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Developmental effects of vasotocin and nonapeptide receptors on early social attachment and affiliative behavior in the zebra finch

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ABSTRACT

Zebra finches demonstrate selective affiliation between juvenile offspring and parents, which, like affiliation between pair partners, is characterized by proximity, vocal communication and contact behaviors. This experiment tested the hypothesis that the nonapeptide arginine vasotocin (AVT, avian homologue of vasopressin) and nonapeptide receptors play a role prior to fledging in the development of affiliative behavior. Zebra finch hatchlings of both sexes received daily intracranial injections (post-hatch days 2–8) of either AVT, Manning Compound (MC, a potent V1a receptor antagonist) or saline (vehicle control). The social development of both sexes was assessed by measuring responsiveness to isolation from the family and subsequent reunion with the male parent after fledging. In addition, we assessed the changes in affiliation with the parents, unfamiliar males, and unfamiliar females each week throughout juvenile development. Compared to controls, MC subjects showed decreased attachment to the parents and MC males did not show the normal increase in affiliative interest in opposite sex individuals as they reached reproductive maturity. In contrast, AVT subjects showed a sustained affiliative interest in parents throughout development, and males showed increased interest in opposite sex conspecifics as they matured. These results provide the first evidence suggesting that AVT and nonapeptide receptors play organizational roles in social development in a bird.

Introduction

Early in the development of species that exhibit parental care, young offspring often form close social and affiliative relationships with family members—they become attached to their parents and siblings. Attachment is commonly defined as a selective social or emotional bond, measured by maintenance of proximity, voluntary contact, or selective or differential behaviors toward the attachment object, as well as distress when separated from it (Ainsworth, 1989; Carter et al., 1995). Depending on the species, the onset of sexual maturity often coincides with interest in non-family members, especially potential mating partners. In species that exhibit both parental care and pair bonding in adulthood, the young seem to transition from an exclusive close relationship with the family to an adult pair relationship similarly characterized by attachment and affiliation.

Zebra finches (*Taeniopygia guttata*) exhibit socially monogamous pair bonds in adulthood and demonstrate a shift in affiliative preferences during juvenile development (Adkins-Regan and Leung, 2006; Immelmann, 1972; Zann, 1996). The young fledge around day 18 post-hatching, but remain dependent on parental feeding until approximately 35 days of age, though they remain in contact with parents until around 48 days of age and sometimes into adulthood (Boogert et al., 2014; Zann, 1996). As the juveniles progress toward reproductive maturity, the objects of their affiliation change from the parents and siblings to potential partners, followed by the formation of permanent pairs.

Upon fledging, zebra finch chicks must be motivated to remain proximal to parents and family only after leaving the nest, which requires both the recognition of the parents and selective behaviors directed toward them. In the wild, zebra finch fledglings are left alone for significant amounts of time, though the parents will return at regular intervals from their foraging bouts to feed the fledglings (Zann, 1996). When alone, the young typically remain inconspicuous by clumping together silently and motionlessly with their siblings (Zann, 1996). However, the fledglings will respond to adult distance calls with their immature vocalization, known as the long tonal call. When their parents arrive, the fledglings are observed to hop toward them, emitting the long tonal call, which often progresses into the begging call (Zann, 1996). Zebra finch fledglings will preferentially respond to the distance calls of their parents, particularly their fathers, though this specificity appears to develop over the course of several days (Mulard et al., 2010). Recognition of the parents by the fledglings is commonly observed in other colonial and nidiculous species, suggesting that this behavior is a widespread phenomenon (swallows (Beecher et al.,

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1981; Leonard et al., 1997; Medvin and Beecher, 1986; Sieber, 1985; Stoddard and Beecher, 1983), jays (McArthur, 1982) and seabirds (Aubin and Jouventin, 2002; Beer, 1969; Charrier et al., 2001; Evans, 1970; Mulard et al., 2008)).

Despite decades of research on the development of early social attachments, such as classic research on filial imprinting and vocal learning in birds, the development of the neural and neuroendocrine mechanisms mediating the formation and maintenance of selective affiliative relationships is still largely a mystery (Hoffman, 1987; Immelmann, 1975; Lorenz, 1937). Nonapeptides in the oxytocin family (mesotocin (MT) and arginine vasotocin (AVT) in birds; oxytocin (OT) and arginine vasopressin (AVP) in mammals) have been implicated as important modulators of social behaviors, though the vast majority of this research has focused on the activational effects of these peptides in adult animals. Nevertheless, convergent neurochemical, anatomical and behavioral evidence suggests that these nonapeptides acting in the reciprocally-connected network of brain nuclei known as the social behavior network are important in the formation and maintenance of selective affiliative relationships with conspecifics across a wide range of vertebrate species (Goodson, 2005; Newman, 1999; O'Connell and Hofmann, 2011).

The primary sources of nonapeptides that act on receptors within the social behavior network derive from the AVP/OT cell groups of the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus, as well as from smaller extrahypothalamic accessory cell groups, including the medial amygdala (meAMY), medial bed nucleus of the stria terminalis (BSTm), lateral septum (LS), olfactory bulb (OB), and suprachiasmatic nucleus (SCN) (Choleris et al., 2013; Laycock, 2009). Importantly, the distribution of nonapeptide cell bodies and their receptors is species specific (Kelly and Goodson, 2014a).

Nonapeptides modulate social behavior across taxa via their actions on many brain regions—including regions involved in sensory processing, learning and memory, reward and motivation, and even motor output at the level of the spinal cord (O'Connell & Hofmann, 2011, Rose & Moore, 2002; Insel & Young, 2001; Ferguson et al., 2000; Goodson & Bass, 2001). In general, a large body of literature suggests that the central activities of nonapeptides have evolved, in part, to modulate the salience of, attention to, or reward value of interactions with conspecifics. A common interpretation of the remarkable diversity of nonapeptide mechanisms across species is that differences in these systems are critically linked to variation in social phenotypes, including affiliation and attachment behaviors (Goodson, 2005; Goodson & Wang, 2006; Insel et al., 1994; Dewan et al., 2011).

Until very recently, most research on the neural mechanisms of attachment and pair bonding has focused on the socially monogamous prairie vole (*Microtus ochrogaster*) (McGraw and Young, 2010). However, there is increasing evidence that the nonapeptides play an important role in affiliative behaviors in birds. Two recent studies showed that antagonists which act primarily at the VT3 (OT-like) receptor increased the latency to pair and decreased pair formation in zebra finches when administered both centrally and peripherally (Klatt and Goodson, 2013; Pedersen and Tomaszycki, 2012). Additionally, pairing for 48 h was found to increase expression of both AVT and MT in the PVN in both sexes and AVT in the BSTm in males (Lowrey and Tomaszycki, 2014). Consistent with this finding, antisense knockdown of MT in the PVN significantly increased the latency to pair in females and reduced affiliative behaviors in zebra finches of both sexes (Kelly and Goodson, 2014b). Knockdown of AVT production in the PVN also reduces gregariousness in both sexes (Kelly and Goodson, 2014b). In several species of birds, there is an increase in the expression of c-Fos, an immediate early gene, in AVT-producing neurons in the BSTm in response to positively-valenced social stimuli, including potential mating partners (Goodson et al., 2009; Goodson and Wang, 2006). Males that failed to reliably court females had fewer AVT neurons in the BSTm than did reliable courters and they failed to show an induction of c-Fos expression in response to exposure to a female conspecific (Goodson et al., 2009). However, partner preference is not induced by central infusions of either AVT or MT in adult zebra finches, suggesting that the prairie vole findings do not generalize to zebra finches (Goodson et al., 2004).

