrogen) and reverse transcription was carried out using Superscript II® Reverse Transcriptase reagents (Invitrogen). Using a forward primer, 5'-GACGCGATGAGGAAGTGGCTAAAA-3', that binds to a region of the *avtr1a2* mRNA 5' to the exon 1/exon 2 boundary and a reverse primer, 5'-TCCGCTGAACACCATGTAGATCCA-3', that binds 3' to the exon 1/exon 2 boundary, 884 bp target sequence was PCR amplified (Platinum Taq DNA polymerase, Invitrogen) in control embryos but not in MO-injected embryos. The knockdown was confirmed by gel electrophoresis (not shown). To examine the specificity of knockdown, *EEF1A1* (eukaryotic translation elongation factor 1 alpha 1, AY422992) was RT-PCR amplified (324 bp) using a forward primer, 5'-CACCAAGGAAGTCAGCGCATACAT-3', and a reverse primer, 5'- ACAACCATACCAGGCTTGAGGACA-3'.

To synthesize mRNAs to rescue phenotypes for *avt* and *avtr1a2* (translation blocking) morphants, DNA constructs were made to encode the complete coding sequence for each of the genes. The nucleotides were changed at the wobble positions as well as 5' UTR to prevent blocking by the translation blocking MOs and a Kozak sequence, 5'-GCCACC-3' (Kozak, 1987) was added before the start codon resulting in changing 9/25 bp

of the *avt* MO target sequence and6/25 bp of the *avtr1a2* MO target sequence. pcDNA3.1 (+) (Invitrogen) was used as a backbone vector. The mRNAs were *in vitro* transcribed (mMESSAGE mMACHINE T7 Ultra Kit, Ambion, Life Technologies) and co-injected with each corresponding translation blocking MO (*avt* mRNA, 0.01 ng/μl, 2.3 nl per embryo; *avtr1a2* mRNA, 0.005 ng/μl, 2.3 nl per embryo) in recently fertilized embryos and assayed for touch-induced swimming at 48 hpf.

Escape swimming assay

Touch-induced swimming was initiated by a light touch to the tail region of embryos with a pair of fine forceps (No. 5, FST, Dumont). Embryos were individually placed in the center of a testing dish with a gridded sheet (0.635 x 0.635 cm) attached to the bottom, and mechanically stimulated. The embryo gets a score of zero if it does not respond after three tail touches were applied. The responses were videorecorded (Handycam, HDR-CX100, Sony®) and viewed in slow motion (VLC media player, VideoLAN Organization) to count the number of grids each embryo entered in response to tail stimulation. The touch-induced swimming assays were performed and analyzed under blind conditions.

c-fos transcription analysis

PTU treated wild type embryos (48 hpf) were each placed in a well of 24-welled-plates filled with 2 ml fish water with PTU at 28.5 ^{o}C in a dark incubator for 2 - 5 hours. Extra care was taken to keep the plates undisturbed to minimize *c-fos* expression due to inadvertent stimulation of the embryos. The plates were then taken out of the incubator into ambient temperature and light for behavioral testing. 20 μl of 5 mM mustard oil (allyl

isothiocyanate, Aldrich Chemistry, 377430) plus 1% DMSO was added from a micropipette to each well (final mustard oil concentration = 50 μM) in experimental condition and 20 μl of fish water containing 1% DMSO in control condition. Mustard oil serves as a noxious stimulus, which induces an increase in locomotor activity in zebrafish larvae (Prober et al., 2008). At 50 μM, mustard oil induces swimming in 2 dpf embryos, as well, compared with control embryos (n = 7 per condition, data not shown). However, at a concentration greater than 100 μM mustard oil induced erratic motor responses such as spinning (not shown). Therefore, 50 μM was used for the *c-fos* transcription analysis. The embryos were kept in the wells for 30 min following the addition of the 20-μl solutions to maximize *c-fos* transcript levels induced by neuronal activation (Lau et al., 2011). The behavioral response was video recorded and analyzed with the VLC media player (0.25x to 0.5x the normal speed) to quantitate the duration and the number of the forward swimming episodes during the first 5 minutes after addition of the mustard oil. Following behavioral testing, the embryos were fixed in 4% PFA (in 1x PBS) overnight and dehydrated for *in situ* hybridization with anti-sense probes for *c-fos* with/without anti-sense probes for *avtrs*.

Light modulation of touch induced swimming

Embryos were injected with STDMO or *avt* MO and kept in an incubator (28.5 ^{o}C). The embryos were dechoryonated at approximately 24 hpf and placed in a dark incubator until 45 to 47 hpf when tail touch was applied under different light conditions. A light source (White Light Transilluminator, FB-WLT-1417, FisherScientific) was covered with two different filters in order to selectively block light of certain wavelengths. The room lights were turned off during the experiment. A red filter (Safelight filter, Kodak, No. 1A) was

used to block a range of wavelengths which include λ_{max} for opn4 (470 nm, Davies et al., 2011) and TMT opsin (460 nm, Koyanagi et al., 2013) that may be important for the sensory-locomotor responses ("red light" condition). Red light did not induce swimming by itself (not shown) but allowed embryos to be video recorded. We found that unfiltered light from the transilluminator induced swimming, but a 40% neutral density filter ("white light" condition) did not (not shown). Under each condition, embryos were touched one minute after placement in the central grid of a circular testing dish (14 cm in diameter) to assay touch-induced swimming (up to three times if they do not respond to the first 2 touches).

Statistical analysis

The statistical analysis was performed using SPSS (IBM® SPSS® Statistics Version 22). The means of the groups for each experiment were compared using One-way ANOVA or independent t-test when the Shapiro-Wilk test indicated that the data was normally distributed. If a given data set contains values that are not normally distributed, non-parametric Mann-Whitney's U test was used to compare the means.

3. Results

3.1. AVTR expressing neurons are activated by sensory stimulation

A small group of neurons in the caudal hindbrain that express *avtr1a1* and *avtr1a2* are hypothesized to be interneurons mediating sensorimotor function in zebrafish (Iwasaki et al, 2013). This hypothesis was in part based upon the finding that RB sensory axons extended in close proximity to the AVTR+ hindbrain neurons. RB axons, which are located in the Lateral Longitudinal Fasciculus (LLF), were labeled with anti-acetylated α tubulin

that labels all axons (Chitnis and Kuwada, 1990). This was confirmed by labeling for *avtr1a1* transcripts in *TG(isl1(ss):GAL4; UAS:GFP)* transgenic zebrafish in which RB sensory neurons specifically express GFP (Palanca et al., 2013). The RB axons were seen to extend in close proximity to *avtr1a1+* hindbrain neurons (Fig. 3.1A).

The hypothesis predicts that AVTR+ hindbrain neurons should be activated by sensory stimulation that normally initiated a motor response in zebrafish embryos. To examine this possibility *c-fos* transcripts were assayed by *in situ* hybridization in wholemounted embryos (57-59 hpf). *c-fos* transcription is a sensitive indicator of neuronal activity in zebrafish (Lau et al, 2011). Control embryos (n=10) did not exhibit any episodes of forward swimming and there was little *c-fos* transcription in their CNS apart from a small number of cells near the nasal placodes with no specific increase in *c-fos* transcription in the hindbrain (Fig. 3.1B). However, embryos (n=24) exposed to 50 mM mustard oil swam extensively (4.8 $\pm$ 3.8 episodes, $p<0.0001$ Mann-Whitney's U test; 8.2 $\pm$ 8.8 seconds, $p<0.0001$ Mann-Whitney's U test) and *c-fos* transcription was induced in selective cells within the CNS including cells in the forebrain, hindbrain and spinal cord of embryos examined (n=17, 18, 17 respectively) (Fig. 3.1C). Co-labeling for *avtr1a1* (7 out of 8 embryos) or *avtr1a2* (7 out of 7 embryos) transcripts along with *c-fos* transcripts showed that AVTR expressing hindbrain neurons were amongst the cells activated during mustard oil initiated swimming (Fig. 3.1D). Cells near the dorsal midline of the caudal hindbrain and anterior spinal cord also expressed *c-fos* (Fig. 3.1C). By their position and size they are likely to be RB sensory neurons a subset of which are known to be stimulated by mustard oil (Prober et al., 2008). Thus the AVTR expressing hindbrain neurons that are potential sensorimotor interneurons are active during sensory initiated swimming.

3.2. Exogenous AVT and AVT knockdown enhances and inhibits, respectively, touch induced swimming

Preoptic axons that include those of *avt*+ neurons can be visualized in *otpb.A:egfp*^{caax} transgenic zebrafish that express a membrane-targeted EGFP in preoptic neurons and their axons were seen to run through the hindbrain into the spinal cord in close vicinity to the AVTR expressing hindbrain neurons (Iwasaki et al., 2013). Since only a subset of preoptic neurons express *avt*, zebrafish were labeled with an antibody against AVT (Herget et al, 2014) to determine whether AVT+ axons of preoptic neurons were in close proximity to the AVTR hindbrain neurons. By 4 dpf, AVT+ axons extend through the hindbrain into the spinal cord (not shown), and AVT+ axons were found to extend close to the $AVTR_{1a1}$+ hindbrain neurons in 6 dpf larvae (Fig. 3.2A) suggesting that AVT could potentially modulate AVTR+ hindbrain neurons and thus sensorimotor responses. This was tested by injecting AVT into the 4th ventricle overlying the hindbrain of zebrafish embryos to see if exogenous AVT could modulate touch-induced swimming. In fact there appeared to be a dose-dependent enhancement of touch-induced swimming by exogenous AVT (Fig. 3.2B). Since exogenous AVT enhanced the sensorimotor response, the effect of knocking down AVT was examined by injecting embryos with translation blocking antisense Morpholino oligonucleotides (MO) targeting AVT. Morphants showed no obvious morphological defects (not shown) but exhibited much less touch-induced swimming compared with control MO injected embryos (Fig. 3.2C). Since anti-AVP (Eaton 2008) and anti-AVT (not shown) appear not to label zebrafish prior to 2 dpf, we assayed the effectiveness of the antisense MO by co-injecting embryos with the antisense MO and RNA that included the *avt* targeted sequence and *gfp*. All 11 of the antisense MO injected embryos (n=11) failed to

express GFP but 8/11 control MO injected embryos exhibited GFP expression (Fig. 3.2D). The specificity of knockdown was examined by co-injecting the antisense MO and RNA for *avt* with the MO targeted sequence changed in the wobble position to prevent binding by the MO. These embryos exhibited partially normal touch-induced swimming and thus were rescued from the morphant phenotype (Fig. 3.2D) suggesting that the morphant phenotype was not an off-target artifact. Thus knocking down AVT inhibits touch-induced swimming. This in combination with the enhancement of touch-induced swimming by exogenous AVT suggests that AVT release presumably by the preoptic neurons normally modulates sensorimotor responses in zebrafish embryos.

3.3. Knockdown of AVTRs suppresses touch induced swimming

Since manipulations of AVT modulates sensorimotor responses by zebrafish embryos, knockdown of AVTRs should also inhibit touch-induced swimming. This was examined by injecting translation blocking antisense MOs against *avtr1a1* or *avtr1a2* into embryos and assaying touch-induced swimming. Indeed *avtr1a1* and *avtr1a2* morphants both exhibited decreased touch-induced swimming (Fig. 3.3A). The antisense MOs were effective since co-injection of RNA containing the target sequences followed by *gfp* with the antisense MOs but not control MOs inhibited GFP expression. All 6 embryos injected with *avtr1a1* MO failed to express GFP while 7/10 injected with control MO expressed GFP; all 12 embryos injected with *avtr1a2* MO failed to express GFP while 8/12 injected with control MO expressed GFP (Fig. 3.3B). The decrease in touch-induced swimming by morphants was likely due to a specific effect on AVTRs since expression of a wobble position modified *avtra1a2* RNA rescued the morphant phenotype (Fig. 3.3C). Additionally,

morphants in which splice blocking MOs targeting *avtr1a2* were injected also exhibited decreased touch-induced swimming (not shown, see Materials and Methods). Occasionally different clutches of embryos exhibited differences in distances swum in response to touch, but morphants and the corresponding controls were always from the same clutch. Thus knocking down AVTRs phenocopies AVT morphants suggesting that AVT signaling mediated by AVTRs modulates sensorimotor responses in zebrafish embryos.

3.4. Some preoptic neurons co-express *avt* and *opn4a* and light modulates touch induced swimming

The finding that AVT/AVTR signaling mediates modulation of sensorimotor responses in zebrafish embryos and that a subset of preoptic neurons express AVT and project their axons into the hindbrain and spinal cord raises the question of how AVT release is regulated. One interesting possibility is that AVT release may be controlled by light since preoptic neurons are known to express deep brain opsins (Tessmar-Raible et al., 2007; Matos-Cruz et al., 2011). *In situ* hybridization confirmed that preoptic neurons express *opn4a* in zebrafish embryos and further showed that a subset of preoptic neurons co-express *opn4a* and *avt* (Fig. 3.4A). *opn4a* was not expressed in the spinal cord nor hindbrain (not shown). To see if light might modulate sensorimotor responses the response of 48 hpf embryos to touch was examined in the presence of a moderate level of white light that by itself did not induce any motor responses or in the presence of red light, to which Opn4a is not responsive (see Materials and Methods). Embryos responded more vigorously to touch in white light but not in red light (Fig. 3.4B). Furthermore, the enhancement of the touch-induced swimming was eliminated in morphants in which *avt*

was knocked down (Fig. 3.4B). Thus touch-induced swimming is modulated by light. One possibility is that embryos are more sensitive to sensory stimulation in the light due to transduction of light by opsin expressing AVT+ preoptic neurons, which causes the release of AVT to enhance the sensitivity of AVTR hindbrain neurons to sensory stimulation.

3.5. AVTR is expressed by a subset of RB mechanosensory neurons in the zebrafish spinal cord

The initial examination of the expression pattern of AVTRs found expression of *avtr1a1* by occasional dorsal spinal neurons by 24 hpf (Iwasaki et al., 2013). By double labeling embryos with a riboprobe against *avtr1a1* and anti-acetylated α tubulin to label axons (Chitnis and Kuwada, 1990) or anti-HNK-1 (zn12) to label Rohon-Beard (RB) neurons (Metcalfe et al., 1990) we showed that the dorsal neurons were RB mechanosensory neurons, which extend central axons in the Dorsal Longitudinal Fasciculus (DLF) of the spinal cord (Fig. 3.5). Expression of *avtr1a1* was observed in a subset of RB neurons, and the number of receptor expressing RBs increased from 3.5 ± 0.65 at 23 hpf (n=4) to 14 ± 3.9 at 48 hpf (n=8). *avtr1a2* was also expressed in the spinal cord by unidentified neurons that appear not to be RBs (not shown). Thus AVT may regulate the responsiveness of the neural circuit for sensory induced swimming at multiple levels.

4. Discussion

Neuropeptides have long been appreciated as modulators of synaptic function and thus neural circuits regulating a wide variety of behaviors. There is extensive evidence that the nonapeptide, AVP/AVT, is important for social and reproductive behaviors yet how

AVP/AVT actually regulates neural circuits is much less clear. The findings that AVT and AVTRs are expressed in the brain of zebrafish embryos (Tessmar-Raible et al., 2007; Eaton et al., 2008; Iwasaki et al., 2013) when the CNS is relatively simple (Eisen et al., 1986; Bernhardt et al., 1990; Chitnis and Kuwada, 1990) provided an opportunity to examine how AVT might modulate the neural circuit that mediates escape swimming in zebrafish embryos. Pharmacological and molecular manipulations in combination with analysis of the stereotyped escape responses of zebrafish embryos were used to examine the hypothesis that AVT/AVTR signaling presumably by the hindbrain AVTR+ neurons regulates motor responses to sensory stimulation. The specificity of morpholino manipulations have recently been questioned (Kok et al., 2015), but in this case knockdown and at least partial rescue were confirmed, and significantly knockdown of both signal and its receptors (*avt*, *avtr1a* and *avtr1b*) all exhibited similar phenotypes. Furthermore increasing AVT resulted in a predicted gain of function. These results argue in favor of the hypothesized regulation of touch-induced swimming by AVT signaling.

Interestingly a moderate level of light was found to enhance touch induced escape swimming, and that some preoptic neurons express both *avt* and *opn4a*. These results suggest a model (Fig. 3.6) in which light activates deep brain opsins and the release of AVT by preoptic neurons onto the hindbrain AVTR+ neurons to enhance their excitability. The hindbrain neurons are activated by sensory input from RB neurons that in turn excite central pattern generators mediating escape swimming. Furthermore the finding that some RB sensory neurons express AVTRs suggests the intriguing possibility that the sensory neurons themselves are regulated by AVT. One way this could be achieved is by expression of AVTRs on the presynaptic terminals of the central axons of these RB neurons within the

hindbrain and spinal cord where AVT+ preoptic axons are located. Thus it might be possible that AVT released by preoptic neurons might modulate the excitability of both presynaptic AVTR+ RB terminals and the postsynaptic AVTR+ hindbrain neurons. Clearly anatomical and physiological analysis of AVTRs in the hindbrain will be required to test whether AVT might regulate the escape swimming circuit at multiple sites as hypothesized.

Regulation of neural circuits at multiple levels is a common neural circuit motif. In the crayfish CNS, for example, activation of particular command neurons triggers the tailflip escape behavior, a behavior incompatible with postural behavior (Kuwada and Wine, 1979). To ensure suppression of the latter behavior during the escape response, the same command neurons initiate inhibition of the postural neural circuit at multiple levels including sensory receptors, motor neurons and muscles. Mechanistically, multi-level regulation of neural circuits can result from the modulatory actions of a single neuropeptide, in some cases released by different neurons. In the *Aplysia* feeding system, the small cardioactive peptide (SCP) is released by esophageal nerve axons to directly activate an egestion modulatory neuron (B65) and an egestion central pattern generator (CPG) neuron (B20) that is postsynaptic to B65, while directly inhibiting the ingestion CPG neuron (B40) (Wu et al., 2010). Also in the *Aplysia* feeding system, allatropin-related peptide (ATRP) released by the CBI-4 projection neuron excites protraction motor neurons to speed up the rhythmic egestion cycle, while ATRP is also released by these motor neurons to enhance the contraction amplitude of the protraction muscles (Jing et al., 2010). In the crab *Cancer borealis* feeding system, tachykinin-related peptide (CabTRP) Ia triggers the gastric mill (chewing) rhythm in the stomatogastric ganglion via its serial release from an extrinsic input pathway (post-oesophageal commissure neurons) and one of the post-

oesophageal commissure neurons' target projection neurons (MCN1) in the commissural ganglia (Blitz et al., 2008; Wood et al., 2000).

The finding that light can modulate touch-induced swimming in 48 hpf zebrafish embryos brings up the question of how light is being sensed by these embryos. This effect of light on touch-induced swimming is likely mediated by deep brain opsins rather than opsins in the visual system for a number of reasons. First, opsins do not begin developing in the zebrafish retina until 50 hpf (Raymond et al., 1995) with the outer segments developing at 60 hpf (Branchek and BreMiller, 1984). Second, electroretinograms are not recorded until 3dpf (Branchek, 1984). Third, visually evoked motor responses are not exhibited until 68-79 hpf (Easter and Nicola, 1996).