Furthermore, there is not yet a complete story regarding the role of nonapeptides in the development of social behaviors in any species (Cushing, 2013). Manipulations of the nonapeptide system during development have indeed been found to affect social behaviors of both juvenile and adult rats, as well as in prairie voles (Bales and Carter, 2003a, 2003b; Boer, 1985; Boer et al., 1994; Bredewold et al., 2014; Schank, 2009; Stribley and Carter, 1999; Veenema et al., 2012; Winslow and Insel, 1993). However, few experiments focus on how nonapeptides might be acting in the brain as social behavior is developing. Yet the paucity of comparative developmental data has not slowed the speculation that nonapeptides may be implicated in the development of social deficit disorders in humans (Carter, 2007; Insel, 2010; Kenkel et al., 2014; Marazziti and Dell'Osso, 2008). To our knowledge, there is only one experiment providing evidence that the nonapeptides underlie differences in social behaviors during development in any non-rodent species: systemic injections of AVT altered approach behavior to an imprinting stimulus in newly-hatched ducklings (Martin et al., 1979; Martin and Van Wimersma Greidanus, 1978). These findings suggest that AVT may very well be important in social development across taxa, though this hypothesis remains to be investigated.

We aimed to test whether AVT and nonapeptide receptors play an organizational role in the development of species-typical affiliative behaviors in a socially-monogamous songbird, the zebra finch. Organizational effects of a hormone typically occur early in development, when they establish the neural and physiological substrate for future behavior (Phoenix et al., 1959). Organizational effects are thought to occur during a sensitive period in development and exert permanent and long-lasting effects for the life of the individual.

In this experiment, we manipulated the nonapeptide system of zebra finch chicks on days 2–8 post-hatching via daily intracranial injections of either AVT, Manning Compound (MC, a potent V1aR antagonist) or saline (vehicle control) and assessed the development of social attachment and affiliative behaviors across juvenile development. We first assessed attachment starting the first day after fledging and then in weekly tests from post-hatch days 30 to 86. We hypothesized that AVT and activity of the nonapeptide receptors would lead to alterations to attachment to the parents, as well as changes in the affiliative preferences for opposite sex individuals as the subjects reached sexual maturity. We predicted that AVT injected birds would show a stronger affiliative preference for the family early in development compared to controls and that this preference would be sustained throughout development. We also predicted that Manning Compound injected birds would not show strong affiliative preferences for any birds, less affiliation overall, and diminished interest in opposite sex birds.

Materials & methods

Breeding conditions

Seventy-two unpaired adult males and females (hereafter "parents") were assigned to one of six breeding aviaries ($1.2 \times 0.9 \times 0.6$ m) and allowed to pair and breed. Offspring hatched within 40 days became the experimental subjects used in the study. Until approximately 40 days of age, subjects were cared for by the parents, which were provided with ad libitum access to finch seed, cuttlebone, grit, water, and supplemented weekly with hard-boiled egg. Parent pairs and nest box occupancy were determined based upon the display of pair maintenance behaviors, including clumping, allopreening, and the occupancy of a nest box together. Observations were performed multiple times by independent observers until pairing status was confirmed. Nests were checked daily and the number of eggs,

number of chicks, and chick mass were recorded. Chicks were marked using colored non-toxic permanent marking pen, re-applied daily.

Genetic sexing

In order to balance the sex ratios across treatment, subject chicks were genetically sexed on the day of hatching using DNA extracted from feather follicle tissue. PCR was performed using primers developed by Soderstrom et al. (2007). Each 10 μL PCR reaction contained 6.3 μL RNAse-free H_20; 1 μL 10X Rxn Buffer (10X PCR–$MgCl_2$); 0.4 μL 50 mM $MgCl_2$; 0.2 μL Deoxynucleotide Solution Mix, 8 μmol each dNTP; 1 μL W + Z Primer Mix containing 2 μM of each primer; 0.1 μL Platinum® Taq DNA Polymerase; and 1 μL of sample DNA. The gene fragments were amplified by PCR using the following cycling conditions: an initial hot-start 4-min denaturation step at 95 °C; followed by 35 cycles of 94 °C for 30 s, 62 °C for 45 s, 72 °C for 45 s; and a final extension at 72 °C for 10 min. Results were visualized on a 1.25% agarose gel.

Intracranial injections

Starting on day 2 post-hatching through day 8, subjects received daily 2 μL intracranial (IC) injections of either 1) AVT (10 ng, (Arg8)-Vasotocin, Bachem 1785.0005); 2) Manning Compound (MC), a potent V1a and mild OT receptor antagonist (50 ng, d(CH2)51,Tyr(Me)2,Arg8)-Vasopressin, Bachem 5350.0005); or 3) 0.9% isotonic saline (Castagna et al., 1998; Goodson et al., 2004; Manning et al., 1989). These dosages were based on those used in intracerebroventricular infusions in adults in Goodson et al., 2004 and scaled by 1/5, based on the changes in brain volume between adults versus juveniles (Ikebuchi et al., 2012). Both AVT and MC are predicted to act at multiple receptor subtypes in the zebra finch brain, including the VT4 (V1aR), VT3 (OT-like), and V2 receptors (Busnelli et al., 2013; Kruszynski et al., 1980; Leung et al., 2009; Manning et al., 2012).

IC injections were performed using a sterile stainless steel insulin syringe (Beckman Dickman, U-100 BD Ultra-Fine Short Lo-Dose™ Insulin Syringes, 31 Gauge, 0.5 mL volume, 8 mm needle length), similar to Bender & Veney (2008). This technique is feasible because the zebra finch hatchling skull is thin, flexible and easily penetrable by a needle. To perform the IC injection, 2 μL was pipetted onto a sterile petri dish using a sterile barrier tip pipette. The bead of liquid was carefully drawn into the tip of the syringe. The needle was then shallowly inserted bevel down at a 45° angle on the top of the cranium at the midline. The chicks continued to behave normally immediately following injections, including normal begging and locomotor behavior. The chicks also gained weight and developed normally (Fig. 1a) and there was no increase in mortality associated with the IC injections or any of the treatments.

The efficacy of intracranial injections was verified by injecting two non-subject chicks (day 2 and day 8) with 2 μL of India ink, diluted 1:10 in 0.9% saline. After four hours, the chicks were euthanized by isoflurane overdose, decapitated, and the whole head was flash frozen and sectioned to determine where in the brain India ink was present (see Supplementary Fig. 1). In both the day 2 and day 8 chicks, India ink was found primarily along the lateral ventricles. There was also more limited staining within the hypothalamus along the third ventricle in the day 8 chick. All procedures were developed with veterinary supervision and approved by Cornell University's Institutional Animal Care and Use Committee.

Chicks of each sex were randomly assigned to a treatment group on day two, following genetic sexing. Chicks within the same clutch were randomly assigned to different treatment groups, such that treatment was unrelated to hatching order. The numbers of birds that completed treatment and survived until fledging are as follows: AVT males ($N = 11$); MC males ($N = 11$); control males ($N = 8$); AVT females ($N = 9$); MC females ($N = 10$); and control females ($N = 11$). One control male was found dead on post-hatch day 38 and one control female was euthanized on post-hatching day 51 due to a leg injury.