An alternative to visual input is deep brain opsins that are expressed in numerous regions of the vertebrate brain (reviewed in Davies et al., 2010; Fernandes et al., 2013). Some deep brain opsins are expressed at appropriate stages of development to mediate light modulation of the touch response in zebrafish embryos. *VAL* opsins are expressed by discrete neurons in the CNS including the hindbrain of zebrafish embryos although expression of *VAL* opsins in the preoptic region was not noted (Kojima et al., 2008). Interestingly, *VAL* and *tmt* opsins are co-expressed by motor neurons of the facial nucleus in larval zebrafish and Medaka fish (Fischer et al., 2013). Whether these opsins are expressed by spinal motor neurons was not reported, but expression of opsins by motor neurons raises the possibility that light enhancement of touch-induced escape swimming may involve direct enhancement of motor neuron excitability. However, this would presumably not include *opn4a* in embryos since there was no expression of *opn4a* by any cells in the spinal cord nor hindbrain in zebrafish embryos. *opn4a*, however, is expressed

by the myotome in zebrafish embryos (Matos-Cruz et al., 2011) which suggests the intriguing possibility that light may regulate motor responses in general by directly increasing the excitability of muscles.

The enhancement of touch-induced swimming by light may be mediated by mechanisms underlying the photomotor response (PMR; Kokel et al., 2013). Since PMR can be induced by directly shining light onto the hindbrain, unidentified opsins in the hindbrain may mediate PMR (Kokel et al., 2013). Thus a hindbrain opsin initiated pathway may be the source of light enhancement of touch-induced swimming. Another possible avenue for light mediated enhancement of touch-induced swimming may involve opsins in AVT+ preoptic neurons. In fact *tmt* and *opn4a* opsins are expressed by preoptic neurons by 48 hpf (Tessmar-Raible et al., 2007; Matos-Cruz et al., 2011) with *tmt* and *avt* coexpressed by preoptic neurons. Furthermore, *opn4a*+ preoptic neurons may be required for dark photokinesis, which is a light seeking behavior initiated by loss of illumination, exhibited by zebrafish larvae (Fernandes et al., 2012). This report found that *opn4a* and *avt* were also coexpressed by a subset of preoptic neurons. Furthermore, another opsin, *VA* opsin, was coexpressed with AVT by avian hypothalamic neurons (Garcia-Fernandez et al., 2015) although so far there are no reports of expression of *VA* or *VAL* opsins in the preoptic neurons of zebrafish. At any rate these findings suggest that light modulation of touch-induced escape swimming in zebrafish embryos are likely mediated by deep brain opsins perhaps ones expressed by AVT+ preoptic neurons.

The finding that AVT modulates sensorimotor circuits in zebrafish is concordant with the evidence that AVP might affect signaling within the mammalian spinal cord. AVP+ neurons project axons into the spinal cord and AVP binding sites are found extensively in

the spinal cord of neonatal mammals (Leong et al., 1984; Kudo et al., 1993; Lakke, 1997; Liu et al., 2003; Rood and De Vries, 2011; Stoop, 2012). Application of AVP induces inward currents in motor neurons even in the presence of TTX suggesting that the effect is a direct one (Liu et al., 2003). However, a direct effect of AVT onto motor neurons in zebrafish embryos is unlikely since AVTRs are not expressed by motor neurons. Interestingly, application of AVP onto isolated spinal cords of mammalian neonates can initiate locomotor-like activity that is enhanced when serotonin activity is increased suggesting that AVP may regulate mammalian spinal circuits (Pearson et al., 2003; Barriere et al., 2004).

Finally neuropeptides that are members of the oxytocin/vasopressin family in the leech were found to regulate motor circuits and reproductive behavior (Wagenaar et al., 2010). (Arg^8)-conopressin, hirudotocin and annetocin each controlled a component of the courtship and cocoon deposition behavior of leeches. Thus regulation of motor circuits by AVP/AVT is found in a wide variety of animals and may represent an ancient function for these neuropeptides.

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Figure Legends

Figure 3.1. *avtr1a1*+ and *avtr1a2*+ posterior hindbrain neurons are active during sensory-induced swimming in 2 dpf embryos. In all panels a dorsal view is shown with anterior up. **(A1)** *avtr1a1*+ neurons labeled by *in situ* hybridization (red, bracket) in the posterior hindbrain (30-31 hpf) are in close proximity of RB sensory axons (green, blue arrow) in a *Isl1(ss):Gal4-VP16;UAS:gfp* transgenic embryo. Scale, 50 μm. **(A2)** A single plane confocal image (blow-up) for the *avtr1a1+* neuron denoted in A1 (white arrow) showing that RB axons (arrow) are very close to the *avtr1a1+* neuron (arrowhead). Scale, 10μm. **(B1-B3)** Control embryos that were not exposed to mustard oil exhibit some *c-fos* expression near nasal pits (arrowheads) but little expression in the hindbrain or spinal cord. **(C1-C3)** Embryos exposed to mustard oil exhibit *c-fos* expression in numerous cells including in the nasal pit area (blue arrowheads) and forebrain (green arrowheads), lateral posterior hindbrain (bracket), and in the dorsal spinal cord (arrowheads). Scale for B1-C3, 100 μm. **(D1-D2)** Dual *in situ* hybridization for *c-fos* and *avtr1a1* or *avtr1a2* shows that chemosensory stimulation activates *avtr1a1*+ (D1) and *avtr1a2*+ (D2) neurons in the posterior hindbrain (arrowheads). Scale, 50 μm.

Figure 3.2. AVT modulates touch-induced swimming in zebrafish embryos (48 hpf). **(A)** A single plane confocal image (lateral view) showing AVT immunoreactive axon branches (arrow and arrowhead) projected from preoptic neurons that are in close proximity to an *avtr1a1*+ neuron cell body (red) in the posterior hindbrain (6 dpf). Scale, 30 μm. **(B)** Microinjection of synthetic AVT into the 4th ventricle enhances touch-induced swimming in an apparent dose-dependent manner (48 hpf). One-way ANOVA, contrast test (* $p < 0.05$). Normality of the data was confirmed by Shapiro-Wilk test. n=9 per group. **(C)** Injection of antisense *avt* MO inhibits touch-induced swimming and co-injection of wobble position modified *avt* mRNAs partially rescued the morphant phenotype. $p < 0.0001$ by Kruskal-Wallis and individual pair-wise Mann-Whitney U tests were performed; *$p < 0.05$, **$p < 0.01$, ***$p<0.0001$. n=22 (control), 25 (*avt* MO) and 21 (*avt* MO + *avt* mRNA). **(D1-D2)** The translation blocking MO against *avt* effectively blocks the translation of a GFP reporter mRNA fused to the MO target sequence seen at 28 hpf (D2), while the control MO does not block the translation (D1). Scale, 400 μm.

Figure 3.3. Knockdown of *avtrs* specifically suppresses touch-induced swimming. **(A)** Injection of *avtr1a1* and *avtr1a2* MOs but not control MO inhibits touch-induced swimming (48 hpf). For comparison of *avtr1a1* morphants (n=23) and control embryos (n=20), independent t-test (* $p < 0.05$) was conducted with normality confirmed by Shapiro-Wilk test; for comparison of *avtr1a2* morphants (n=21) and control embryos (n=15) Mann-Whitney U, exact 2-tailed test (** $p < 0.01$) was conducted. **(B1-B4)** Morpholinos against *avtr1a1* and *avtr1a2* effectively block the translation of a GFP reporter mRNA fused to the targeted sequence (B2 and B4), while the control morpholino does not block the translation of each reporter mRNA (B1 and B3) at 22-26 hpf. **(C)** Injection of *avtr1a2* MO (n=22) inhibits touch-induced swimming compared with control MO injections (n=32) and coinjection of wobble position modified *avtr1a2* mRNA (n=26) rescues the morphant

phenotype at 48 hpf. *** $p < .0001$ (independent t-test, normality confirmed by Shapiro-Wilk test), ** $p < 0.01$ (Mann-Whitney U, exact 2-tailed test). Scale B1-4, 400 μm.

Figure 3.4. AVT is required for light-dependent modulation of touch-induced swimming. **(A)** Dual *in situ* hybridization shows that a subset of preoptic neurons co-express *opn4a* (purple) and *avt* (red) (48–50 hpf). Ventral view with anterior up. Scale, 50 μm. **(B)** Moderate white light enhanced touch-induced swimming compared with red light of embryos injected with standard MO (control). ** $p < 0.01$ by Mann-Whitney U, exact 2-tailed test (n = 10 per condition). In contrast, white light did not enhance touch-induced swimming over red light of *avt* morphants. N.S. = not significant by Mann-Whitney U, exact 2-tailed test (n = 8 per condition).

Figure 3.5. *avtr1a1* is expressed by a subset of sensory RB neurons in zebrafish embryos. **(A)** Dorsal view (anterior left) of a 25 hpf embryo double labeled with a riboprobe against *avtr1a1* (red) and Zn-12 antibody (green) for RB neurons showed that *avtr1a1* is expressed in a subset of RB neurons (arrowheads). Scale, 50 μm. (Inset) A single plane confocal image for the *avtr1a1*+ RB cell denoted by a blue arrowhead in (A) projecting a central axon (arrow) into the DLF. Scale, 10 μm. **(B)** Dorsal view (anterior up) of the posterior spinal cord of a 48 hpf embryo double labeled with a riboprobe against *avtr1a1* (red) and anti-acetylated α-tubulin (green) to label RB neurons showed that a subset of RB neurons are *avtr1a1*+ (arrowheads). Scale, 30 μm.

Figure 3.6. Model for the regulation of sensorimotor responses by AVT/AVTR signaling in the CNS of zebrafish embryos. AVT is released onto AVTR+ hindbrain neurons by preoptic neurons expressing opsins upon light stimulation to modulate the motor responses to sensory stimulation of neurons in the spinal cord.

Fig. 3.1 . *avtr+* hindbrain neurons respond to chemosensory stimuli.

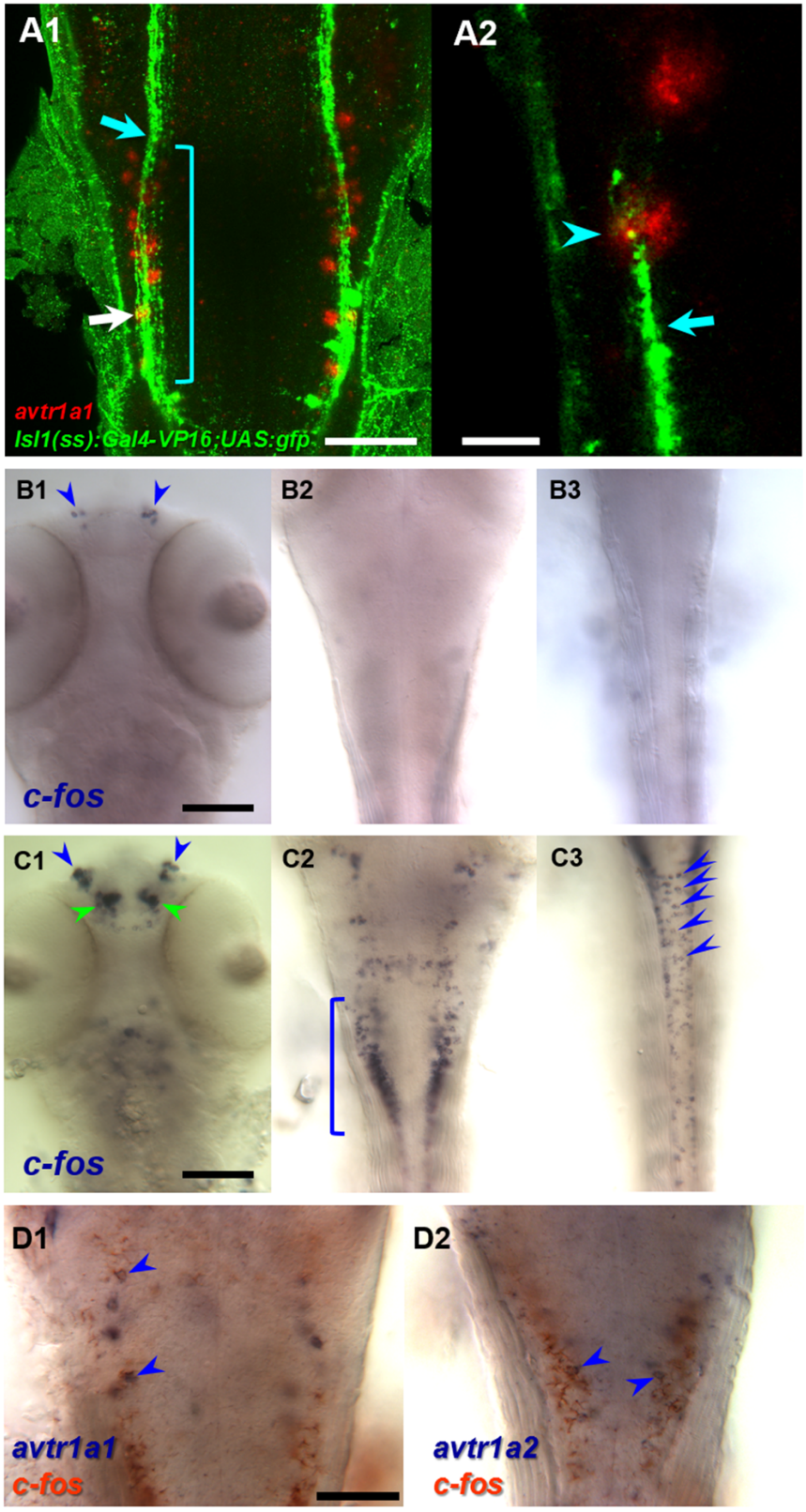


Fig. 3.2. anti-sense morpholino knockdown of *avt* disrupts sensorimotor behaviors.

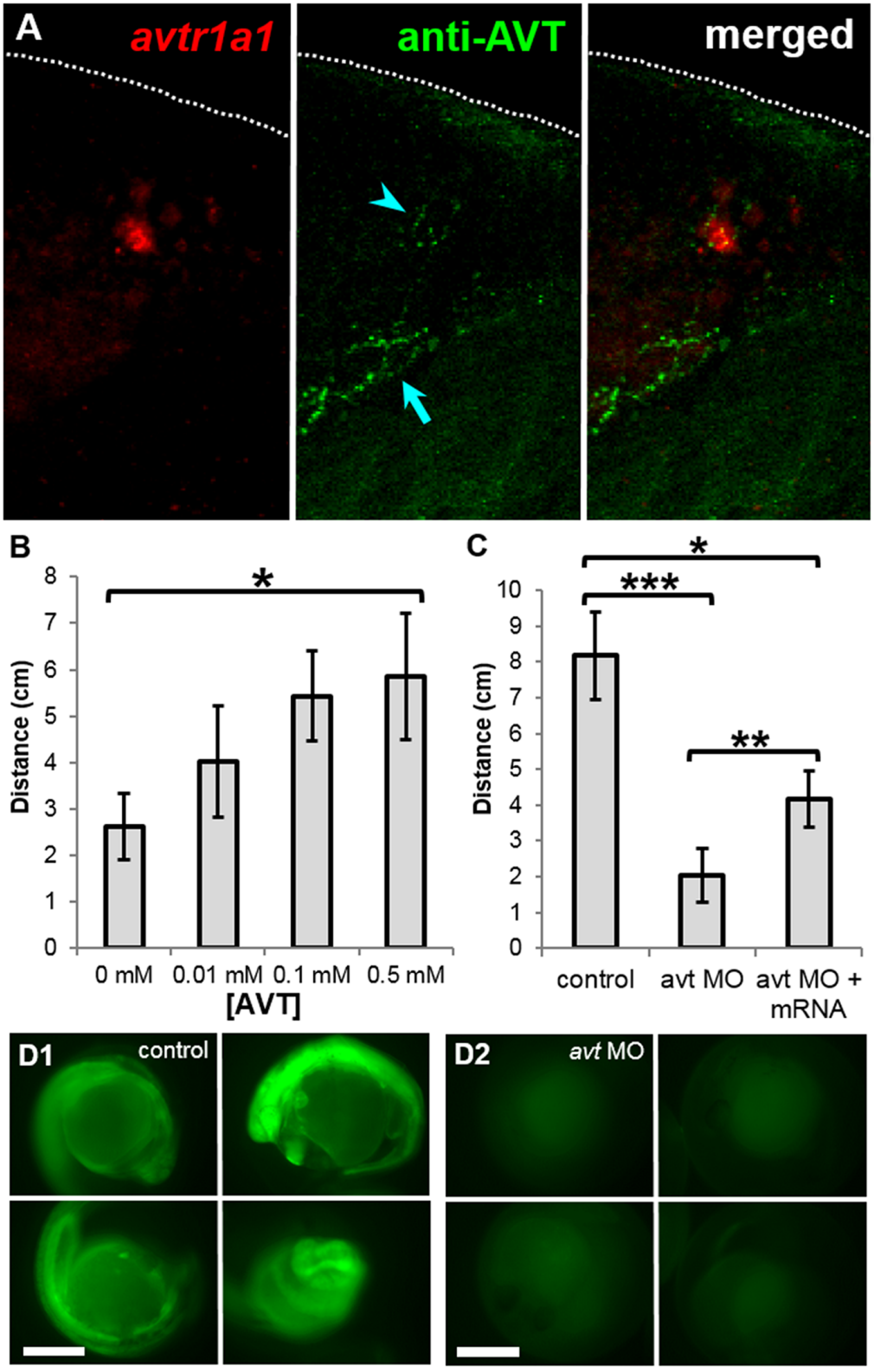


Fig. 3.3. Morpholino knockdown of *avtrs* disrupts sensorimotor behaviors.

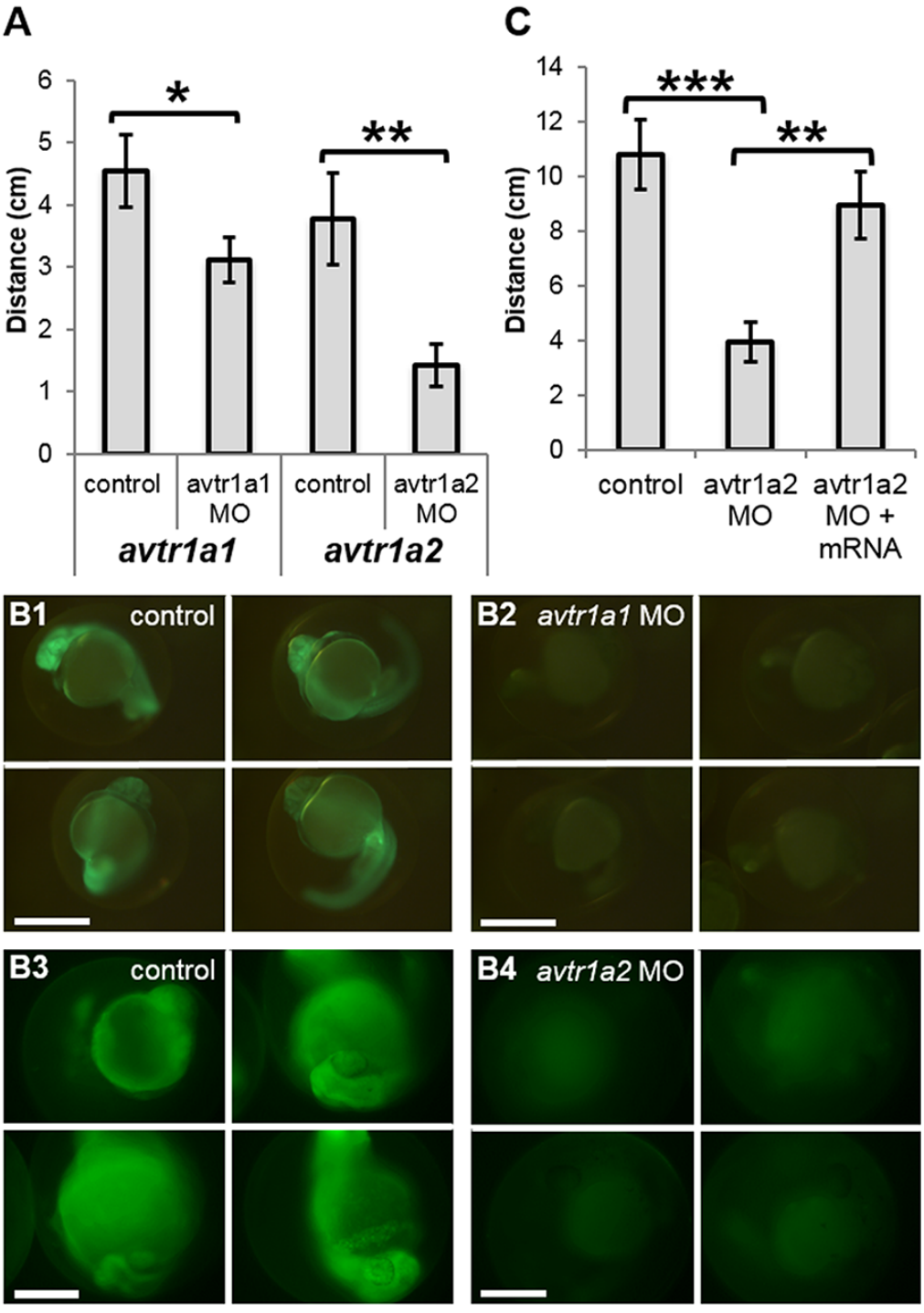


Fig. 3.4. *opn4a+ avt+* preoptic neurons may mediate light-dependent touch response.

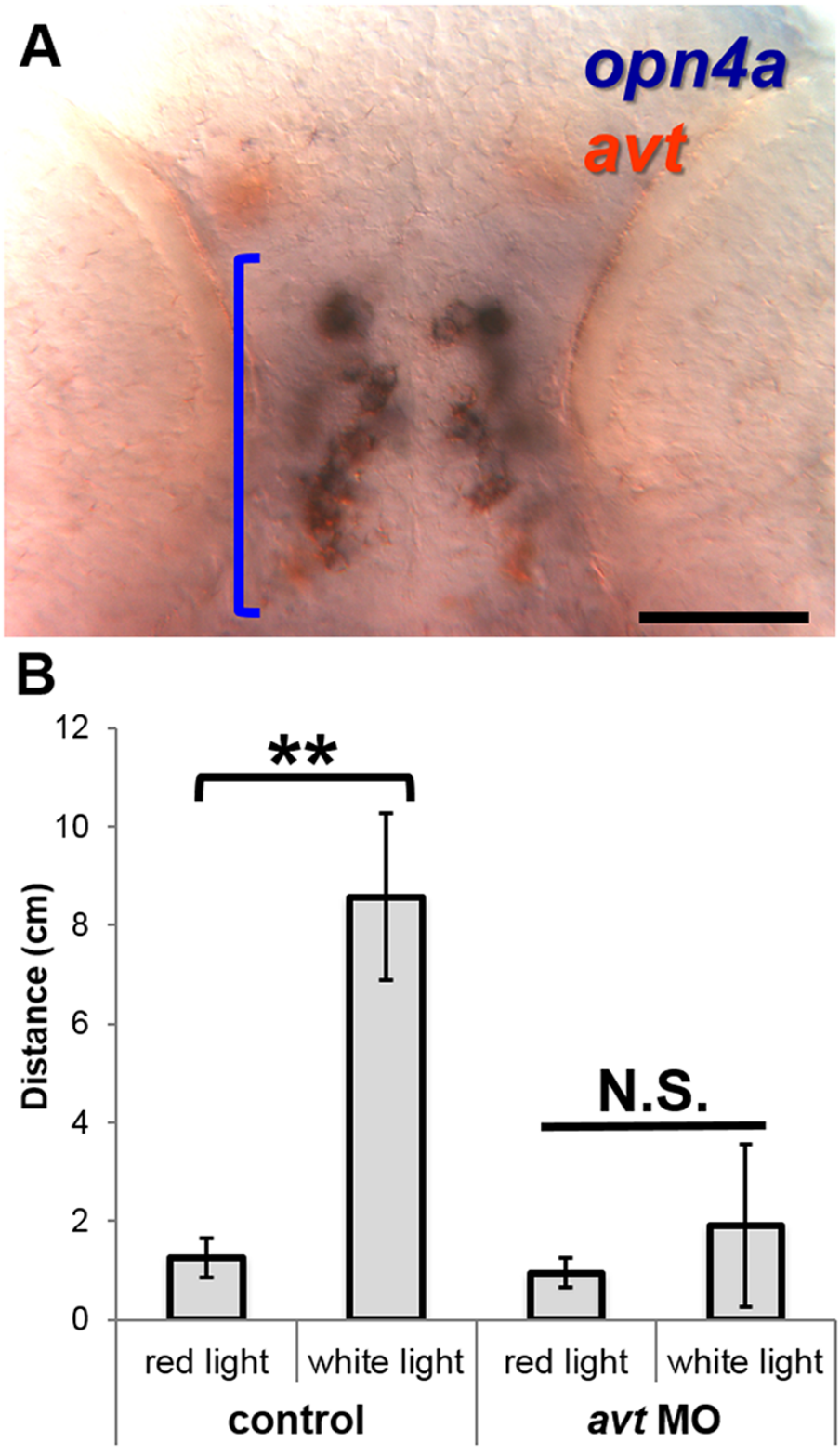


Fig. 3.5. *avtr1a1* is expressed in spinal mechanosensory cells

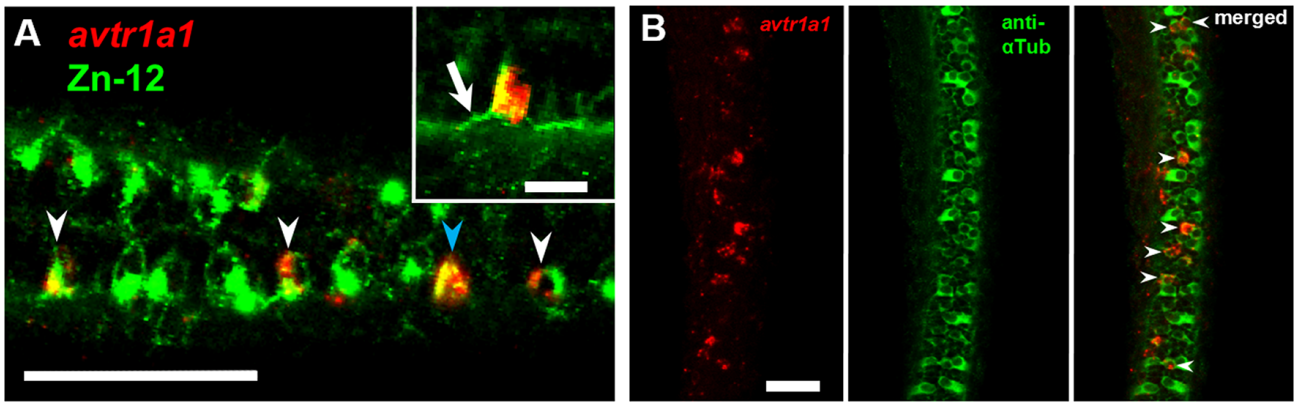
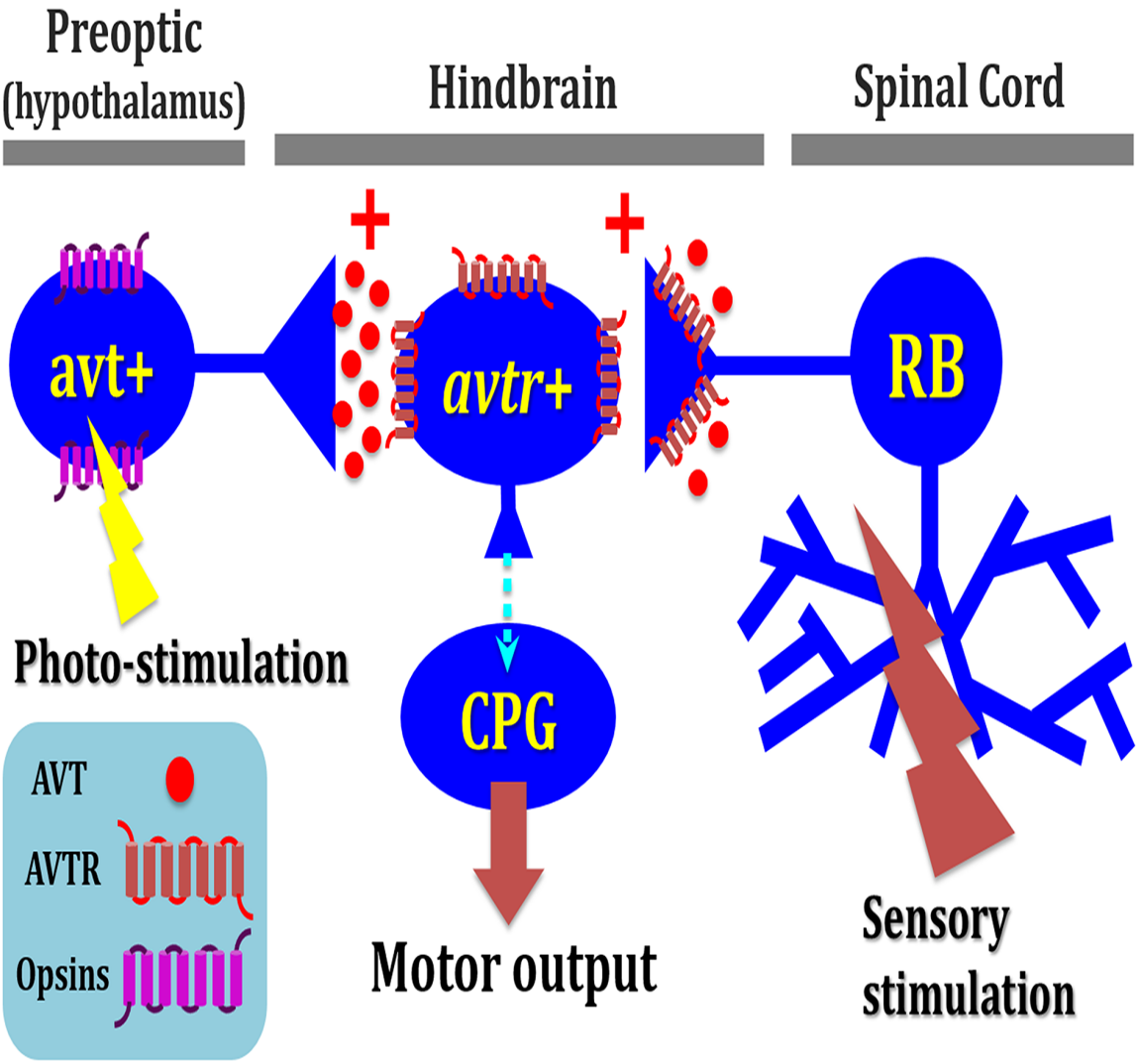


Fig. 3.6. A Model for vasotocinergic modulation of sensorimotor behaviors



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[2] Chapter III was prepared for publication with the following authors:
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*KI conducted c-fos assays, anti-sense morpholino oligonucleotide knockdown experiments including synthesis of DNA constructs and rescue/ISH RNAs, light assays, analyzed all data; MCS, WNY, PES assisted in conducting behavioral experiments and behavioral scoring. PES assisted in conducting *in situ* hybridization for Fig. 3.5A. MT assisted in making DNA constructs for morpholino experiments. NMP conducted immunolabeling and in situ hybridization for Fig. 3.2A using RNA probes synthesized by KI. KI did rest of the experiments.

Chapter IV. Social modulation of aggression-related behaviors and the potential roles of *avtr* signaling in modulating adult behaviors

1. Introduction

The biological basis of social behaviors has been of interest to many. However, the biological mechanism for social behaviors has been notoriously elusive as there were no reliable technology until recently to probe for neural circuits in the brain that control social behaviors. However, recent advances in molecular genetics have started to allow scientists to identify just such circuits. For instance, the optogenetic manipulations of genetically defined cell subpopulations in specific brain nuclei in the mammals led to the discovery of neuron groups that can modulate complex social behaviors (reviewed by Miczek et al., 2015). The scientists in fact demonstrated that one can induce specific social behaviors simply by activating neuron groups localized in specific brain regions like the amygdala and ventromedial hypothalamus (Hong et al., 2014; Lin et al., 2011). Scientists are now equipped with molecular genetic tools to uncover the causal relationship between brain circuits and specific social behaviors. Moreover, studies on zebrafish demonstrate that neural circuits containing the habenular nucleus (Ha) are required for adaptive responses critical for survival like fear and avoidance responses (Agetsuma et al., 2010; Amo et al., 2014). These studies strongly suggest that the vertebrate brain contains separate neural circuits to regulate specific type of behaviors. Although it is only beginning to be understood how upstream executive brain regions like prefrontal cortex (PFC) regulate the

the activities of these neural circuits (Adhikari et al., 2015), animals can clearly select certain behaviors over others depending on the social situation.

If animals in a same species share identical behavioral neural circuits as the aforementioned studies suggest, every individual should be capable of performing the same repertoire of social behaviors. However, individuals in the same species often act very differently from others when they are placed in the same social situations. Each individual appears to preferentially select specific behaviors over others, and their behavioral decision can differ markedly from that of others in the social group. What accounts for the great variability in social behaviors seen between individuals within the same species? It turns out that the functions of neural circuits are often modulated by neuromodulators, and behavioral outputs can be changed significantly depending upon the neuromodulation in effect at the moment (Bargmann, 2012; Marder, 2012; Marder et al., 2014). The efficiency of modulation of neural circuitry by neuromodulators may differ significantly between individuals and this may well lead to great variability in how they act in similar social situations.

Among neuromodulators, neuropeptides have attracted increasing attention in recent years for their ability to modulate behaviors (Asahina et al., 2014; Beets et al., 2012; Dolen et al., 2013; Dolen & Malenka, 2014; Flavell et al., 2013; Garrison et al., 2012; Hergarden et al., 2012; Huber et al., 2005; Kim et al., 2013; Leinwand & Chalasani, 2014; Taghert & Nitabach, 2012; Woods et al., 2014). Neuropeptides can alter an individual's behaviors for an extended period of time (Burbach, 2011), and as a result, the individual can show a behavior that's markedly different from its conspecifics in the social group. For instance, arginine vasopressin (AVP), a neuropeptide synthesized in the hypothalamus, has

been known to play an important role in shaping aspects of social behaviors in mammals. Among three known receptor types for the neuropeptide, V1a receptor plays pivotal roles in regulating social behaviors. The expression pattern of V1a receptor in the brain appears to determine the social interaction of animals (Donaldson et al., 2010; Gobrogge et al., 2009; Lim et al., 2004; Young et al., 1999). V1a-mediated AVP signaling shapes the monogamous bonding behaviors of prairie voles by modulating the functions of a reward pathway involving ventral pallidum (VP) that contains a greater amount of V1a receptors in the brain of monogamous species of voles compared to that of promiscuous species of voles (Barrett et al., 2013; Lim et al., 2004a; Lim et al., 2004b; Lim et al., 2004c). Importantly, virus-mediated RNA interference down regulation of pallidal V1a receptor impairs social bonding behavior in voles (Barrett et al., 2013). This demonstrates that the differential V1a expression in the ventral pallidum leads to a variability in individual social behaviors. However, the molecular, genetic basis underlying such an effect is still not well understood partly because these studies focus on large, highly complex mammalian brains, which makes it technically challenging to study neural circuits.

In non-mammalian species, a homologous neuropeptide called arginine vasotocin (AVT) modulates social behaviors. Like its mammalian homologue AVP (Gobrogge et al., 2009; Haller, 2013), AVT is also linked to aggressive social interactions across species including teleosts (Filby et al., 2010; Huffman et al., 2014; Larson et al., 2006; Lema et al., 2015; Yokoi et al., 2015); however, the underlying mechanism remains unclear except that AVT modulates social behaviors via V1a-like receptor signaling (Yaeger et al., 2014).

In the current study, we first describe how aggressive behaviors, consistently linked to *avt* signaling, can be modulated in a large social group of adult male zebrafish. Two AVT

receptors homologous to mammalian V1a receptor (*avtr1a1* & *avtr1a2*) have been cloned and found to be expressed widely in the brain of zebrafish embryos, suggesting a role in modulating early behaviors (Iwasaki et al., 2013). In contrast, the expression pattern of *avtrs* in adult zebrafish brains is currently unknown. However, such information is essential to better understand the roles of *avt* signaling in adult social behaviors. The current study therefore examines the expression patterns of *avtrs* in adult zebrafish brains via whole adult brain *in situ* hybridization technique to gain insights into how the neuropeptide signaling may modulate vertebrate social behaviors. Based on the obtained data on adult brain expression of *avt* receptors, the possible mechanism by which *avt*/*avtr* signaling modulates social interaction is discussed.

2. Materials & Methods

Animal maintenance

Adult zebrafish were housed in a fish breeding facility that maintained a 14 hr (light): 10 hr (dark) light cycle and followed the University of Michigan Animal Care and Use protocols. Fish were fed daily with dry pellets and brine shrimp. To obtain zebrafish embryos, pairs of adult breeders were isolated overnight in a clear fish cage with a divider between the fish. The next morning, the divider was removed to allow breeding for approximately 1 to 2 hours. Embryos were collected and kept in a fish tank (28.5 ^{o}C) til the fish reaches 2-3 weeks post fertilization (wpf), at which the fish were placed and kept in a fish breeding system til the desired developmental stage.

Brain dissection & preparation for in situ hybridization

At a desired developmental stage (72-100 days post fertilization) at which zebrafish adults reached a body size (1.2-1.7 cm in length) with brains small enough to penetrate with RNA probes and semi-translucent for easier microscopic observation post-labeling that can be reliably dissected for whole brain *in situ* hybridization, zebrafish adults were anesthetized in 0.04% Tricane (Ethyl 3-aminobenzoate methanesulfonate, Sigma-Aldrich) for 10 minutes immediately prior to brain dissection. The sex was first confirmed by the presence of a testis or an ovary. The brain was dissected out (Gupta & Mullins, 2010) and fixed in 4% paraformaldehyde overnight. The fixed embryos were subsequently dehydrated in a series of methanol solutions (25, 50, 75, 100%) and kept at -20 ^{o}C for at least 2 hours before *in situ* hybridization was conducted.

Whole-brain in situ hybridization of adult zebrafish

Dissected brains were labeled for *avtr1a1* or *avtr1a2* mRNAs via *in situ* hybridization (ISH) as previously described (Iwasaki et al., 2013). Sense RNA probes for *avtr1a1* were used for a negative control. The RNA probes (anti-*avtr1a1*-DIG, anti-*avtr1a2*-DIG, and sense-*avtr1a1*-DIG) were synthesized *in vitro* using *avtr1a1* and *avtr1a2* cDNA clones (Iwasaki et al., 2013). For male brains, brains were selected randomly from a mixture of dehydrated brains from 5 separate clutches (1.3-1.7 cm in length, 72-110 dpf) and assigned to each of the three conditions [anti-*avtr1a1 (N=4)*, anti-*avtr1a2 (N=5)*, and sense-*avtr1a1* control (N=4)] to minimize selection bias. Moreover, all three RNA probes

were diluted in DEPC-treated water to 1.2 ng/µl prior to labeling, and *in situ* hybridization for the three conditions were conducted simultaneously side by side to keep all conditions identical except for the difference in RNA probe types. The color reaction (substrate: NBT/BCIP, Roche) was run for 9 hours for all conditions.