Zebra finches typically become independent of parental feeding at 40 days of age and reach sexual maturity between 60 and 90 days of age (Zann, 1996). Thus, after approximately 40 days of age (39.8 ± 5.4 days), subjects were removed from their natal aviary and housed in same-sex aviaries in a separate room from the parents. Each same-sex aviary contained birds of the same treatment to control for possible social interactions between birds in different treatments. For a timeline of the experiment, see Fig. 1b.

Social isolation tests

The day after subjects were first observed having fledged from the nest, we assessed subjects' responses to social isolation and subsequent reunion with the male parent. In the wild, male parents take a more active role in caring for the fledglings, because the female parent is more involved in incubation if the pair starts a second clutch (Zann, 1996). The social isolation tests (SI tests) were performed in a testing apparatus (60 × 41 × 36 cm) in a separate room from the breeding cage. Two aviaries of paired adults were in the room but were behind a curtain to provide ambient colony noise. A nest box was attached to both the right and left sides of the testing apparatus. The test nest boxes were filled with coconut fibers, imitating the structure of a nest. After one minute of acclimation, we recorded behavior in isolation for nine minutes total. Next, the male parent was placed in the aviary

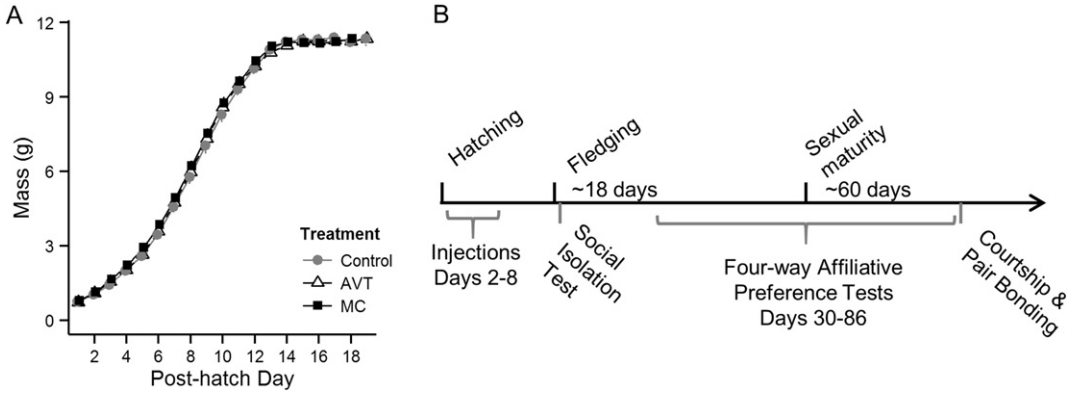

Fig. 1. Growth curve and experimental timeline. Panel A) depicts the mean ± SE chick mass (g) for each day post-hatching until fledging. Control subjects are depicted with circles and a solid gray line, AVT with triangles and dashed black line, and MC as squares and solid black line. The growth curves are highly overlapping across treatments and there are no statistically significant differences between groups. Panel B) shows the experimental timeline with developmental events (hatching, fledging, and timing of reproductive maturity) above the line and experimental events (injections, social isolation tests, and four-way proximity tests) below the line.

with the fledgling for five additional minutes. The video was scored for the number of perch hops, saccadic head movements, and long tonal calls performed by the subject per minute.

Four-way tests

Preferences for being proximal to parents or unfamiliar conspecifics were assessed weekly from days 30 to 86 in four-choice proximity tests with two males, two females, the parent pair, or no conspecifics as the four stimulus choices, similar to Adkins-Regan & Leung (2006). For testing, the subject was removed from its aviary and placed alone in a testing cage (61 × 61 × 41 cm) in a separate testing room, which was flanked on three sides by cages containing pairs of stimulus birds. The three stimulus cages (61 × 36 × 45 cm) were positioned next to the subject's cage. One stimulus cage contained the subject's parents, one contained two unfamiliar adult females, and one contained two unfamiliar adult males. The stimuli were placed first into the apparatus and allowed to acclimate for at least one minute prior to the introduction of the subject. Subjects were allowed to acclimate in the apparatus for one minute prior to recording. Tests were 15 min long and were videotaped from behind a blind with no human in the room. The testing cage contained three stimulus proximity zones, delineated by perches and tape on the floor. Each corner of the stimulus cage was blocked by wire mesh, creating a cross-shaped testing apparatus, with a perch near each of the four sides of the cage (see Adkins-Regan & Leung, 2006). The remainder of the cage (the center portion and the zone nearest to the video camera) was considered a neutral (non-proximity) zone. The same pool of 20 males and 20 females was used as stimuli in a random order for each subject, and the stimuli were unfamiliar to the subject at the time of presentation. The position of each stimulus set was varied randomly to control for possible position preferences within the apparatus. The total time that the subject's head was in each of the three proximity zones was recorded. Proximity is a valid indicator of family and pairing interest in this species, because these relationships are marked by close physical proximity (Clayton, 1990). The testing period, with nine weekly tests, covered the majority of the juvenile period, allowing us to measure changes in affiliative preferences across juvenile development.

All tests were recorded with a Canon Vixia HFM31 HD camera and a Sennheiser ME66 microphone. Digital videos were coded using ELAN annotation software (http://tla.mpi.nl/tools/tla-tools/elan/) by trained assistants who were blind to the subject's treatment. In addition, all researchers were blinded to treatment throughout the experiment, until after data collection and coding was complete.

Statistical analyses

To test the effect of the treatment (a categorical variable with three levels) on the behavioral response of chicks in the social isolation tests the day after fledging (perch hops, head saccades, and vocalizations as dependent variables), we used a random slope linear mixed model (LMM), where not only the intercept but also the slope of the regression line is allowed to vary between individuals. In this model, Sex, Treatment, and Test (isolation versus with male parent) were specified as independent variables. Random variables were individual ID (57 levels), nested within Family ID (17 levels). The interaction effect considered was Treatment*Test, which indicates whether treatment changes behavior in the presence of the male parent.

To test the effect of treatment on behavior in the four-way tests, we again used random slope LMMs, where the dependent variables were either the number of zone changes, time spent in any proximity zone, or time spent in each of the three proximity zones (parents, opposite sex conspecifics, or same sex conspecifics), again with individual-specific random intercepts and slopes. For the model investigating the effect of treatment on the number of zone changes and time in any proximity zone, the fixed factors were Sex, Treatment, and Post-hatch day (9 weekly tests, day 30 to day 86, modeled as a continuous variable). Random factors were individual ID (57 levels), nested within Family ID (17 levels). The interaction effects considered were Treatment*Post-hatch day and Sex*Treatment*Post-hatch day. Because we are most interested in how treatment affects the change in affiliative interest across development, these interactions are the key terms of interest. In the models testing the effect of treatment on time in individual proximity zones, we additionally included the time in any proximity zone as a fixed factor.

In order to better understand the qualitative differences between the treatment groups either between tests or across development in the four-way test, we also occasionally report the results of LMM within each treatment group to test the effect of tests or days on behavior. In these models, only Sex and either Test (in the social isolation test) or Post-hatch day (in the four-way tests) were included as independent variables and the random factors were Individual ID nested within Family ID. We also performed one-sample t-tests to determine whether subjects in each treatment group spent more time than expected by chance (greater than 150 s) in the proximity zone nearest to the parents on day 30 only. These results were used to describe patterns of change within treatment groups, which is helpful for interpreting the results of the larger models.

All statistical analyses were performed with R software (R Development Core Team 2007). To perform the LMM analysis, we used the *lmer* function of the lme4 package (Bates et al., 2014). To test whether the effect of treatment or an interaction term was significant, we used likelihood ratio tests using the *anova* function to compare the full model to a reduced null model with only the factor of interest removed. These results generally can be interpreted as indicating whether there is evidence that one of the treatment groups differs from the others. In addition, we calculated both marginal and conditional R^2 measures of effect size for the model using the *r.squaredGLMM* function in the MuMIn package (Bartoń, 2015; Johnson, 2014; Nakagawa and Schielzeth, 2013). In generalized linear mixed effects models, including random slopes models, marginal R^2 ($R^2_{\mathrm{GLMM}(m)}$) is a measure of the variance explained by the fixed effects and conditional R^2 ($R^2_{\mathrm{GLMM}(c)}$) is the variance explained by both the fixed and random effects. The results of these full model tests are presented in the text.

Because Treatment is a categorical variable with three levels, we further explored treatment effects in two ways. First, for each of the models, we present the effect estimates, standard errors, t-values, and *p*-values for all of the fixed effects in tables. *p*-Values for fixed effects were obtained using the Kenward-Roger approximation to calculate corrected degrees of freedom (Kenward-Roger in the pbkrtest package) (Højsgaard, 2014). The results are presented as the effect of either AVT or MC compared to the control group. If there was a statistically significant interaction between Treatment and either Test or Post-hatch day, we performed post hoc tests on the interaction terms only using the *testInteractions* function in the phia package (Rosario-Martinez et al., 2015). In addition, we discuss any observed sex differences, where relevant.

Results

Social isolation tests

A total of fifty-six fledglings were tested in the SI test. Tests were not performed for two subjects and the video files were corrupted for another two subjects. Consistent with Zann's (1996) observations, recently fledged zebra finches were relatively inactive in isolation. The average number of perch hops was only 1.9 ± 3.2 perch hops per minute. However, MC birds showed the highest number of perch hops in isolation (3.3 ± 4.8 perch hops per minute) and decreased their perch hop rate when reunited with the male parent (LMM, within-MC: $X^2(1) = 9.96$, $p = 0.0016$, $R^2_{\mathrm{GLMM}(m)} = 0.17$, $R^2_{\mathrm{GLMM}(c)} = 0.43$) (Fig. 2a). There was a statistically significant interaction between Treatment and Test,

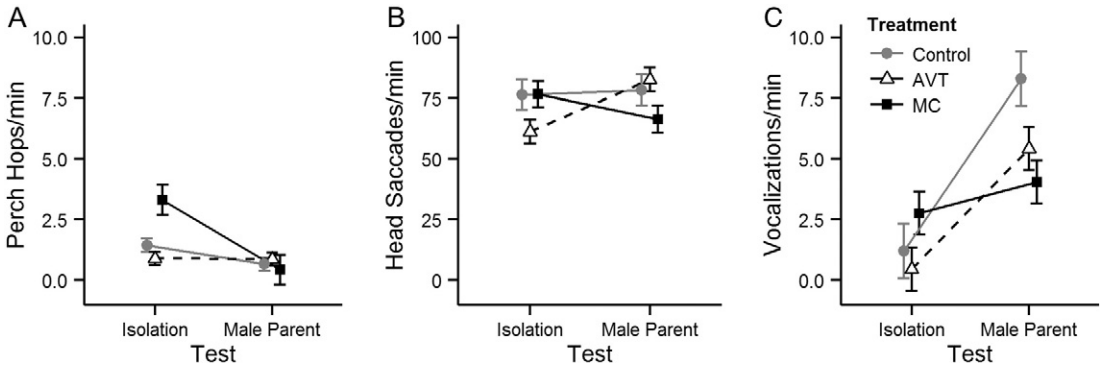

Fig. 2. Perch hops, saccadic head movements and vocalizations in social isolation tests. Mean ± SE of the number of A) saccadic head movements, B) perch hops, and C) vocalizations per minute in the social isolation test in isolation compared to when the male parent was present. Control subjects are depicted with circles and a solid gray line, AVT with triangles and dashed black line, and MC as squares and solid black line.

controlling for sex and for ID nested within Family ID as random factors in a linear mixed model (LMM: $X^2(2) = 9.81$, $p = 0.0074$, $R^2_{GLMM(m)} = 0.17$, $R^2_{GLMM(c)} = 0.29$; Table 1a). Post hoc tests suggest that AVT and MC birds significantly differed from each other in their slope and there was a marginally-significant difference between MC and control (AVT-MC: $X^2(1) = 9.58$, $p = 0.0059$, MC-control: $X^2(1) = 4.99$, $p = 0.051$).

Saccadic head movements are thought to be a good proxy of visual scanning and vigilance in birds because movements of the head are necessary for birds to search for objects of interest in their environment (Fernández-Juricic, 2012). AVT birds showed the lowest rate of saccadic head movements in isolation, but showed a significant increase in the head saccade rate when the male parent was present (LMM, within-AVT: $X^2(1) = 6.28$, $p = 0.012$, $R^2_{GLMM(m)} = 0.11$, $R^2_{GLMM(c)} = 0.47$) (Fig. 2b). However, neither MC nor control birds showed a statistically significant change between isolation and the male parent test. In the overall model, there was also a statistically significant interaction between Treatment and Test (LMM: $X^2(2) = 7.00$, $p = 0.030$, $R^2_{GLMM(m)} = 0.10$, $R^2_{GLMM(c)} = 0.42$; Table 1b). There was also a statistically significant effect of sex, with males showing a slightly higher rate of saccadic head movements (Table 1b). Post hoc tests suggest that AVT and MC birds have statistically different slopes ($X^2(1) = 7.41$, $p = 0.019$).

Both AVT and control birds vocalized at a higher rate in the presence of their male parent compared to isolation, whereas MC birds showed no change in their vocalization rate (LMM, within-AVT: $X^2(1) = 12.02$, $p = 0.00053$, $R^2_{GLMM(m)} = 0.27$, $R^2_{GLMM(c)} = 0.34$; within-MC: $X^2(1) = 0.83$, $p = 0.363$, $R^2_{GLMM(m)} = 0.02$, $R^2_{GLMM(c)} = 0.23$; within-control: $X^2(1) = 12.81$, $p = 0.00035$, $R^2_{GLMM(m)} = 0.32$, $R^2_{GLMM(c)} = 0.34$) (Fig. 2c). There was a statistically significant interaction between treatment and test (LMM: $X^2(2) = 6.99$, $p = 0.030$, $R^2_{GLMM(m)} = 0.23$, $R^2_{GLMM(c)} = 0.28$), with MC and control birds differing in their slopes ($X^2(1) = 7.14$, $p = 0.023$; Table 1c).

Table 1
Social isolation test linear mixed model (LMM) results.
Summary of the linear mixed models with the number of saccadic head movements, perch hops, and vocalizations performed by the subject as the dependent variables. The fixed effects of Sex, Treatment, Test (isolation versus male parent) as well as the interactions are shown. Individual ID nested within Family ID was included as a random effect. To test the significance of each parameter within the model, we used the Kenward–Roger approximation to get approximate degrees of freedom and the *t*-distribution (SE = standard error, bold numbers indicate significance, * refers to an interaction term).