Following *in situ* hybridization, microscopic images for the labeled brains were taken both from ventral and dorsal sides (some lateral images were also taken for confirmation of certain brain structures) using a microscope with DIC settings (Leitz, Orthoplan 2) prior to vibratome sectioning in order to capture *avtr*+ brain regions which can be identified from intact, semi-translucent whole brains (Wullimann et al., 1996). The pre-sectioning images were used to identify *avtr*+ cells in the following brain regions; telencephalon (Tel), habenula (Ha), anterior/posterior parvocellular preoptic nuclei (PPa/PPp), suprachiasmatic nuclei (SC), and the most-caudal posterior hindbrain. Subsequently, the brains were embedded in low-melting point agarose (7 %) and sectioned (cross-sections, 150 µm in thickness) using a vibratome. Obtained brain slices were mounted in 70 % glycerol and cross-sectional images were taken using a microscope with DIC settings (Leitz, Orthoplan 2). Both pre-sectioning (both ventral and dorsal) and post-sectioning (cross-sectional) microscope images were examined to identify brain regions (Wullimann et al., 1996) that expressed *avt* receptors.

Pair aggression assay

Ten size-matched (by body length and weight) pairs of male wild-type adult zebrafish were selected and isolated (as pairs) in a fish tank (covered with a black plastic sheet except for the front side) with an opaque divider (to prevent them from interacting

with or seeing each other) for 5 days. The mean age for the paired fish was 14.6 months post fertilization. Average size for the paired fish was 3.5 cm (error within pairs ≤ 0.1 cm). Average weight for the paired fish was 391.5 mg (error within pairs ≤ 60 mg). The sides of the tanks were covered with a black plastic sheet to prevent the fish from seeing other fish. The fish were fed once per day with brine shrimp. After five days of isolation, the dividers were removed to let each pair of fish interact with its partner. Either top or bottom tail fin was cut with a razor blade to allow for fish identification during behavioral analysis. The tail fin cut did not bias the fish to be either the dominant (winner) or the subordinate (loser) (it was confirmed post-experiment that 50 % of the fish with the top fin cut became winners). The responses were recorded (Handycam, HDR-CX100, Sony®) for 6 minutes immediately after the divider removal. The recording at approximately same time of the day (11 am to 12 pm) was done for 2 more days, and the behaviors shown during the interaction were scored manually by playing the videos in slow motion (VLC media player, VideoLAN Organization) according to pre-defined behaviors; (i) circling, (ii) mutual ramming, (iii) chasing, and (iv) freezing as follows (chasing was described by Larson et al. (2006), but it was redefined in detail in the current study to make the manual scoring as consistent as possible between experimenters).

(i) Circling (measured in seconds):

Circling occurs when two fish swim in circles in one place, closely following each other's body. It is often associated with mutual ramming (see below). Circling is counted separately from chasing, as both fish mutually engage in circling.

(ii) Mutual ramming (number of events counted):

Mutual ramming toward each other results in quick swimming away from each other. The number of ramming events is counted instead of measuring duration (ramming often lasts less than one second).

(iii) Chasing (seconds):

Chasing is counted when one fish chases after the other (unidirectionally). The fish chased swims away from the chaser. Chasing includes the initial quick swimming toward the fish chased, ramming by one fish (excluding circling or mutual ramming as these acts are mutual), and biting. When the winner temporarily swims away within a second but comes right back to chase, it is considered continuous chasing. Only if the winner turns away for more than a second, is it considered the end of chasing. Chasing typically occurs within 2-fish-lengths between the winner and the loser. Chasing occurs when one fish follows the other fish regardless of the speed (speed is not a factor when considering chasing).

(iv) Freezing (seconds):

Freezing occurs when one fish stops forward swimming. Freezing often involves lowering of the tail and slowly sinking vertically toward the bottom of the tank.

It is important to note that circling and mutual ramming require both parties' participation and therefore can be thought of as a mutual aggression. On the other hand, chasing is a unidirectional aggressive act by the dominant toward the subordinate. Freezing is exclusively shown by the subordinate fish as the dominance hierarchy develops. The dominant fish (winner) and subordinate fish (loser) were determined based on chasing behaviors; only winners show chasing on day 2 & 3 of pair interaction. Moreover,

only the losers show freezing over the course of 3 days of pair interaction while the winners show no freezing.

The fish were not fed during the pair interaction to prevent the dominant fish from becoming larger in size/weight by dominating the food, which may give the winners an advantage in fighting.

Social group aggression assay

At the end of recording for Day 3 of the pair aggression assay, each fish within each pair was placed separately into a size-matched larger social group for 3 days (N = 5 male adult zebrafish; the difference in body length between a pair fish and its corresponding social group fish ≤ 0.28 cm, the difference in weight between a pair fish and its corresponding social group fish ≤ 46 mg). The behaviors each fish showed immediately after placement into a new large social group was recorded 5 min. The fish were recorded for 5 min on the subsequent 2 days as well, and the pre-defined behaviors were scored manually for the social group setting. Immediately after the last day of the social group assay, the experimental fish (winners or losers) were dissected to confirm the male sex by the presence of testes (all ten pairs were confirmed to consist of male fish). The male naïve fish to make the large social groups were chosen based on male specific physical characteristics of having a slim body (due to the lack of oocytes that make the females' bellies bulging and large) with a more pink and yellow belly.

3. Results

3.1. Aggressive interaction and development of a dominant relationship within pairs of adult male zebrafish

Immediately after dividers were removed, two mutually aggressive acts were observed; circling and mutual ramming. These mutual aggressive acts were only seen on day 1 of pair interaction (Fig. 4.1A and 4.1B). In fact, circling behavior quickly diminishes to near zero during the 1st 6 minutes after divider removal (Fig. 4.1C) while mutual ramming gradually increased during the same period (Fig. 4.1D). This indicates that circling is a more transient phenomenon that dissipates quickly during the first minutes of aggressive interaction while ramming develops and dissipates more slowly. Further experiments are necessary to determine exactly when the ramming gets eliminated. Such information will provide a more complete view of how antagonistic interaction transforms over time.

Unlike mutually aggressive acts, chasing increased over the course of 3 days of pair interaction (Fig. 4.2). By day 2 of pair interaction, dominant relationships were established clearly in which the dominant fish showed aggressive chases while the subordinates did not. Instead, the losers showed a rapid increase in freezing behaviors by day 2 of pair interaction (Fig. 4.3) while the winners showed no such behaviors on any of the three days of pair interaction.

3.2. Social modulation of aggressive behaviors; decrease in aggressiveness/submissiveness upon placement into a large social group

To investigate whether the social environment of zebrafish modulates aggressive behaviors, we examined the behavior of winner or loser zebrafish in a social context. First

we observe that in a group of 5 naive adult males, there was little to no aggressive behaviors within the group (data not shown). Interestingly, individual winners showed little chasing behavior immediately after they were placed into a group of 5 other male zebrafish (Fig. 4.4). The level of chasing remained low during the next 2 days after placement into the social group (1.3 and 0.9 seconds in 5 min respectively). Similarly, individual losers stopped freezing as soon as they were placed in a larger social group (Fig. 4.5). Freezing by losers remained low during the next 2 days as well (0 and 1.8 seconds in 5 min respectively). A mild level of chasing was directed toward the winners and losers by other fish (non-experimental fish) in the social groups upon placement of winners and losers into the groups (2 and 0.8 seconds in 5 minutes), but they appear to be minuscule compared to the intense chasing shown by the winners (89.9 seconds on average on Day 3 of pair interaction) between pairs of fish (Fig. 4.2). The level of chasing directed toward the winners and losers by other fish in the groups remained low (less than 6.1 seconds in 5 min) for the next 2 days (Fig. 4.6). This further confirms that aggression remains suppressed in the social groups.

3.3. Adult brain expression of avtrs

a) avtr1a1 expression in adult brains

The goal of this part of the current study was to gain insights into the biological mechanism by which neuropeptide signaling modulates social behaviors like aggressive acts. For *avt*/*avtr* signaling to modulate social behaviors as shown by past studies, we reasoned that *avtrs* must be expressed in key brain regions that are known to be important for regulating social behaviors, such as the habenular nucleus (Ha) that regulate fear

responses (Agetsuma et al., 2010; Amo et al., 2014). To test this possibility, we conducted whole adult brain *in situ* hybridization to probe for mRNAs for *avtr1a1* and *avtr1a2* in the brain of both male and female adult zebrafish.

We found that *avtr1a1* is expressed in various brain regions (Table 1; $N_{male\ brains} = 4$, $N_{female\ brains} = 2$). Importantly, the list includes brain regions known to affect social behaviors across species such as telencephalic regions (Tel), parvocellular preoptic regions (PP), suprachiasmatic nucleus (SC), and dorsal habenula (Had). *avtr1a1* is expressed by cell groups in multiple regions (Dm/Vd, Vv, Dp; see Table 1 for abbreviations) in the dorsal and ventral telencephalic regions (Fig. 4.8B, 4.8D, 4.8F). Moreover, *avtr1a1* is expressed in the diencephalic regions as well. The anterior and posterior parts of parvocellular nuclei (PPa and PPp) and the suprachiasmatic nucleus (SC) all express *avtr1a1* (Fig. 4.8H, 4.8J). It is intriguing that PPa and PPp both contain neuroendocrine cells that synthesize and release arginine vasotocin (AVT) (Larson et al., 2006). Fig 8J clearly shows the expression of *avtr1a1* in the suprachiasmatic nucleus which lies above where the optic tracts (OT) cross (i.e. optic chiasm).

Importantly, the results clearly demonstrate the presence of *avtr1a1* in the dorsal habenula (Had) (Fig. 4.8L-M, 4.8O-P), a brain region known to regulate fear responses in zebrafish (Agetsuma et al., 2010). As a matter of fact, Had is one of the regions that showed the most intense signal for *avtr1a1* mRNA in both male and female brains. Fig. 4.8O-P show that *avtr1a1* is localized to a small region in the Had adjacent to the dorsal sac (SD) which also shows intense *avtr1a1* signal. The habenular commissure (Chab) between the two dorsal habenular nuclei also expresses *avtr1a1* (Fig. 4.8P). The function of SD is currently unknown although its direct physical connections with the dorsal habenula

suggest a potential effect on fear responses. Interestingly, *avtr1a1* is completely absent in the ventral Ha (Hav) which is essential for zebrafish to avoid potential dangers (Amo et al., 2014). *avtr1a1* is also expressed in the posterior hindbrain. Specifically, *avtr1a1* is expressed in cell groups in the vagal lobe (LX) (Fig. 4.8R) and unidentified cells in the ventro-medial posterior hindbrain (Fig. 4.8T). The receptor is also seen in the rostral most rostral part of the spinal cord (Fig. 4.8V). None of the sense-*avtr1a1* control brains (N = 4) showed signal in these structures (Fig. 4.8A, C, E, G, I, K, N, Q, S, U).

Individual variability is seen in the *avtr1a1* expression in the telencephalic regions as only 50 percent of the examined brains showed *avtr1a1* expression in Dm/Vd and Vv for both male and female brains, and in Dp for male brains (Table 1). In the diencephalic regions, all brains examined showed *avtr1a1* in PPa and SC for both sexes and in PPp for male brains (cross sections to confirm PPp were not obtained for female brains). All brains examined for both sexes showed *avtr1a1* expression in the dorsal habenula (Had) while only male brains showed the receptor expression in the adjacent dorsal sac (DS). *avtr1a1* expression in the habenular commissure (Chab) was confirmed by vibratome sections for male brains. In the posterior hindbrain regions, *avtr1a1*+ cells were found in the vagal lobe (LX), ventro-medial posterior hindbrain, and ventro-medial rostral spinal cord for all brains examined for both sexes.

b) avtr1a2 expression in adult brains

On the other hand, *avtr1a2* was also found to be expressed in various brain regions (Table 2; $N_{male\ brains} = 5$, $N_{female\ brains} = 3$), some of which overlap with *avtr1a1*+ regions. Fig. 4.9B shows that *avtr1a2* is expressed in cell groups in PPp and SC that are located between

the diencephalic ventricle (DiV) and the optic tracts (OT). *avtr1a2* is also expressed in the thalamic nucleus located immediately below the habenula (Ha) (Fig. 4.9D). The dorsal habenula (Had) also expresses *avtr1a2* (Fig. 4.9F & 4.9G) but the expression of *avtr1a2* is more widespread in the nucleus than that of *avtr1a1*. Both SD and Chab are also positive for *avtr1a2* (Fig. 4.9F & 4.9G). In the hindbrain, *avtr1a2* is found in the cerebellar corpus (CCe) and facial lobe (LX) (Fig. 4.9I), vagal lobe (LVII) (Fig. 4.9K), and unidentified cell groups in the ventro-medial posterior hindbrain (Fig. 4.9M). The controls brains (N = 4) showed no specific labeling in these structures (Fig. 4.9A, C, E, H, J, & L).

Unlike *avtr1a1*, *avtr1a2* was not found in the telencephalic regions for either sex (Table 2). All brains examined for both sexes showed *avtr1a2* expression in posterior parvocellular preoptic nucleus (PPp) and suprachiasmatic nucleus (SC). 80 percent of male brains examined and 67 percent of female brains examined showed *avtr1a2* expression in the thalamic nucleus while *avtr1a1* was not found in the region. The dorsal habenula (Had), habenular commissure (Chab), and dorsal sac (SD) all showed *avtr1a2* expression in every brain examined for both sexes. In the hindbrain regions, *avtr1a2*+ cells found in cerebellar corpus (CCe), facial lobe (LVII), Vagal lobe (LX), and ventral posterior hindbrain region in every brain examined for both sexes. *avtr1a1* was also found in LX but not in CCe and LVII, suggesting functional differentiations between the receptor subtypes in the hindbrain.

4. Discussion

An earlier study demonstrated that pairs of adult male zebrafish develop a dominance hierarchy (Larson et al., 2006). Our results confirmed this finding and showed

that after an initial period of mutual aggression (e.g. circling and ramming), a dominance hierarchy quickly developed. The dominant status of the winners is established by the second day of interaction, but the winners continue to show aggressive chases toward the subordinates til the third day despite the fact that the losers stopped fighting completely by day 2 and engage in freezing behaviors. This indicates that the dominant fish makes efforts to maintain the dominant status once it is established. These findings are interesting given that zebrafish exhibit extensive shoaling and form tight social groups (Gerlai, 2014; Green et al., 2012; Mahabir et al., 2013; Miller & Gerlai, 2012). In accord with their shoaling behavior, we found that groups of male zebrafish exhibited little to no aggressive behavior (data not shown). Furthermore, both individual dominant and submissive zebrafish immediately ceased their aggressive and submissive behaviors, respectively, when placed into a group of male zebrafish. These findings suggest that social context can apparently exhibit a powerful effect on aggression by zebrafish. The basis for this apparent inhibition of aggression is unknown. Mammalian brains possess neural circuits that can antagonistically regulate two opposing social and anti-social behaviors (Hong et al., 2014). Such antagonistic mechanism may be present in fish brain as well to antagonistically control aggressive acts and shoaling behaviors depending on the social situations the fish is placed in (Fig. 4.7). The dorsal habenula (Had) modulates fear responses and when it's genetically inactivated, zebrafish tends to choose freezing to a conditioned fear stimuli (Agetsuma et al., 2010). We found expression of both *avtr1a1* and *avtr1a2* in the dorsal habenula, and it is possible that *avtr* signaling modulates the behavioral functions of Had during social interactions. In mice, a subregion in the ventromedial hypothalamus (VMH) was identified as an aggression locus that can induce aggressive attacks by males (Lin et al.,

2011). However, the locus of aggression is not identified in fish brain and it is not known if the neural circuit for aggression can be directly modulated by neuropeptide signaling. In fruit flies, a neuropeptide tachykinin modulates the activity of FruM+ neurons directly involved in intermale aggression (Asahina et al., 2014). However, it is not known if this or other neuropeptides can modulate aggression-inducing neurons in the vertebrate species.

The current study shows the presence of *avtr1a1* and *avtr1a2* in several brain regions that are known to be important for regulating complex social behaviors such as telencephalic regions, habenula, preoptic nuclei, and suprachiasmatic nucleus (Table 3). The expression of *avtr1a1* in telencephalon (Tel) is intriguing in that this region is equivalent to human neocortex that controls executive behavioral functions; however, in zebrafish brain, the behavioral roles of each subregion in Tel is largely unexplored. In fact, the telencephalic subregions are just beginning to be defined spatially using molecular markers (Ganz et al., 2012). The neuroendocrine cells in the anterior hypothalamus are linked to aggressive behaviors (Filby et al., 2010; Gobrogge et al., 2009; Larson et al., 2006), and the expression of both *avtr* types in the region suggests a potential involvement of *avtr* in affecting functions of neuroendocrine cells in the region. *avtr1a1* and *avtr1a2* expression in the suprachiasmatic nucleus (SCN) suggest their modulatory roles in SCN-dependent circadian rhythm (Moore & Whitmore, 2014), which likely affects the state of aggressiveness.

Although little is known about the vertebrate neural circuitry modulating aggression in different social situations, studies implicate several candidate sensory cues in modulating social behaviors. Vinegar flies are known to rely heavily on chemosensory cues to recognize and interact with others in social situations (reviewed by Billeter & Levine,

2013). The transient social group interaction vinegar flies exhibit is in fact olfactory-cue dependent (Schneider et al., 2012). Cis-Vaccenyl Acetate (cVA), a male-specific pheromone regulates intermale aggression in vinegar flies, and the pheromone also regulates the size of a social group by regulating the level of in-group aggression (Wang & Anderson, 2010). Olfaction-dependent communication is also essential for zebrafish to survive in social groups as zebrafish utilize mixtures of olfactory cues to decide how to interact with others including reproductive partners (Sorenson et al., 1998). In fact, the development of olfactory sensitivity to certain odorants starts very early during the larval stage (Li et al., 2005), and zebrafish larvae exhibit behavioral responses to different odorants (Vitebsky et al., 2005). Sensitivity to odorant cues is essential for the survival of larvae as the cues convey important information such as food source or presence of other fish (Vitebsky et al., 2005). For instance, the predator avoidance behavior in juvenile zebrafish relies on the functional olfactory detection of conspecific skin extracts (Blechinger et al., 2007). Moreover, zebrafish may utilize pheromonal cues from the conspecifics to stay close to the shoaling group as the pheromonal cues from the conspecifics can act as attractants (Bloom et al., 1977). However, the right compositions of the pheromonal cues that can keep the members in the shoaling group for an extended period of time is rather complex and still remains unknown as zebrafish can respond differently to various chemosensory cues depending on the concentrations of the cues and the number and the sex of the conspecifics that produce them (Bloom et al., 1977). In fact, fish can distinguish conspecific bile acids from those of other sources as and may use endogenous bile acids as migratory pheromone (Li & Sorensen, 1997). For instance, the olfactory epithelium of sea lamprey is particularly sensitive to lamprey-specific bile acids, petromyzonol sulfate and allocholic acid (Li &

Sorensen, 1997). The olfactory organ of adult zebrafish also shows a differential sensitivity to bile acids depending on the conjugation, with taurine-conjugated bile acid the most effective stimulant (Michel & Lubomudrov, 1995). However, the bile acids used in the study were derived from mammalian sources (Michel & Lubomudrov, 1995), and therefore, it is not clear if the endogenous bile acids can act as a conspecific attractant for zebrafish. Moreover, zebrafish olfactory bulb responds to a wide variety of other odorants including amino acids which activate glomeruli in distinct regions within the olfactory bulb (Michel & Lubomudrov, 1995, Li et al., 2005), and a systematic investigation is necessary to identify the social odorants that induce shoaling/schooling which likely consist of mixtures of odorant molecules (Sorenson et al., 1998). Thus, it is plausible that the olfactory cues play an important role in modulating social behaviors like aggression in a school of fish. Importantly, the social behavioral functions of the olfactory system may be modulated by a neuropeptide as vasopressin+ interneurons in the rat olfactory bulb impair social recognition (Tobin et al., 2010).

In fact, olfactory bulbs (OB) send direct projections to the right habenula, and the particular odors detected in OB may affect the behavioral neural circuits consisting of the habenula and its downstream targets including the interpeduncular nucleus (Miyasaka et al., 2013). As a matter of fact, the right dorsal habenula (Had), specifically the medial subnucleus of the right Had ($_{R}Had_{M}$) which projects axons to the interpeduncular nucleus (IPN), responds to a bile salt and modulates the response of larval zebrafish to the odorant (Krishnan et al., 2014). The presence of both *avtrs* in Had therefore suggests a possible involvement of *avtrs* in modulating odor-induced behavioral responses. However, it is still not clear whether both Had subnuclei or either subnucleus expresses *avtr1a1* and *avtr1a2*

(although the wide expression of *avtr1a2* may suggest its presence in both subnuclei), and this can be resolved by checking the expression of *avtrs* in the habenula of transgenic fish, narp:*Gal4VP16; UAS:DsRed2* (specific to the lateral subnucleus of Had) and *brn3a-hsp70:GFP* (specific to medial subnucleus of Had) (Agetsuma et al., 2010). This will allow us to determine whether *avtr* signaling participates in the $_{R}Had_{M}$-IPN pathway important for odor-evoked behavior (Krishnan et al., 2014) or the Had_{L}-IPN pathway important for regulating fear responses (Agetsuma et al., 2010).

It is important to point out that the freezing behaviors shown by the subordinate fish in the pair aggression assay show some of the characteristics that may mimic depression-like symptoms in humans; low baseline activity, hypolocomotion, droopy tail, reduced shoaling (Nguyen et al., 2014). In fact, zebrafish may be a useful model to study the biological basis of psychiatric disorders like depression because of rapid advances in molecular, genetic techniques to probe for behavioral neural circuitry (Jones and Norton, 2015; Nguyen et al., 2014; Parker et al., 2013). Thus a better understanding of the mechanisms that control freezing may be useful for understanding the debilitating disorder. Interestingly, the depression-like responses shown by the losers in the current study results as a direct consequence of aggressive interaction with its partner. This is consistent with a rodent study that suggests that mice develop experience-dependent aversion to social contact following repeated social defeats (Berton & Nestler, 2006). Better understanding of how social defeats leads to depression-like symptoms may help identify the direct cause for depression-like responses. Moreover, the present study suggests that some of the depression-like characteristics of the loser zebrafish can be dramatically eliminated once the fish is placed in larger social group. This suggests that

placement into social groups may reverse the depression-like symptoms at least in fish. How social context can modulate an individual's anti-social behaviors is poorly understood. Here the genetically amenable zebrafish may be useful in elaborating both the neural and genetic mechanisms regulating anti-social behavior and social regulation of anti-social behavior. Importantly, another major psychiatric disorder, autism, has been linked to a polymorphism in the promoter region for V1a receptor (reviewed by Frank & Landgraf, 2008; Hammock & Young, 2006). Therefore, understanding how *avt*/*avtr* signaling modulates social behaviors in zebrafish may hold therapeutic potential as well.

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Figure Legends.

Figure. 4.1. (A & B) Mutually aggressive acts, circling (A) and ramming (B), are seen on day 1 of pair interaction. $^{**}p < 0.01$; $^{***}p < 0.0001$ by Mann-Whitey U test (exact-2-tailed test). (C) Circling is a transient behavior that quickly diminishes to near zero during the 6 minutes after the divider removal. X-axis indicates the number of minutes after divider removal. Behaviors were analyzed for 6 minutes. (D) In contrast, ramming increases gradually during the 1st 6 minutes after the divider removal. $^{*}p < 0.05$, M-W U test, exact 2-tailed test. This suggests that the paired fish transitions from less aggressive interaction (e.g. swim in circle around each other) into fully aggressive fights that requires a physical contract between the fish. However, ramming will also decrease significantly by the second day of interaction.

Figure. 4.2. Chasing increases over the course of 3 days of pair interaction. The dominant relationship is clearly established by day 2 in which the dominants show intense chasing toward the nonaggressive subordinates . $^{***}p < 0.0001$, $^{*}p < 0.05$ by Mann-Whitney U test (exact-2-tailed)

Figure. 3. Freezing also increases over the course of 3 days of interaction. The losers engage in excessive freezing by day 2 while the winners do not show such behaviors. ** $p < 0.01$ by M-W U test (exact-2-tailed).

Figure. 4.4. Previously dominant fish stopped chasing immediately after placement into a large social group of 5 other male zebrafish. The high level of aggression before placement into a group diminishes to near zero after the social placement. $^{***}p < 0.0001$ by Mann-Whitney U test (exact-2-tailed)

Figure. 4.5. Freezing by the losers decrease dramatically immediately after placement into a large social group as well. $^{**}p < 0.01$ by M-W U test (exact-2-tailed).

Figure. 4.6. Aggression directed toward winners and losers by other fish in the naïve groups remain low following placement of the experimental fish into the groups.

Figure. 4.7. A model proposing an antagonistic control of aggressive acts (chasing/freezing) and shoaling behaviors. The selection of the behaviors may be affected by sensory cues from the conspecifics in the social group such as visual and olfactory cues. The act of swimming with conspecifics in the shoaling group may be another key factor that helps suppress aggression within a larger social group as well.

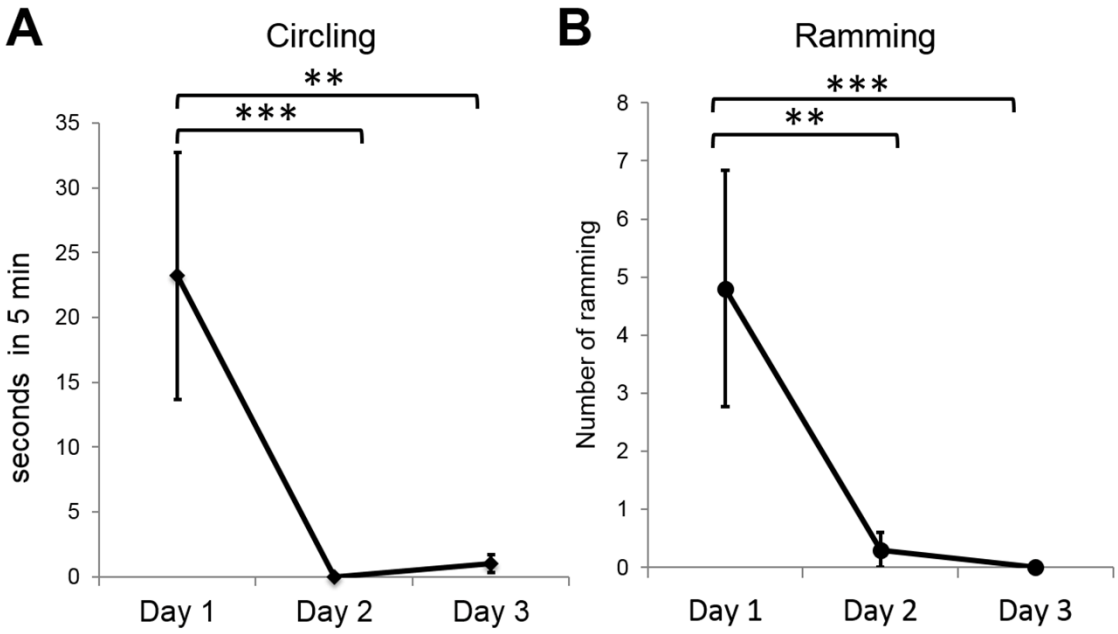
Figure. 4.8. Brain regions found to express *avtr1a1* via whole adult brain *in situ* hybridization as listed in Table 1. Male brains were shown here. Microscopy images for figures A-H, K-M, Q-V were taken immediately after *in situ* hybridization (before sectioning) to retain the anatomical/spatial information as to where *avtr1a1* is expressed. The brain regions were identified according to the zebrafish adult brain atlas by Wullimann et al. (1996). For the identification of brain regions with precision, images were taken dorsally and ventrally for every brain obtained, and the side view images were also taken

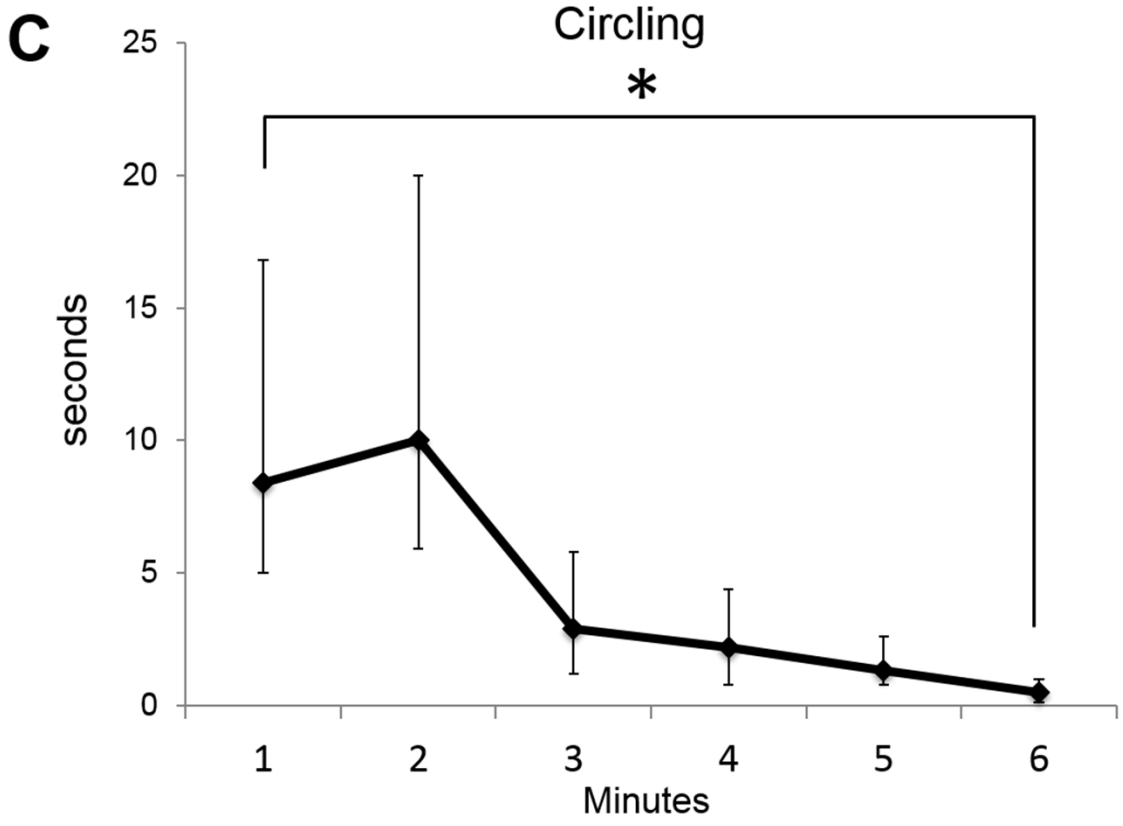
for some brain structures for confirming anatomical position of the *avtr1a1*+ cells. Figures I-J, N-P, were taken after the brains were sectioned (150 μm) with a vibratome for further confirming the anatomical structures for *avtr*+ cells that cannot be reliably identified pre-sectioning, such as the subregions within the habenula (Ha) and structures attached to Ha (e.g. SD). Scale bars indicate 100 μm for all images . Fig. 4.8A-B shows the ventral side of the telenchapalic (Tel) regions, while Fig. 4.8C-F shows the ventral sides. Fig. 4.8G-H shows PPa and SC on the ventral surface of the brains while Fig. 4.8I-J shows the cross section views of the SC as well as PPp that sits immediately above SC. Optic tracts (OT) were used as an anatomical landmark to identify SC (SC sits above the optic chiasm). Fig. 4.8K-M show the dorsal view of the habenula (Ha) that's located on the dorsal region between the telencephalon and the more posterior regions. Fig. 4.8L. shows *avtr1a1*+ dorsal sac (SD) of unknown functions that is connected physically to the dorsal habenula (Had). Fig. 4.8M shows the localization of *avtr1a1*+ cells in a small subregion within Had that is adjacent to SD. The most intense *avtr1a1*+ signal was seen in this *avtr1a1*+ Had subregion. To further confirm the location of the *avtr1a1*+ cells in a subregion within Ha, brain sections (150 μm) containing Ha was examined closely. As indicated in Fig. 4.7M, the cross sections (Fig. 4.8N-P) also clearly shows the localization avtr1a1+ cells in a Had subregion immediately adjacent to SD which is also *avtr1a1*+ (O). The images demonstrate the striking lack of the receptor in the rest of Ha including the ventral portion of Ha (Hav). Intriguingly, *avtr1a2* expression is more sparsely seen in Ha as shown in Fig. 4.9 below. Fig. 4.8Q-R shows the presence of *avtr1a1*+ cells in the vagal lobe (LX) dorsally while S-T (bracket) shows *avtr*+ cells ventrally in the posterior hindbrain (ventro-medial posterior hindbrain). Fig. 4.8U-V indicates the presence of *avtr1a1*+ cells ventrally in the rostral portion of the spinal cord (bracket).

Figure. 4.9. Brain regions found to express *avtr1a2* in the current study via whole brain *in situ* hybridization. Scale bars indicate 100 μm for all images. Fig. 4.9A-B shows a cross-sectional view (slice thickness is 150 μm) of PPp and SC, both of which expresses *avtr1a2*. Optic tract (OT) and diencephalic ventricle (Div) were used as landmarks to identify brain regions. Fig. 4.8C-D, shows the cross-sectional view of the thalamic nucleus located below the habenula (Ha). Thalamic nucleus expresses *avtr1a2*. Fig. 4.9E-G, shows the cross-sectional view of Habenula and dorsal sac (SD) attached to Ha. SD and Had express *avtr1a2*. Fig. 4.9H-M shows the dorsal view (H-K) and the ventral view (L-M) of the posterior hindbrain of an intact brain (before sectioning). *avtr1a2* is expressed in CCe (I), Facial lobe (I), Vagal lobe (K), and a group of cells located in the medial ventral posterior hindbrain (M) (bracket).

Figures (& Tables)

Fig. 4.1. Circling and ramming are only seen on day 1 of pair interaction




C
Circling
*
25
20
15
10
5
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seconds
1
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3
4
5
6
Minutes