Predictors	Estimate	SE	*t*	*p*
Perch hops				
Intercept	1.181	0.556	2.125	**0.037**
Sex (Male)	0.479	0.408	1.174	0.244
Treatment (AVT)	−0.593	0.679	−0.874	0.385
Treatment (MC)	1.766	0.690	2.560	**0.012**
Test (male parent)	−0.791	0.678	−1.166	0.247
Treatment (AVT)*Test (male parent)	0.765	0.947	0.808	0.422
Treatment (MC)*Test (male parent)	−2.089	0.935	−2.234	**0.028**
Head saccades				
Intercept	67.328	8.796	7.654	**7.52E-12**
Sex (Male)	15.117	6.801	2.223	**0.028**
Treatment (AVT)	−15.412	10.216	−1.509	0.134
Treatment (MC)	1.591	10.673	0.149	0.882
Test (male parent)	2.063	9.308	0.222	0.825
Treatment (AVT)*Test (male parent)	19.391	12.634	1.535	0.128
Treatment (MC)*Test (male parent)	−13.783	12.736	−1.082	0.281
Vocalizations				
Intercept	0.824	1.253	0.658	0.513
Sex (Male)	0.785	0.946	0.830	0.410
Treatment (AVT)	−0.797	1.613	−0.494	0.623
Treatment (MC)	1.533	1.593	0.962	0.340
Test (male parent)	7.101	1.595	4.451	**0.000**
Treatment (AVT)*Test (male parent)	−2.120	2.196	−0.965	0.338
Treatment (MC)*Test (male parent)	−5.800	2.170	−2.673	**0.010**

Four-way proximity tests

All three treatment groups showed an increase in the number of zone changes (activity level) but at a decreasing rate over the course of the nine four-way tests (Fig. 3a). However, there was no difference between the treatment groups, when controlling for proportion of time in any proximity zone, Sex, and ID nested within Family ID as a random effect. Similarly, there was an increase in the proportion of time spent in any zone of proximity across the nine four-way tests (Fig. 3b). The best model for the proportion time spent in any zone of proximity included Treatment as a predictor (LMM: $X^2(2) = 6.83$, $p = 0.033$, $R^2_{GLMM(m)} = 0.06$, $R^2_{GLMM(c)} = 0.47$). However, none of the pairwise comparisons between treatment groups reached statistical significance in post hoc tests, suggesting that this treatment effect is very weak. Nevertheless, because of the change in proportion of time spent in any zone of proximity across days, we controlled for this total time in all future analyses. There were no sex differences in either proportion of time spent in any proximity zone or the number of zone changes.

Control subjects were the only group that decreased significantly in the time spent in the proximity zone nearest the parents across days (within-control, LMM: $X^2(2) = 6.79$, $p = 0.034$, $R^2_{GLMM(m)} = 0.04$, $R^2_{GLMM(c)} = 0.34$) (Fig. 4). Furthermore, both AVT and control subjects, but not MC subjects, spent more time in the parent proximity zone than would be expected by chance on day 30 (one-sample t-test, AVT: $t(18) = 1.79$, $p = 0.045$; MC: $t(19) = 0.78$, $p = 0.22$, control: $t(15) = 2.47$, $p = 0.013$). However, neither AVT nor MC subjects show a change in the time spent in proximity to parents across development. This treatment effect, however, is weak because the interaction between Treatment and Day was not significant in the full model, when controlling for the proportion of time spent in any proximity zone, Sex, and ID nested within Family ID as a random effect (LMM: $X^2(4) = 3.66$, $p = 0.45$, $R^2_{GLMM(m)} = 0.20$, $R^2_{GLMM(c)} = 0.31$). There

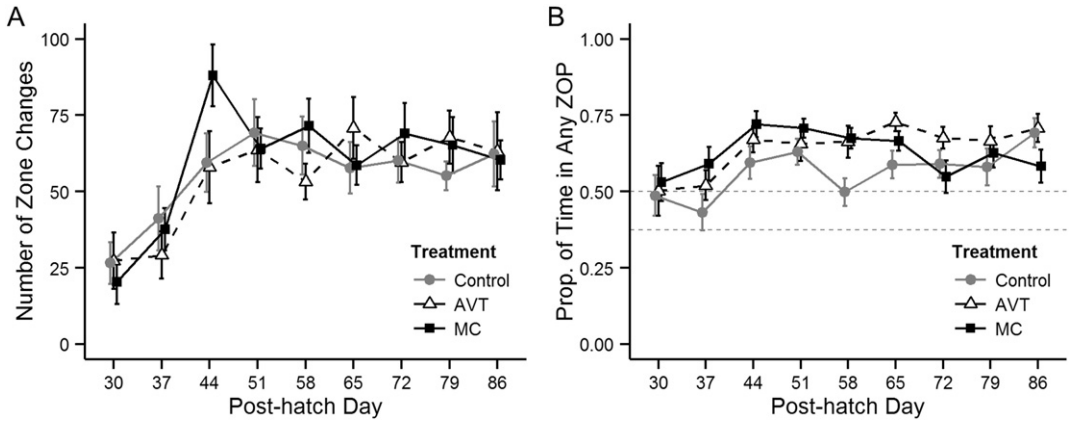

Fig. 3. Zone changes and time spent in any zone of proximity (ZOP). Mean ± SE of A) the number of zone changes across testing days during the four-way proximity tests, and B) the proportion of total test time spent in any of the three zones of proximity (ZOP, as opposed to the neutral zone). In panel B, the dashed gray horizontal lines depict the boundaries delineating what can be considered random activity in the apparatus (0.375 if determined by perch use or 0.5 if by total area use). Thus, means outside the boundary of these lines suggest that the time spent in the proximity zone differs from random chance. Control subjects are depicted with circles and a solid gray line, AVT with triangles and dashed black line, and MC as squares and solid black line.

were also no observed sex differences in time spent in the parent proximity zone. However, a simpler model including the time spent in any proximity zone, Sex, and Post-hatch day was significant (LMM: $X^2(1) = 4.65$, $p = 0.031$, $R^2_{GLMM(m)} = 0.20$, $R^2_{GLMM(c)} = 0.31$; Table 2).

Control and AVT birds of both sexes showed a significant increase in time spent in the proximity zone nearest opposite sex conspecifics across test days, whereas MC birds showed no change in the time spent with opposite sex birds (LMM, within-AVT: $X^2(2) = 8.35$, $p = 0.015$, $R^2_{GLMM(m)} = 0.05$, $R^2_{GLMM(c)} = 0.17$; within-MC: $X^2(2) = 0.78$, $p = 0.678$, $R^2_{GLMM(m)} = 0.01$, $R^2_{GLMM(c)} = 0.29$; within-control: $X^2(2) = 9.75$, $p = 0.0076$, $R^2_{GLMM(m)} = 0.07$, $R^2_{GLMM(c)} = 0.18$) (Fig. 5a and b). The best overall model included a three-way Sex*Treatment*Post-hatch day interaction term (LMM: $X^2(8) = 22.47$, $p = 0.0041$, $R^2_{GLMM(m)} = 0.32$, $R^2_{GLMM(c)} = 0.43$; Table 2). Post hoc tests reveal that both AVT and control birds were statistically different from MC birds (AVT-MC: $X^2(1) = 5.65$, $p = 0.043$, MC-control: $X^2(1) = 5.98$, $p = 0.043$).