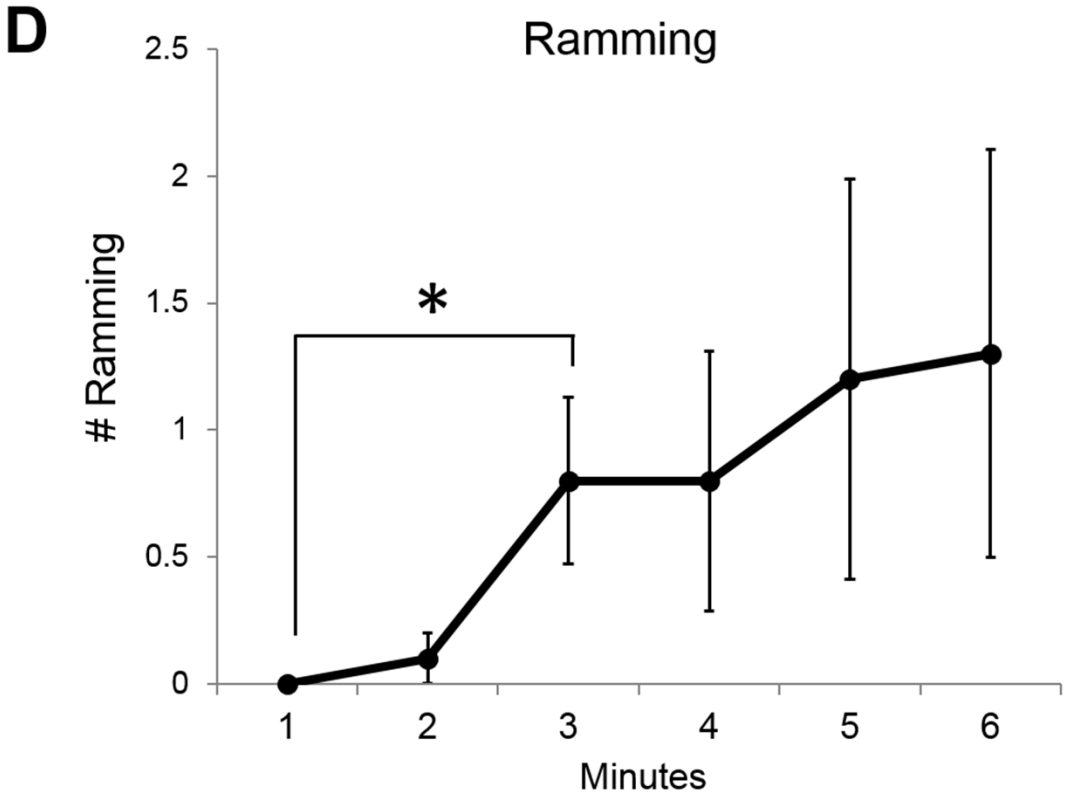

D
Ramming
2.5
2
1.5
1
0.5
0
Ramming
*
1
2
3
4
5
6
Minutes

Fig. 4.2. Chasing shown by winners and losers over the course of three days

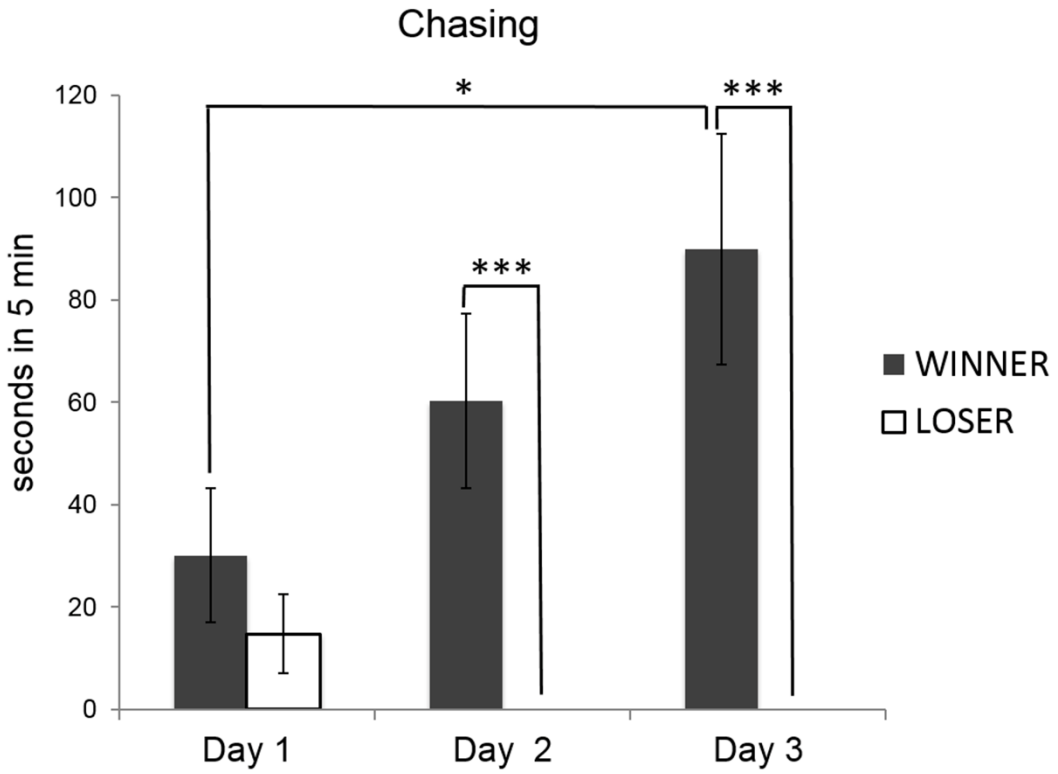


Fig. 4.3. Freezing shown by winners and losers over the course of three days

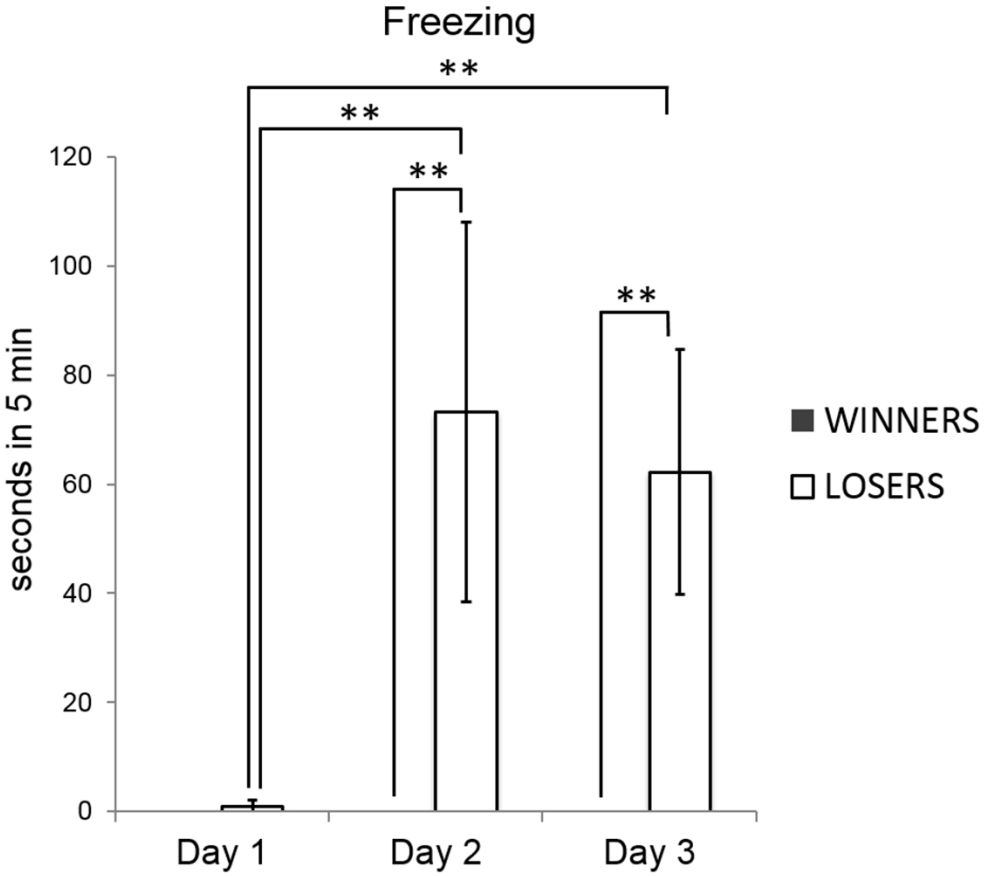


Fig. 4.4. Chasing by winners decreases dramatically upon placement into larger social groups

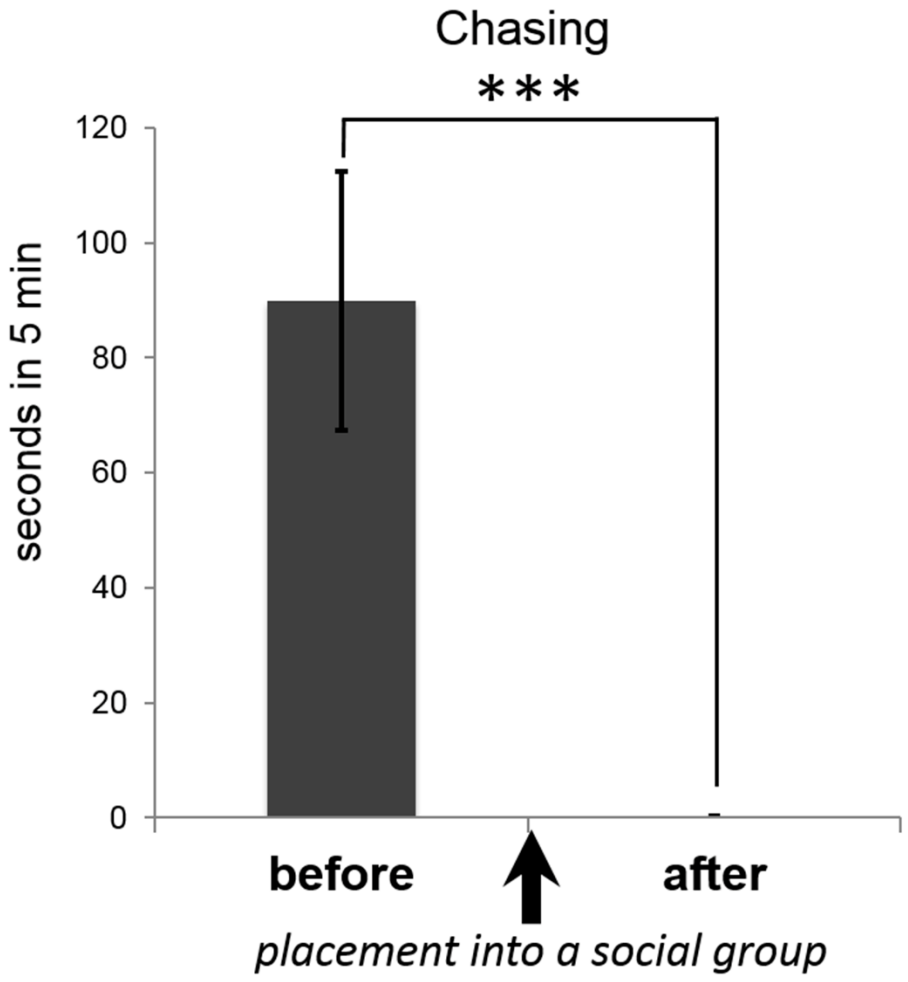


Fig. 4.5. Freezing by losers decreases dramatically upon placement into larger social groups

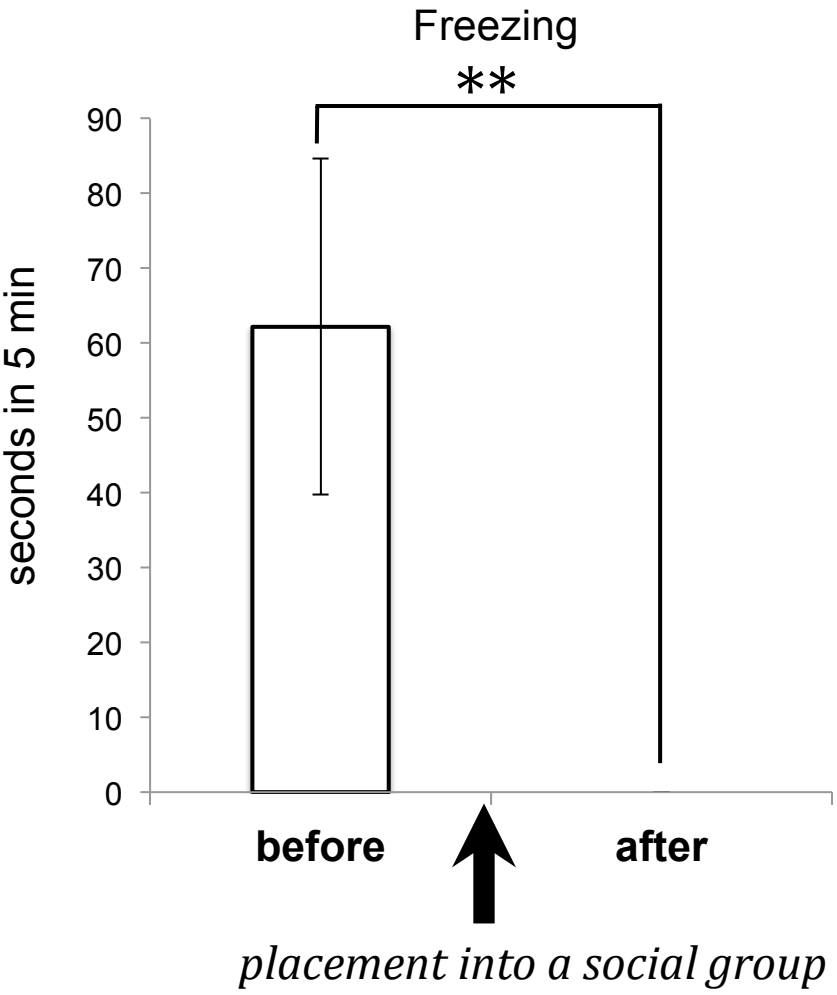


Fig. 4.6. Low aggression directed toward winners/losers upon placement into social groups

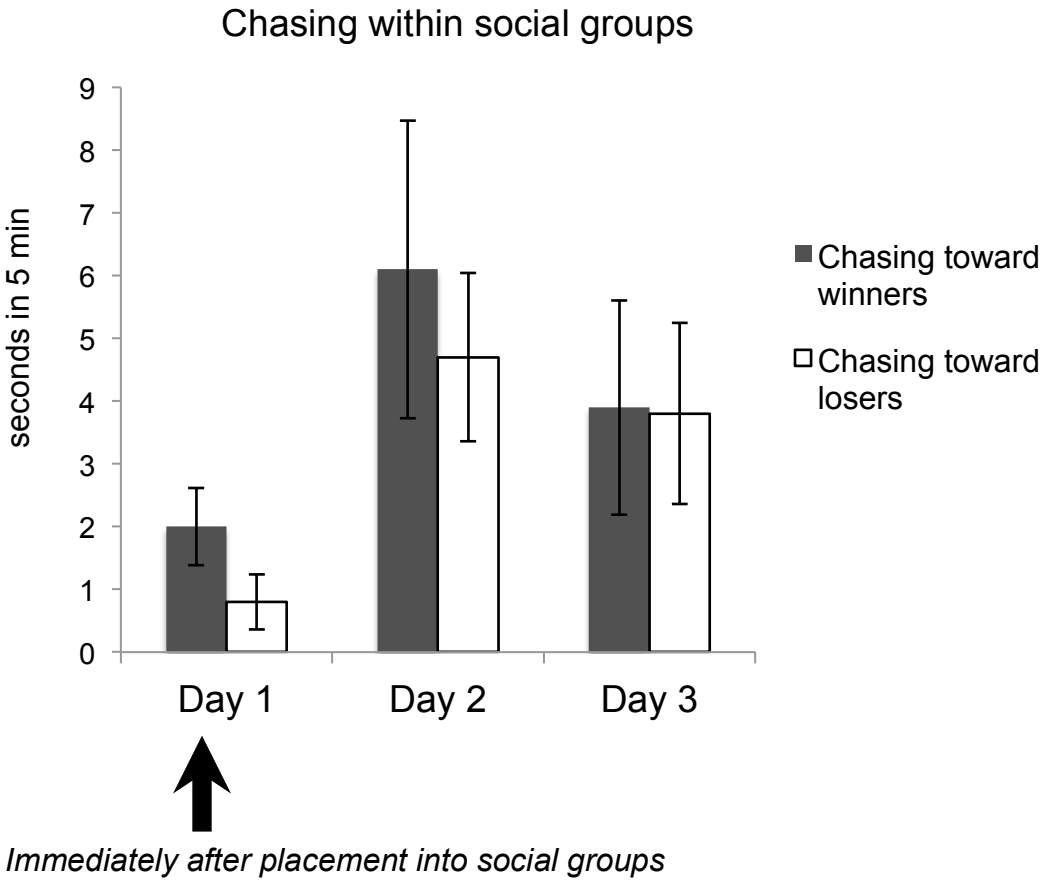


Fig. 4.7. A model proposing an antagonistic control of aggression and shoaling

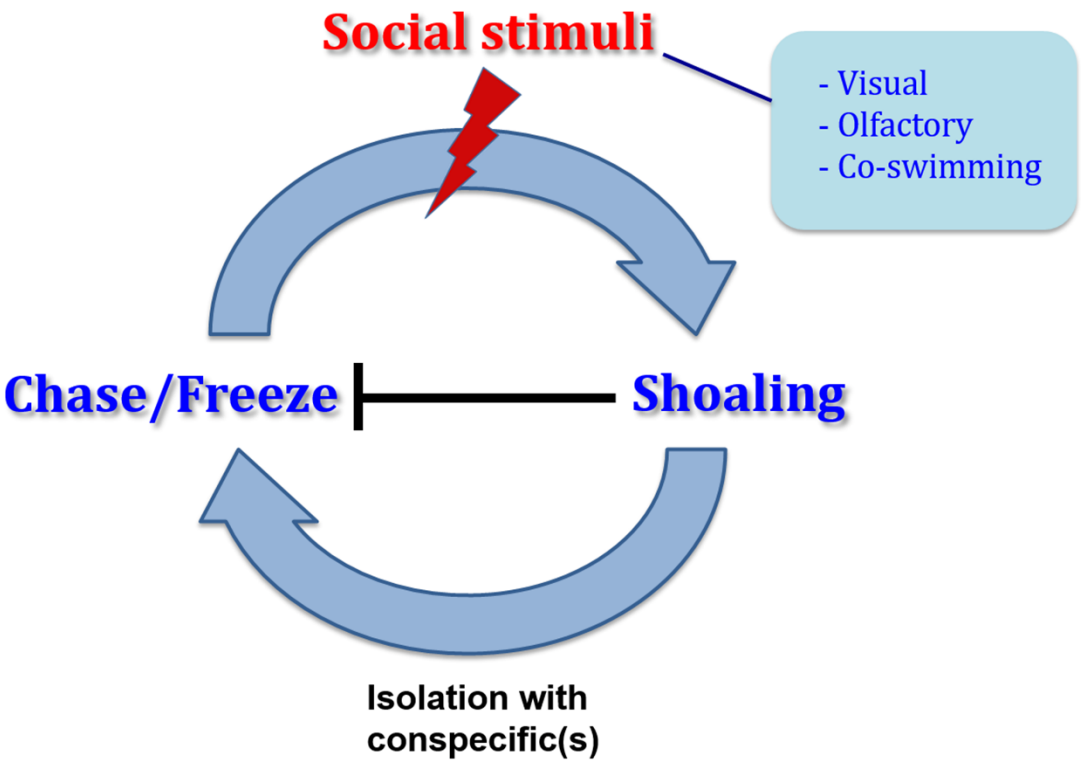


Table 1. *avtr1a1*+ adult brain regions. The numbers indicate the percentage of the brains examined that showed *avtr1a1* expression in each region (N_{male} = 4, N_{female}=2).

avtr1a1+ brain regions	Male	Female
Dm/Vd	50	50
Vv	50	50
Dp	50	100
PPa	100	100
PPp	*100	NA
SC	100	100
SD	100	0
Had	100	100
Chab	**100	NA
Vagal lobe (L X)	100	100
Ventro-medial posterior hindbrain (unidentified)	100	100
Ventro-medial rostral spinal cord (unidentified)	***100	***100

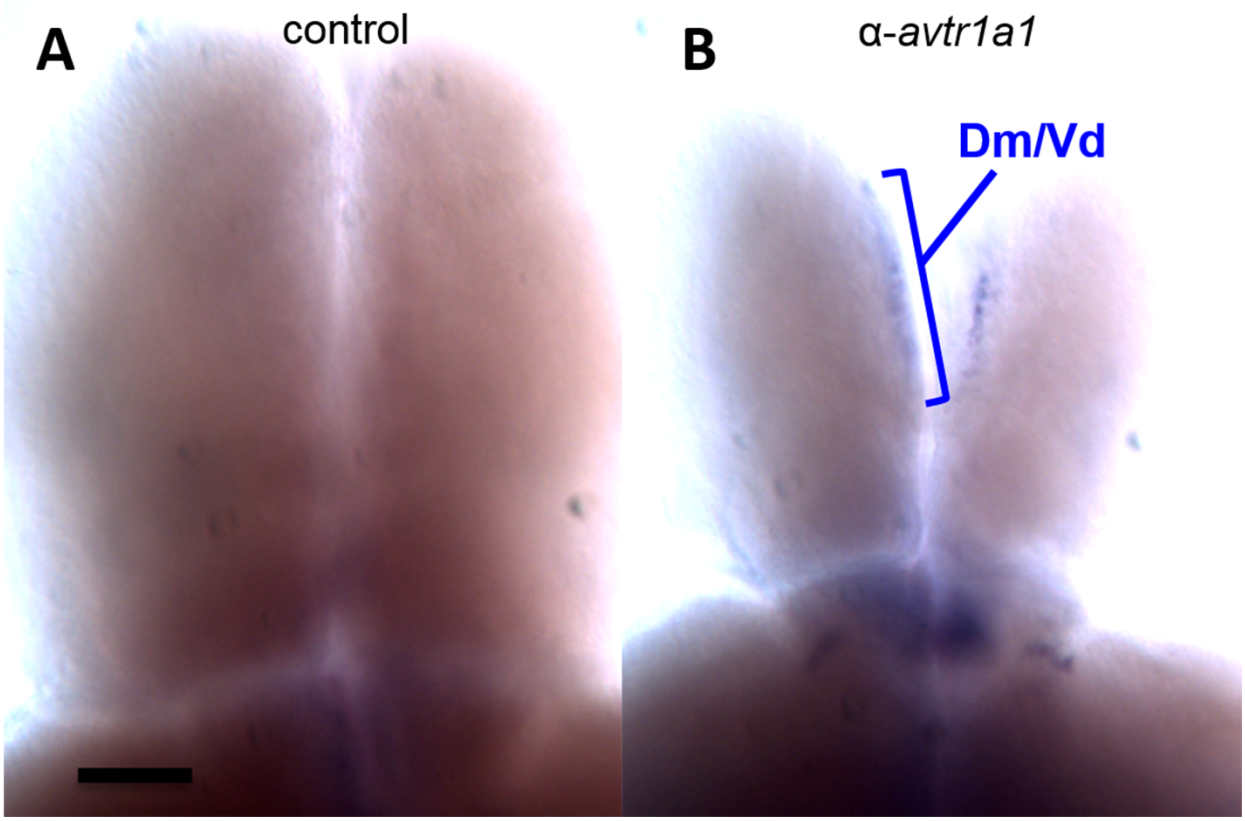
*3 male brains were examined (no slice was obtained for female brains). **3 male brains were examined. **Half of the brains in each sex had rostral spinal cord (N=2, 1 respectively)

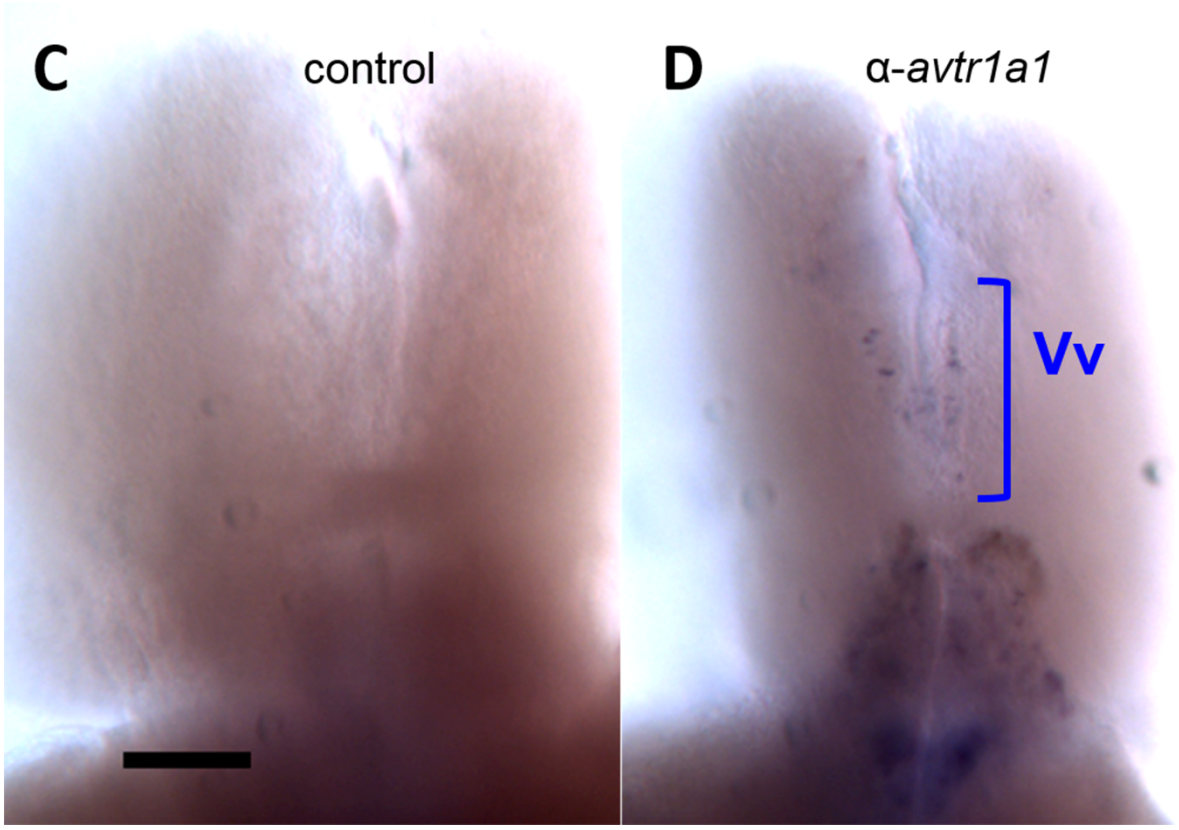
List of anatomy terms used above.

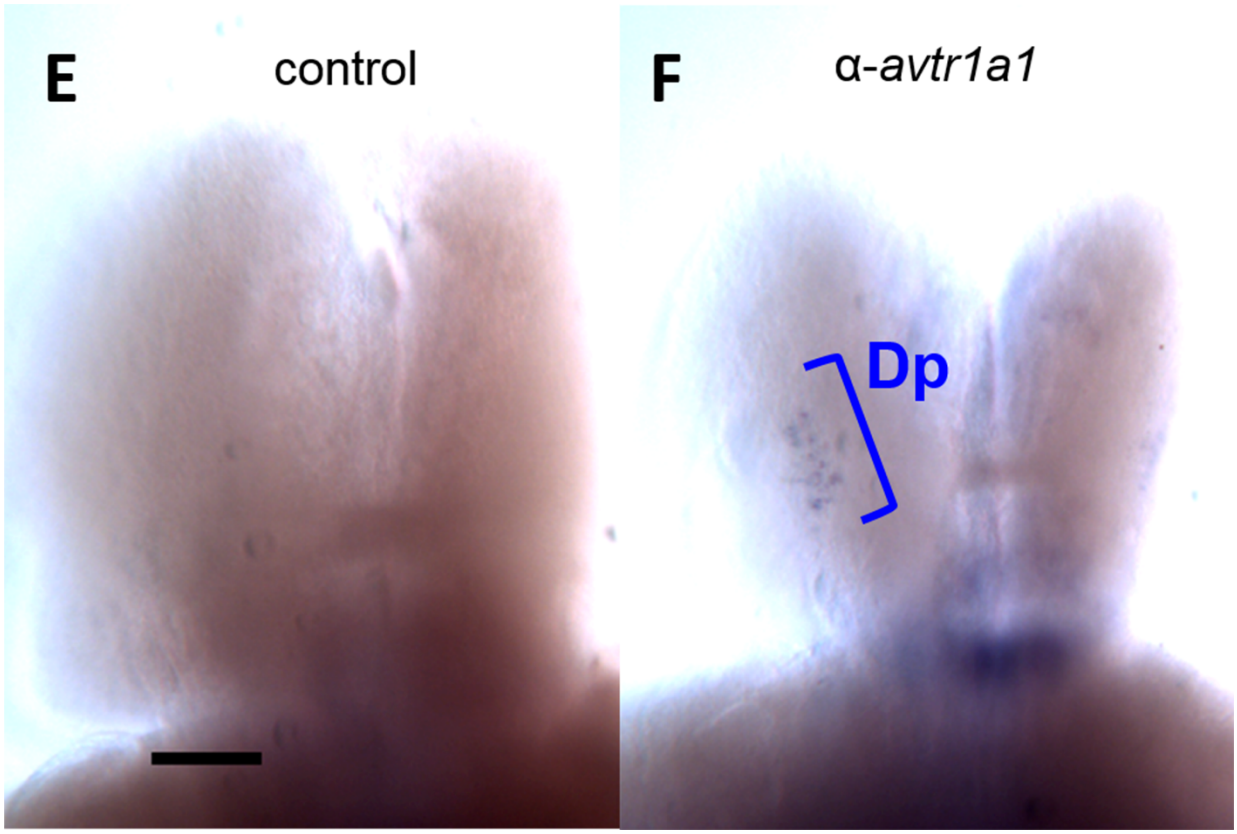
Abbreviation	Anatomical terms
Dm	Medial zone of dorsal telencephalon
Vd	Dorsal nucleus of ventral telencephalon
Vv	Ventral nucleus of ventral telencephalon
Dp	Posterior zone of dorsal telencephalon
PPa	Parvocellular preoptic nucleus, anterior part
PPp	Parvocellular preoptic nucleus, posterior part
SC	Suprachiasmatic nucleus
SD	Dorsal sac
Had	Dorsal habenula (Ha)
Chab	Habenular commissure

Fig. 4.8. *avtr1a1*+ adult brain regions.

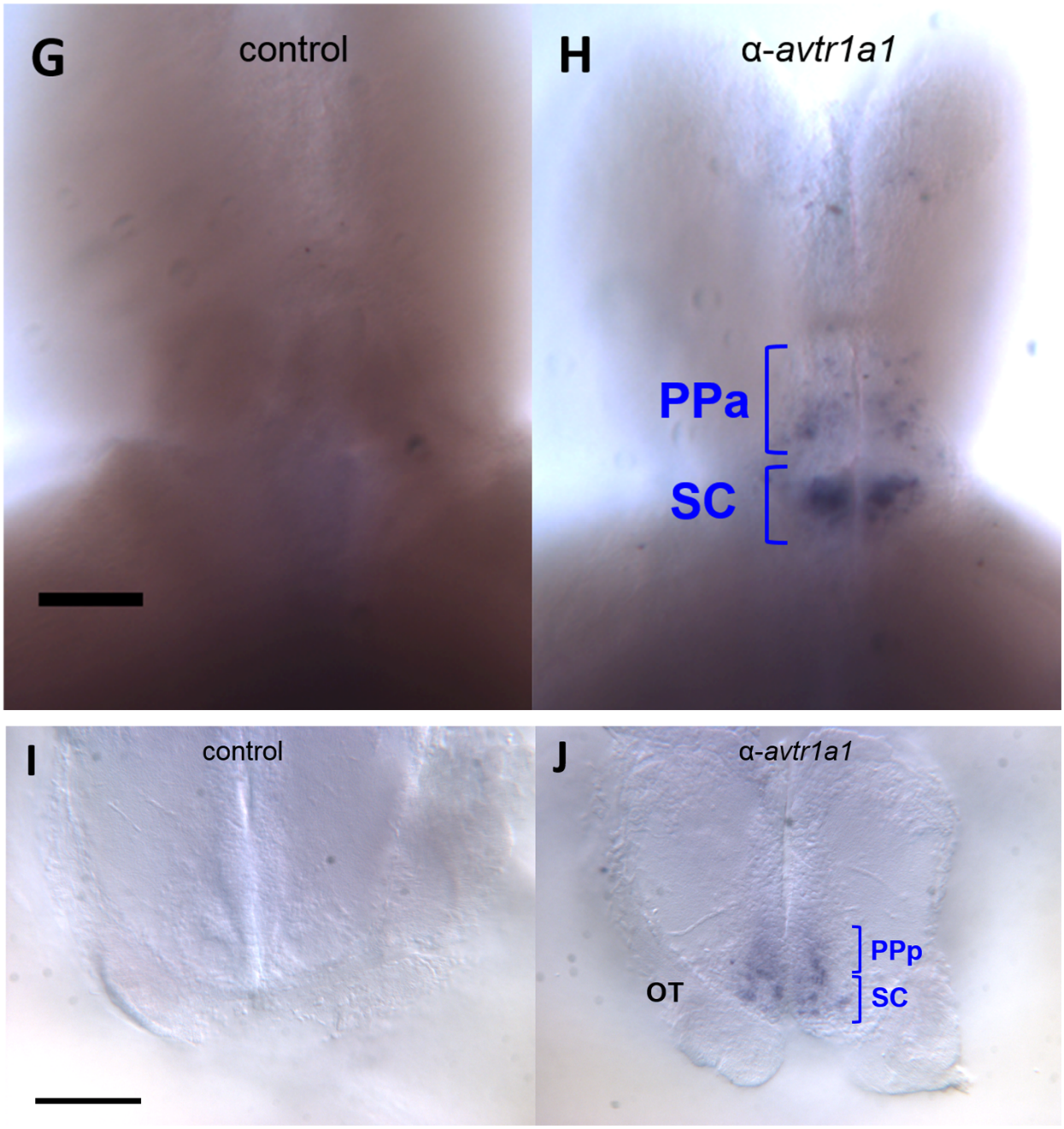
Telencephalic regions (A-F).



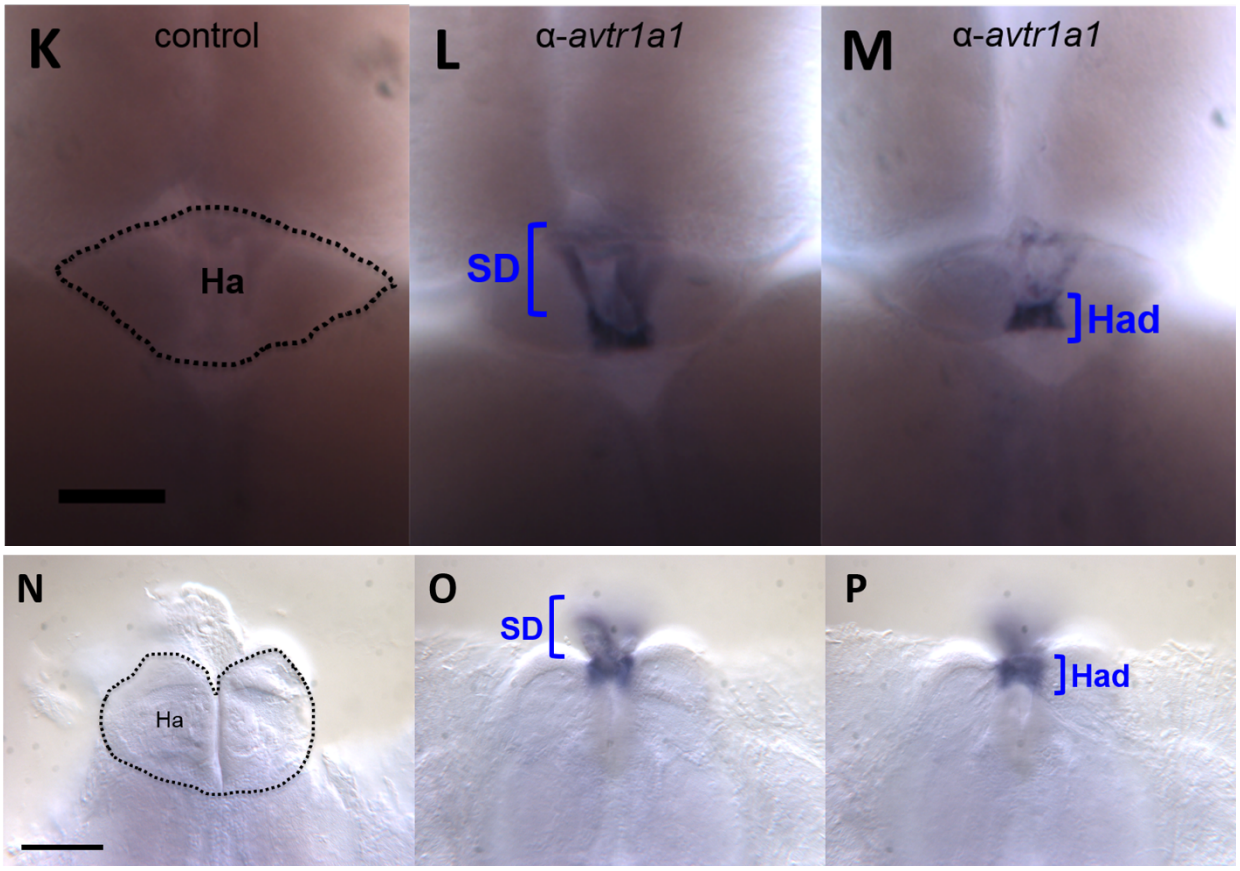

C
control
D
α-avtr1a1
Vv


E
control
F
α-avtr1a1
Dp

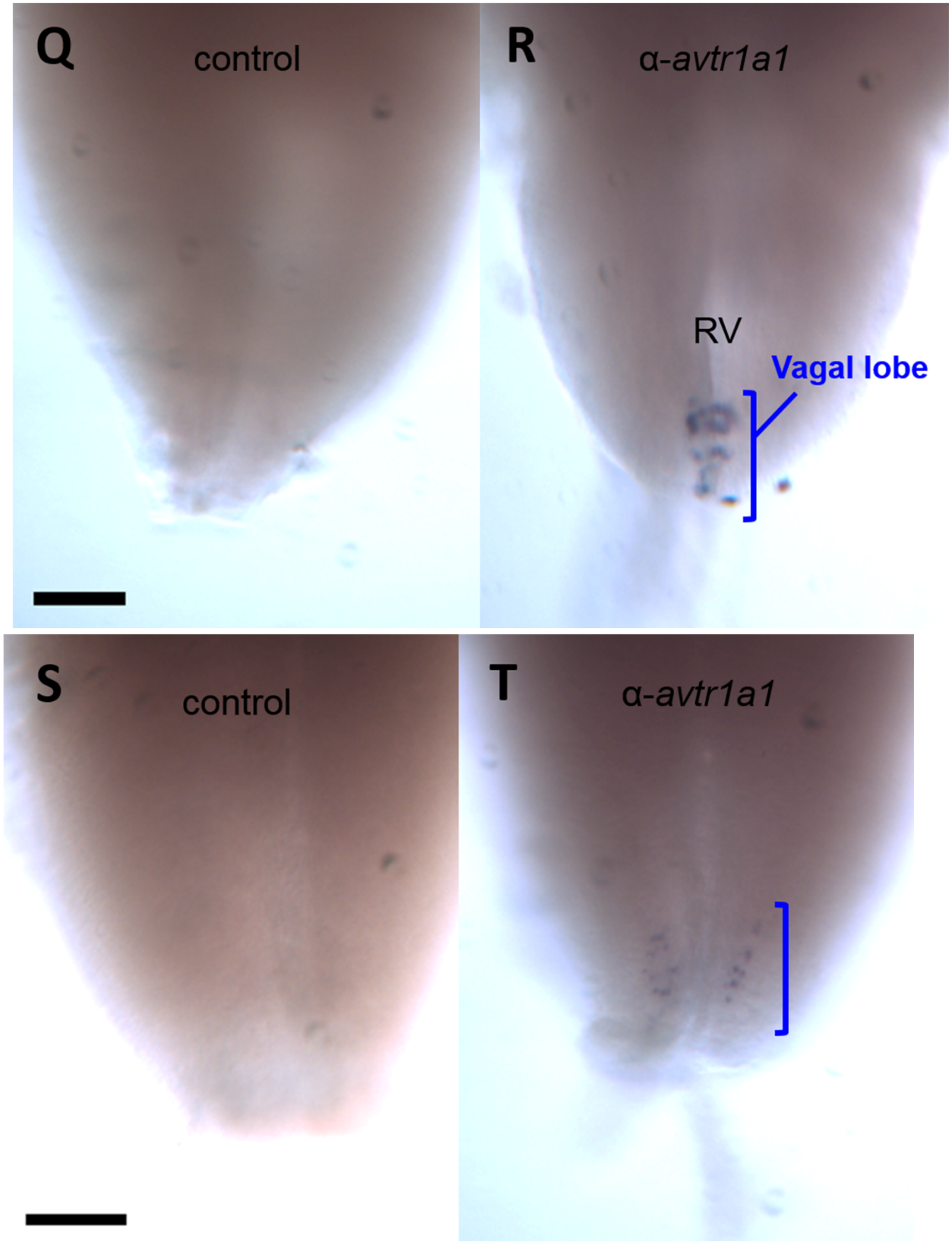
Diencephalic regions (dorsal view of an intact brain, G-H; cross-sectional view, I-J).



Habenula (dorsal view of an intact brain, K-M; cross-sectional view, N-P).



Posterior hindbrain (Q-T).



Rostral spine (U-V).