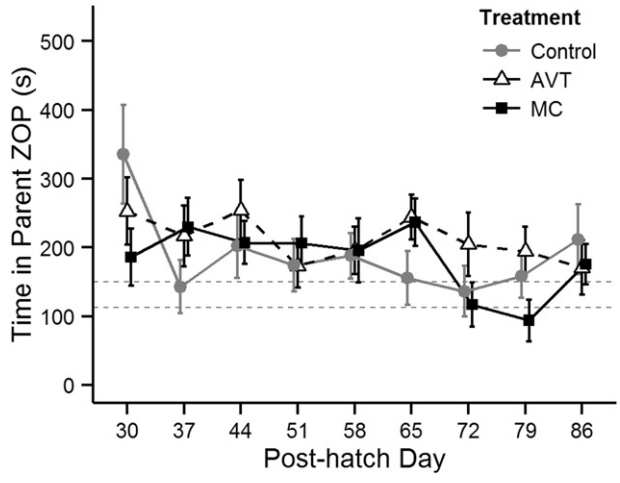

Fig. 4. Proximity parents in male and female subjects. Mean ± SE of the time in seconds spent in the zone of proximity (ZOP) with the male and female parent across testing days during the four-way proximity tests with both sexes of subjects combined. Each four-way test was 900 s long. In both panels, the dashed gray horizontal lines depict the boundaries delineating what can be considered random activity in the apparatus (112.5 s if determined by perch use or 150 s if by total area use). Thus, means outside the boundary of these lines suggest that the time spent in the proximity zone differs from random chance. Control subjects are depicted with circles and a solid gray line, AVT with triangles and dashed black line, and MC as squares and solid black line.

In addition, there was a significant sex difference observed, with males and females showing a different pattern of increase in the time in the opposite sex proximity zone across development. In males, both AVT and control males showed an increase in time spent in the female proximity zone which peaked on day 51 before subsequently decreasing, whereas MC males showed no increase in time spent in the female proximity zone across development (LMM: $X^2(4) = 11.05$, $p = 0.026$, $R^2_{GLMM(m)} = 0.30$, $R^2_{GLMM(c)} = 0.40$; Fig. 5a; Table 3). In contrast, females in all treatment groups gradually increased in the time spent in the male proximity zone, which was highly associated with the increase in total time spent in any proximity zone. Although treatment was found to be a significant predictor of the time females spent in the male proximity zone, none of the pairwise group comparisons reached significance (LMM, within-Females: $X^2(2) = 6.15$, $p = 0.046$, $R^2_{GLMM(m)} = 0.34$, $R^2_{GLMM(c)} = 0.49$; Fig. 5b; Table 3).

Neither control nor AVT birds showed a change in the time spent in the same sex proximity zone across the test days (Fig. 6a and b). However, there was a highly significant quadratic effect of Post-hatch day in the MC group (within-MC, LMM: $X^2(2) = 15.19$, $p = 0.0005$, $R^2_{GLMM(m)} = 0.08$, $R^2_{GLMM(c)} = 0.19$). Only females in the MC group exhibited a peak in time spent in proximity to same sex conspecifics on day 51 (Fig. 6b). Indeed, there was a significant effect of treatment within females (within-Female, LMM: $X^2(2) = 12.59$, $p = 0.0018$, $R^2_{GLMM(m)} = 0.20$, $R^2_{GLMM(c)} = 0.24$; Table 3), but no significant effects of treatment within males. Thus, there was highly significant interaction between Treatment and Post-hatch day and Sex in the full model (LMM: $X^2(8) = 32.21$, $p = 8.55 \times 10^{-5}$, $R^2_{GLMM(m)} = 0.24$, $R^2_{GLMM(c)} = 0.30$; Table 2). Post hoc tests indicated that AVT and MC birds differed from each other in slope ($X^2(1) = 6.89$, $p = 0.026$).

Discussion

To our knowledge, these results provide the first evidence that nonapeptides are involved in social development in a songbird species. Compared to control subjects, birds that had V1a (and potentially VT3/OT-like) receptors antagonized early in life showed a significantly altered behavioral pattern throughout development. As fledglings, MC subjects were twice as active in isolation and in fact decreased their activity levels when in the presence of the male parent. MC subjects were only minimally responsive to the presence of the male parent, with no change in the number of vocalizations emitted in the presence of the male parent compared to isolation and no change in their visual scanning rate. In the four-way proximity tests, MC birds were slightly more affiliative over all, but they demonstrated no clear affiliative interest in their parents versus unfamiliar conspecifics at

Table 2
Linear mixed model (LMM) results for four-way affiliative preference tests.
Summary of the linear mixed models with the time spent in the parent zone of proximity (ZOP), opposite sex ZOP, or same sex ZOP in the four-way affiliative preference tests as the dependent variables. The fixed effects are proportion of time in any ZOP, Sex, Day, Day^2, Treatment, as well as the interactions. Individual ID nested within Family ID was included as a random effect. The LMM models were selected based on model comparisons using likelihood ratio tests. To test the significance of each parameter within the models, we used the Kenward–Roger approximation to get approximate degrees of freedom and the *t*-distribution (SE = standard error, bold numbers indicate significance, * refers to an interaction term).

	Parents				*Opposite sex*				*Same sex*			
Predictors	*Estimate*	*SE*	*t*	*p*	*Estimate*	*SE*	*t*	*p*	*Estimate*	*SE*	*t*	*p*
Intercept	111.879	38.260	2.924	**0.004**	−71.131	70.852	−1.004	0.316	−121.231	65.558	−1.849	0.065
Prop time in any ZOP	311.852	30.691	10.161	**0.000**	398.042	32.394	12.288	**0.000**	227.494	26.593	8.555	**6.66E-16**
Sex (male)	9.587	18.513	0.518	0.605	−168.186	105.444	−1.595	**0.112**	355.901	97.257	3.659	**2.99E-04**
Post-hatch day	−39.597	12.846	−3.082	**0.002**	7.599	30.548	0.249	0.804	51.692	27.317	1.892	0.059
Post-hatch day^2	2.659	1.231	2.159	**0.032**	0.339	2.928	0.116	0.908	−4.460	2.613	−1.707	0.089
Treatment (AVT)					56.859	97.918	0.581	0.562	23.497	90.531	0.260	0.795
Treatment (MC)					7.552	96.226	0.078	0.937	53.197	88.837	0.599	0.550
Sex*Treatment (AVT)					26.602	142.089	0.187	0.852	−36.890	131.116	−0.281	0.779
Sex*Treatment (MC)					258.159	140.782	1.834	0.068	−282.676	129.748	−2.179	**0.030**
Sex*Day					87.482	47.198	1.853	0.065	−124.060	42.030	−2.952	**3.41E-03**
Sex*Day^2					−9.341	4.589	−2.036	**0.043**	10.399	4.086	2.545	**0.011**
Treatment (AVT)*Day					−9.810	43.576	−0.225	0.822	−29.398	38.894	−0.756	0.450
Treatment (MC)*Day					−34.375	43.436	−0.791	0.429	6.254	38.685	0.162	0.872
Treatment (AVT)*Day^2					0.836	4.209	0.199	0.843	2.665	3.748	0.711	0.478
Treatment (MC)*Day^2					4.515	4.238	1.065	0.287	−1.377	3.771	−0.365	0.715
Sex*Treatment (AVT)*Day					7.855	63.615	0.123	0.902	32.555	56.668	0.574	0.566
Sex*Treatment (MC)*Day					−85.854	63.521	−1.352	0.177	101.847	56.530	1.802	0.073
Sex*Treatment (AVT)*Day^2					−1.966	6.181	−0.318	0.751	−2.132	5.499	−0.388	0.698
Sex*Treatment (MC)*Day^2					6.371	6.192	1.029	0.304	−9.191	5.505	−1.670	0.096

any point in development and neither male nor female MC birds showed an increase in interest in opposite sex individuals as they matured.