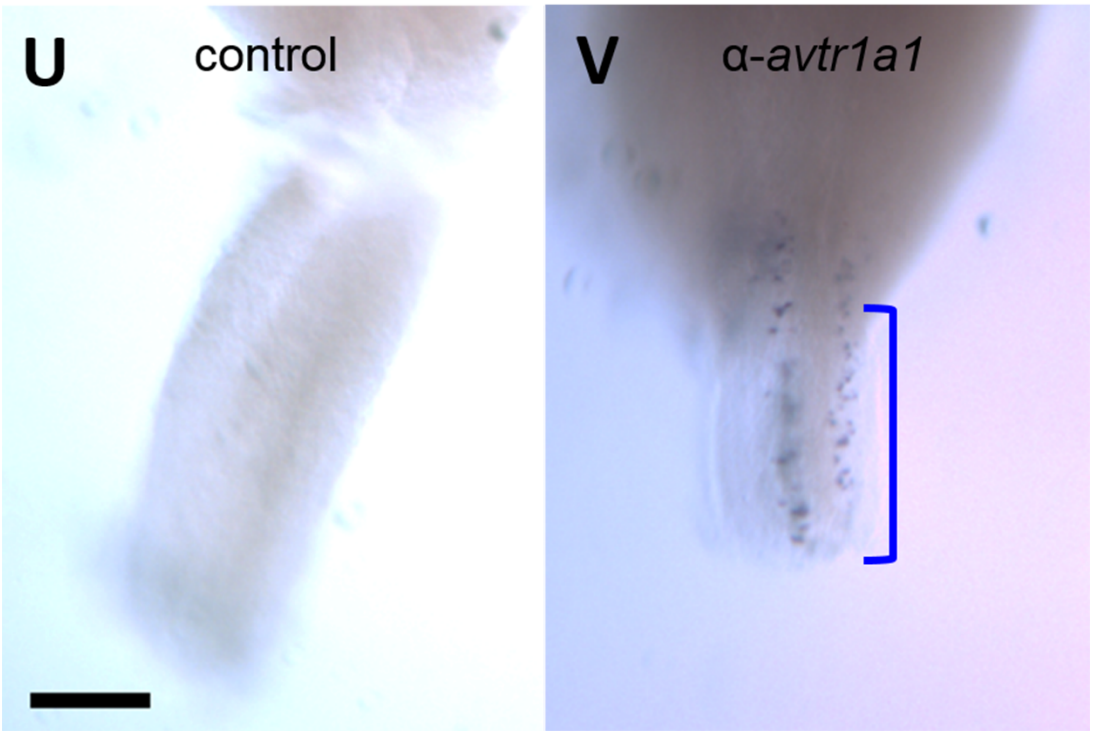


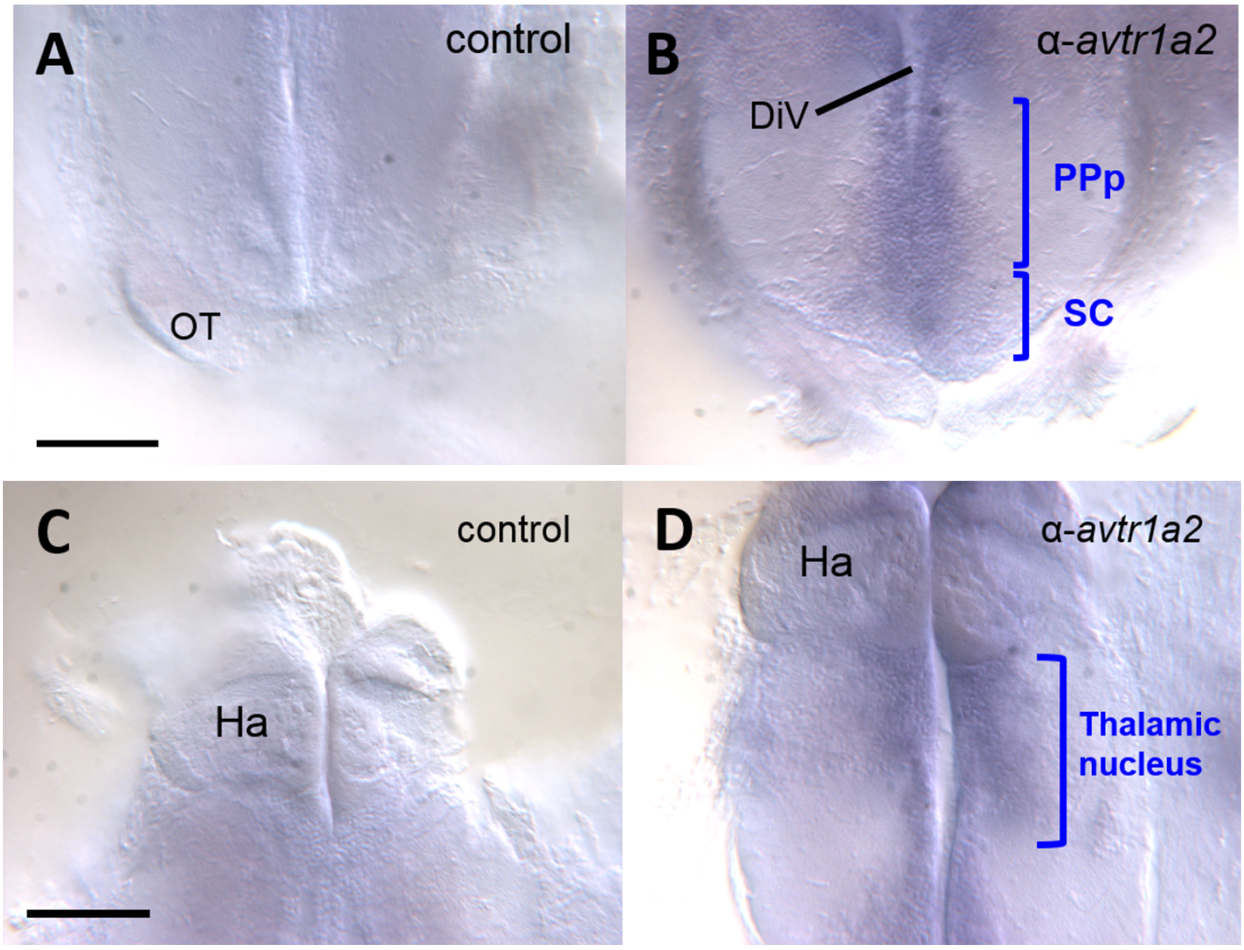
Table 2. *avtr1a2+* adult brain regions. N_{male} = 5, N_{female} = 3. Subregions within thalamic nucleus, ventromedial (VM), intermediate (I), or anterior (A), are not distinguished.

avtr1a2+ brain regions	*Male*	*Female*
PPp	*100	100
SC	*100	100
Thalamic nucleus (VM/I/A)	80	67
SD	100	100
Had	100	100
Chab	**100	100
Cerebellar corpus (CCe)	100	100
Facial lobe (L VII)	100	100
Vagal lobe (L X)	100	***100
Ventral posterior hindbrain cells	100	100

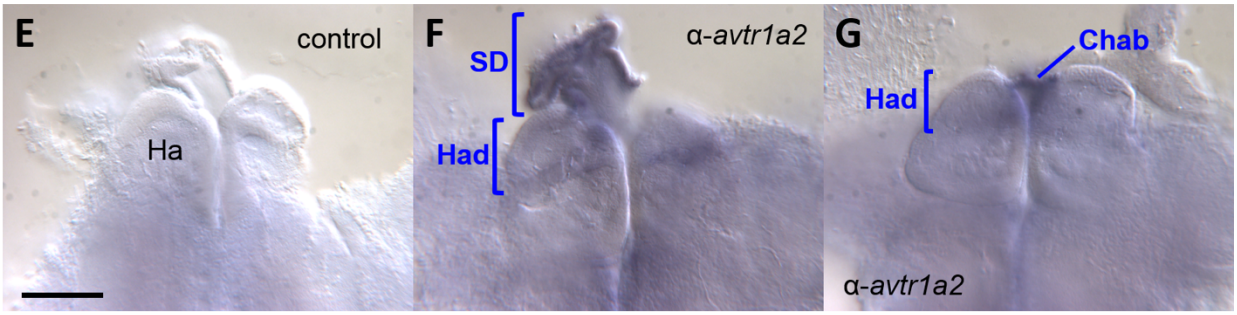
*3 male brains gave intact sections to examine PPp+SC. **4 male brains were examined.
***2 female brains were examined.

Fig. 4.9. *avtr1a2*+ adult brain regions

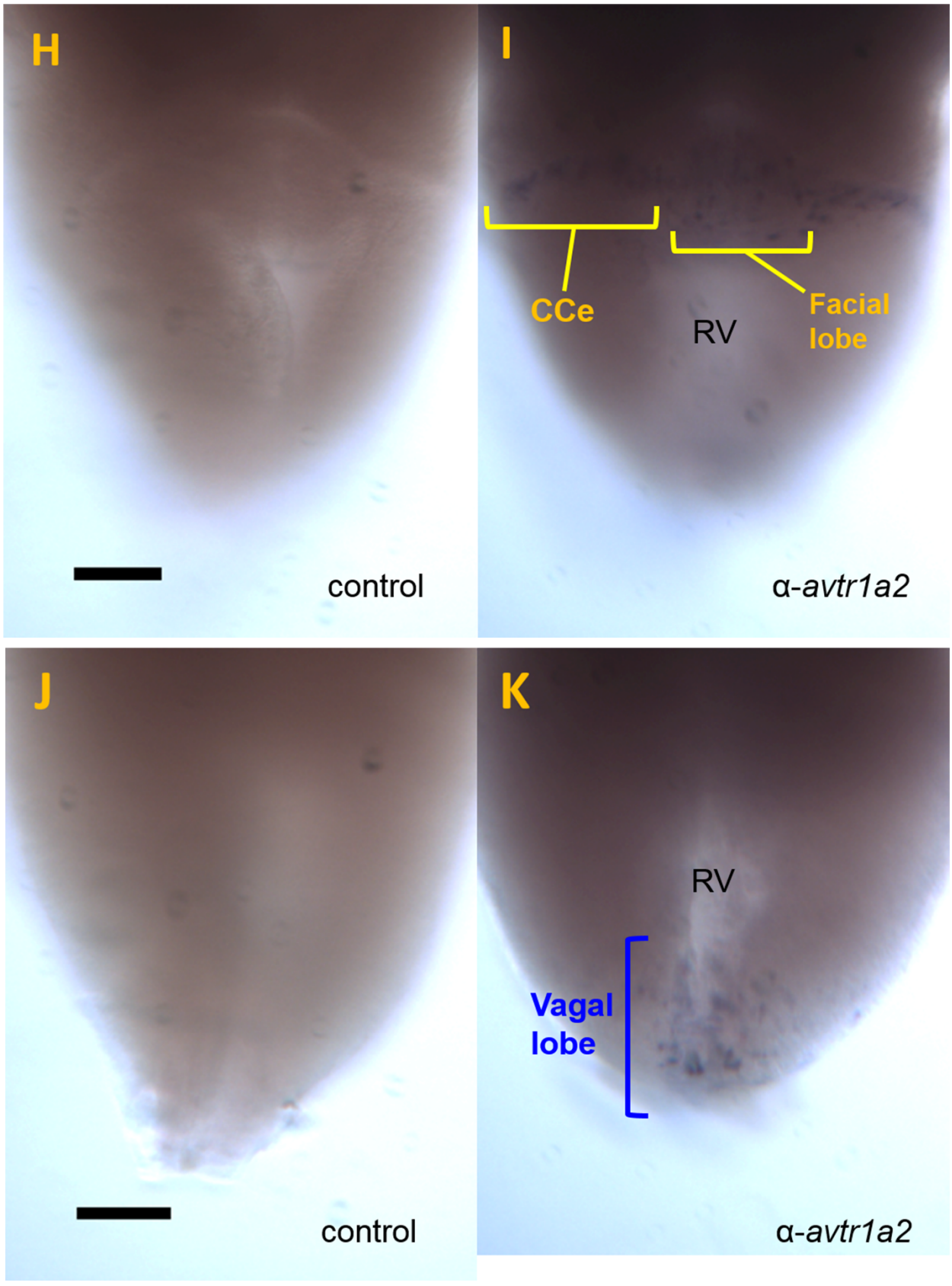
Diencephalic regions (A-D, cross-sections).



Habenula (E-G, cross-sections).



Posterior hindbrain (H-M, dorsal/posterior view of an intact brain).



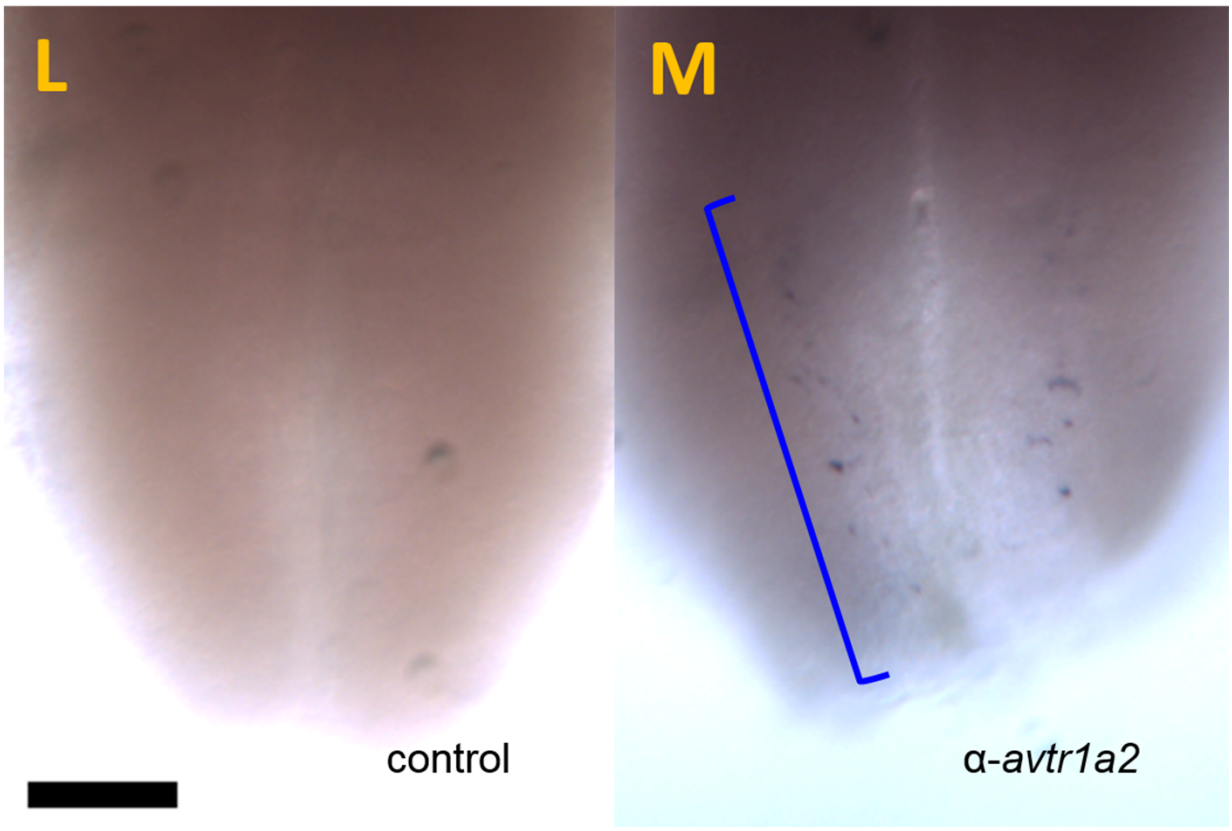

L
M
control
α-avtr1a2

Table 3. *avtr*+ brain regions linked to social behaviors by past studies

avtr+ brain regions	*avtr* types	Behaviors linked to the region	Studies
Preoptic nucleus (PPa/PPp)	*1a1 & 1a2*	Aggression	Larson et al., 2006 Filby et al., 2010
Suprachiasmatic nucleus (SC)	*1a1 & 1a2*	Circadian rhythm	Moore & Whitmore, 2014
Dorsal habenula (Had)	*1a1 & 1a2*	Fear response (*Had_L) Odor-evoked response (*$_RHad_M$)	Agetsuma et al., 2010 Krishnan et al., 2014

* Had_L = lateral subnucleus of dorsal habenula; $_RHad_M$ = medial subnucleus of the right dorsal habenula

3

[3] For chapter IV, K. Iwasaki and J. Kuwada designed experiments. K. Iwasaki conducted experiments for behavioral data collection, synthesized all RNA probes, conducted all labeling experiments, analyzed obtained data; M. Samaha conducted manual behavioral scoring of pair and group aggression assay data.

Chapter V. Conclusion

Thus, the current study first investigated the expression patterns of V1a-like receptors, *avtr1a1* and *avtr1a2*, in the brain of zebrafish embryos (Chapter II) to better understand the behavioral functions of *avt* signaling, which was the goal of the research. We found that both receptors are expressed as early as 1 day post fertilization (dpf) in select groups of cells in the hindbrain and the dorsal spinal cord that were predicted to be part of the sensory-motor circuitry. These cells include the posterior hindbrain neurons that project axons into the medial longitudinal fasciculus (MLF) and the mechanosensory neurons in the spinal cord.

Based on the anatomical position of *avtr*+ cells, we hypothesized that avt signaling modulates sensory-motor responses shown by zebrafish embryos. We designed a series of functional experiments to test the hypothesis (Chapter III). As we predicted, the pharmacological increase of AVT in the posterior hindbrain led to the enhancement of touch-evoked swimming response, while the anti-sense morpholino knockdown of *avt*, *avtr1a1*, and *avtr1a2* disrupted the response. We further showed that *avtr1a1*+ and *avtr1a2*+ hindbrain neurons responded to chemosensory stimulation that evoked forward swimming response. The results are consistent with the model that *avt*/*avtr* signaling modulates early sensorimotor responses at multiple levels in the central nervous system of

zebrafish. These experiments provide a mechanistic insight into how the neuropeptide signaling may alter embryonic behaviors.

However, AVT has been also linked repeatedly to complex social behaviors in adults across species although the underlying mechanism has been poorly understood. We first investigated one such behavior, namely aggressive interaction between paired males, and how aggression becomes modulated when the fish is placed into a larger social group (Chapter IV). To our surprise, the aggression-related behaviors (e.g. chasing by the dominant fish and freezing by the subordinate fish) diminished to near zero as soon as each fish was placed into a larger social group of 5 male zebrafish. For avt signaling to modulate aggressive behaviors, the receptors (*avtr1a1* and *avtr1a2*) must be expressed in brain regions that are known to control social interactions. To answer such possibility, we conducted whole adult brain *in situ* hybridization to examine the expression of *avtrs* in the brains of adult zebrafish. The avt receptors are in fact expressed in several brain regions important in regulating social interaction. For instance, both *avt* receptors are expressed in the preoptic nucleus in the anterior hypothalamus that contains neuroendocrine cells that release AVT. The AVT release in this region has been linked to aggression across species, and the presence of AVTRs suggests that AVT may modulate functions of neuroendocrine cells. Notably, the dorsal habenula (Had) which modulates fear responses as well as odor-dependent behaviors express both receptors strongly although further experiments need to be done to determine which subnucleus in the habenula expresses the receptors. This suggests that avt signaling may alter adult social behaviors by modulating the dorsal habenula-interpeduncular behavioral pathways that regulate fish responses to external stimuli. Transgenic fish lines are available to identify the subnucleus expressing avt

receptors and in future experiments, we may be able utilize the transgenics to examine neuronal activities of the dorsal habenula to sensory stimulation while *avt* signaling is manipulated. Moreover, the obtained data gives us a rationale for generating mutants via CRISPR-Cas9 mutagenesis techniques (Hwang et al., 2013; Jao et al., 2013) for *avt* and *avt* receptor genes for future experiments to further investigate the behavioral roles of AVT signaling.

Thus, the current study provide mechanistic insights into how a neuropeptide signaling can modulate behaviors of vertebrate species by utilizing the simpler brain and behaviors of zebrafish as well as the available molecular techniques to probe into the signaling. Further research on the topic will hopefully help better understand the mechanism of neuropeptidergic control of complex social behaviors of vertebrate species including humans for the hope of developing better treatments of psychiatric disorders that are associated with the neuropeptide genes the current study investigated.

References

Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh J-RJ, Joung JK (2013) Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat Biotechnol 31:227–229.

Jao L, Wente SR, Chen W (2013) Ef fi cient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. 110:1–6.