AVT birds, on the other hand, seemed to show the most specific affiliative behaviors throughout development, though they were more similar to controls than were MC birds. AVT subjects were less active overall in isolation than both control and MC birds, and showed an increase in both visual scanning rate and vocalizations when in the presence of the male parent. Consistent with our predictions, they also showed elevated and sustained affiliative interest in parents throughout development, spending more time with their parents than would be expected by random chance in 7 of 9 periods (control and MC subjects showed this pattern in only 4 of 9 periods). AVT males also showed a normal increase in time spent in proximity to opposite sex birds between days 30 and 51, similar to controls.

In this study, intracranial injections of AVT or a V1aR/OTR antagonist did not affect growth or survival in chicks prior to fledging. We did not directly assess feeding or drinking behavior, but there were no obvious differences between groups in activity levels during the four-way tests. The lack of such an impact may be because the injections appear to influence primarily non-hypothalamic AVT-sensitive cell groups, minimizing detrimental consequences related to water balance and thermoregulation.

However, one possible explanation for the observed effects of the experimental manipulations on social behavior could be that the injections affect the regulation of the hypothalamic-pituitary-adrenal (HPA) axis. AVP/AVT serves as a releasing factor, along with corticotropin-releasing factor (CRF), for the production of adrenocorticotropic hormone (ACTH) in the anterior pituitary (Buckingham, 2009). Perch hops and other measures of activity level have been found to be correlated with HPA activity and anxiety in zebra finches (Remage-Healey et al., 2008; Schweitzer et al., 2014; Woodgate et al., 2010). Thus, it is possible that the higher activity level in MC birds during the social isolation tests were indicative of a change in the HPA-related functions of AVT throughout development. However, it is important to note that there were no differences between treatment groups in the number of zone changes during the four-way tests.

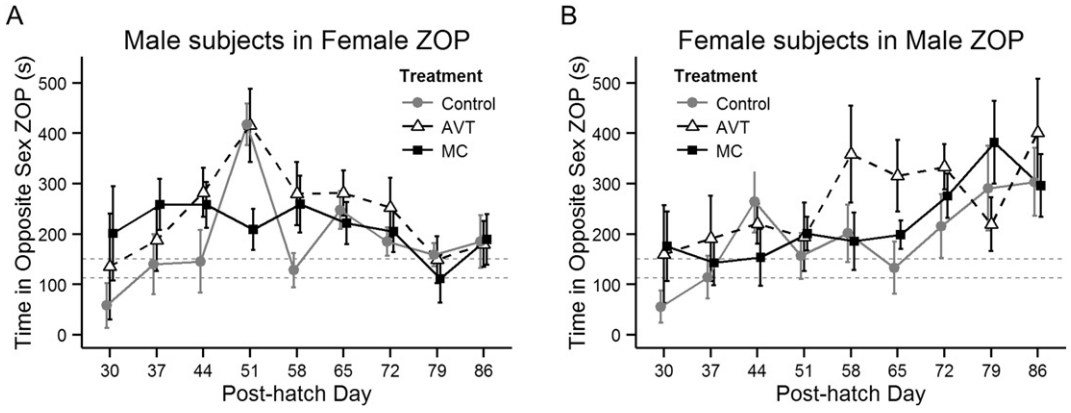

Fig. 5. Proximity to opposite sex conspecifics in male and female subjects. Mean ± SE of the time in seconds spent in the zone of proximity (ZOP) with two opposite sex conspecifics across testing days during the four-way proximity tests in A) male subjects and B) female subjects. Each four-way test was 900 s long. In both panels, the dashed gray horizontal lines depict the boundaries delineating what can be considered random activity in the apparatus (112.5 s if determined by perch use or 150 s if by total area use). Thus, means outside the boundary of these lines suggest that the time spent in the proximity zone differs from random chance. Control subjects are depicted with circles and a solid gray line, AVT with triangles and dashed black line, and MC as squares and solid black line.

Table 3
Linear mixed model (LMM) results for four-way affiliative preference tests for opposite and same sex conspecifics within sex.
Summary of the linear mixed models with the time spent in the opposite sex zone of proximity (ZOP) (top panel) or same sex ZOP (bottom panel) in the four-way affiliative preference tests as the dependent variables. The fixed effects are proportion of time in any ZOP, Day, Day^2, Treatment, as well as the interactions. Individual ID nested within Family ID was included as a random effect. The LMM models were selected based on model comparisons using likelihood ratio tests. To test the significance of each parameter within the models, we used the Kenward–Roger approximation to get approximate degrees of freedom and the *t*-distribution (SE = standard error, bold numbers indicate significance, * refers to an interaction term).

	Males				Females			
Predictors	Estimate	SE	t	*p*	Estimate	SE	t	*p*
Opposite sex								
Intercept	−227.842	85.659	−2.660	**8.57E-03**	−59.679	49.796	−1.198	0.232
Prop time in any ZOP	369.021	44.700	8.255	**4.11E-14**	436.845	45.877	9.522	**0.000**
Post-hatch day	95.677	35.724	2.678	**8.13E-03**	−8.563	18.181	−0.471	0.638
Post-hatch day^2	−8.984	3.515	−2.556	**0.011**	2.131	1.709	1.247	0.214
Treatment (AVT)	75.109	106.968	0.702	0.484	20.237	36.358	0.557	0.578
Treatment (MC)	288.610	106.503	2.710	**7.42E-03**	−40.737	36.442	−1.118	0.265
Treatment (AVT)*Day	0.438	45.984	0.010	0.992				
Treatment (MC)*Day	−120.512	46.056	−2.617	**0.010**				
Treatment (AVT)*Day^2	−1.284	4.502	−0.285	0.776				
Treatment (MC)*Day^2	10.653	4.512	2.361	**0.019**				
Same sex								
Intercept	10.632	27.149	0.392	0.696	−68.711	34.435	−1.995	0.047
Prop time in any ZOP	289.513	40.075	7.224	**3.05E-11**	147.461	32.901	4.482	**1.26E-05**
Post-hatch day					48.201	13.849	3.481	**6.18E-04**
Post-hatch day^2					−4.305	1.339	−3.214	**1.53E-03**
Treatment (AVT)					−24.643	21.720	−1.135	0.258
Treatment (MC)					56.980	21.915	2.600	**0.010**
Treatment (AVT)*Day								
Treatment (MC)*Day								
Treatment (AVT)*Day^2								
Treatment (MC)*Day^2								

Nevertheless, these results suggest that nonapeptides in the brain play an important role during sensitive periods in development and are involved in altering the neural pathways necessary for species-typical social behavior. It has long been recognized that both humans and animals of many different taxa show social attachments in which separation leads to increased feelings of stress or anxiety (Carter, 1998; Panksepp et al., 1997). The social isolation test used in the present study is in many ways analogous to the "Strange Situation" used to assess social attachment in human infants (Ainsworth and Bell, 1970). In this test, attachment to the caregiver is assessed by measuring the behavioral response of the infant to separation from the caregiver, the presence of a stranger, and then reunion with the caregiver. Secure attachment is characterized by exploration and secure-base behavior when the caregiver is present, a marked increase in anxiety when separated from the caregiver, and then a reduction in distress upon their return. Insecurely attached infants, particularly the anxious-avoidant subtype, seem indifferent to the presence of the caregiver and do not exhibit typical stranger anxiety.

Although we did not directly test the response of the juveniles to an unfamiliar conspecific in the social isolation tests, the subjects could hear and interact vocally with the unfamiliar birds located in the room. Both AVT and control subjects showed a low vocalization rate and were less active in isolation compared to when the male parent was present, a pattern typical of secure attachment, as well as evidence of the ability to distinguish between the vocalizations of the parents and unfamiliar conspecifics. Based on the life history of the zebra finch, we would also predict this pattern of minimal activity in the absence of parents, followed by an increase in vocalizations and activity level when the parents return (Zann, 1996). In contrast to this expected pattern, MC subjects vocalized more during this isolation from the

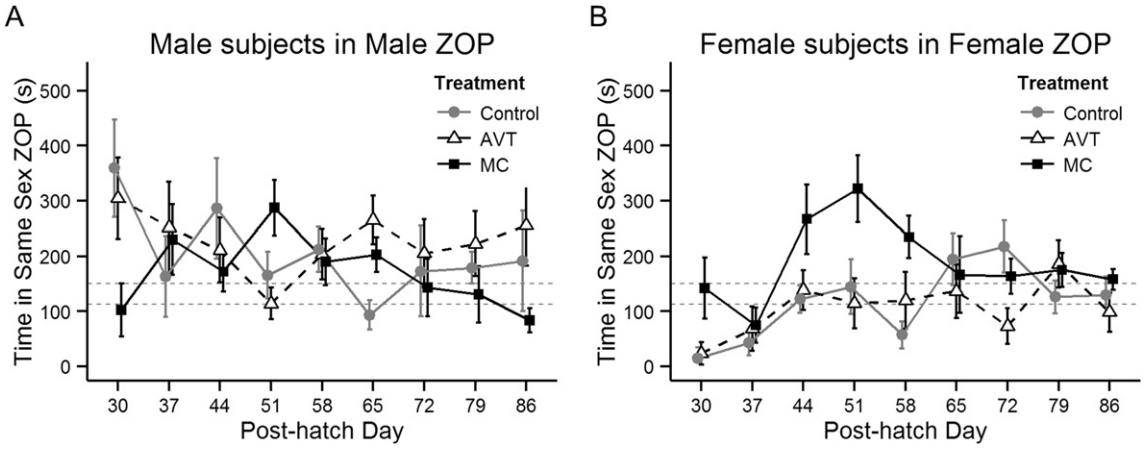

Fig. 6. Proximity for same sex conspecifics in male and female subjects. Mean ± SE of the time in seconds spent in the zone of proximity (ZOP) with two same sex conspecifics across testing days during the four-way proximity tests in A) male subjects and B) female subjects. Each four-way test was 900 s long. In both panels, the dashed gray horizontal lines depict the boundaries delineating what can be considered random activity in the apparatus (112.5 s if determined by perch use or 150 s if by total area use). Thus, means outside the boundary of these lines suggest that the time spent in the proximity zone differs from random chance. Control subjects are depicted with circles and a solid gray line, AVT with triangles and dashed black line, and MC as squares and solid black line.

family than AVT birds and were more active than both control and AVT juveniles.

Thus, there are several possible interpretations of MC subjects' behavior during isolation. One possibility is that they are simply more distressed by separation from their parents and family and are actively searching for their parents and family. However, their increased activity level in absence of their parents may also be indicative of a weaker, less specific, or even insecure attachment to their parents. It is possible, for example, that they are more active because they have not developed the ability to distinguish between the calls of their parents and the unfamiliar adults that are in the room (i.e. the strangers), and thus locomote and vocalize more in response to unfamiliar adult distance calls. This is consistent with observations that the specificity of the fledgling responses to adult distance calls seems to develop over time (Mulard et al., 2010; Zann, 1996). Alternatively, MC subjects may simply not be distressed by separation from their parents, and are exhibiting more exploratory behaviors. Our data do not allow us to distinguish between these possibilities. However, the data from the four-way proximity tests suggests that the most parsimonious explanation is that MC has somehow affected the specificity of the attachment to the parents, though we cannot rule out that this effect is not mediated by generalized effects on HPA axis regulation. Nevertheless, our results are consistent with the idea that zebra finch juveniles form specific affiliative bonds with the parents and also provide the first evidence that this attachment relationship may be mediated by the nonapeptide system in birds.

There are several possible mechanisms by which these alterations of the nonapeptides early in development may have changed the processing of social information. One possibility is that AVT and MC have permanently altered social recognition abilities in the zebra finches. The nonapeptide system is known to be very important in social recognition in rodents. For example, oxytocin knockout mice fail to recognize familiar conspecifics and infusion of oxytocin into the medial amygdala restores social recognition (Ferguson et al., 2001, 2000). Neurons in the meAMY also show selectivity to stimulus odors from conspecifics of different sexes, as well as a striking sexual dimorphism in processing of olfactory information (Bergan et al., 2014). Mice with a null mutation for V1aR display a profound impairment in social recognition, which can be restored by re-expression of V1aR in the lateral septum (Bielsky et al., 2004). Furthermore, viral vector-mediated gene transfer of the prairie vole V1aR into the lateral septum of the rat led to a dramatic improvement in social discrimination (Landgraf et al., 2003).

Zebra finch fledglings typically respond exclusively to the distance calls of their parents within a few days after fledging, suggesting that the specificity of this vocal response develops with experience (Mulard et al., 2010). The present finding that MC birds emit significantly more long tonal calls in the absence of their parent and show no affiliative interest in their parents even at day 30 suggest that MC birds may be either impaired or delayed in their recognition of their parents. Indeed, it is possible that MC injected birds suffer from an impairment in social recognition more broadly and have a more difficult time differentiating between the identities of different individuals that they encounter because of changes to how nonapeptides modulate these critical brain regions.

Another potential explanation is that MC subjects do not have any impairment in recognition per se, but have an impairment in assigning value or importance to different individuals. This interpretation would be consistent with the observation that the AVT neurons in the extended medial amygdala are sensitive to social valence in mammalian species (Newman, 1999; Sheehan et al., 2001). In zebra finches, AVT neurons in the BSTm have been found to be sensitive to social valence (Goodson et al., 2009, 2004; Goodson and Wang, 2006). There is also evidence that neurons in the extended medial amygdala are involved in the formation and maintenance of pair bonds in both zebra finches and prairie voles (Curtis and Wang, 2003; Svec et al., 2009). The extended medial amygdala and the lateral septum are integral parts of both the social behavior network and the mesolimbic reward network, so it is also possible that the present results are a function of alterations to the nonapeptide-sensitive neurons in these regions which are thought to be critical for assigning a reward value or valence to conspecifics (O'Connell and Hofmann, 2011).

A third explanation is that the early manipulations of the AVT system alter attentiveness to or the salience of social stimuli only at the time of injection, leading to downstream differences in socially-relevant behaviors later in life. In this view, AVT does not directly alter the production of or sensitivity to nonapeptides. Instead, by acting in regions that process social information, AVT may act to increase the salience of these social stimuli at a critical point during development, such that the developing bird learns to more strongly or easily form an association between features of their parents and reward. Thus, stimulation or inhibition of the AVT system, coupled with salient social experiences occurring at the time of the injections, could have led to changes in social behaviors via domain-general learning mechanisms like associative learning. In this view, the injections did not exert their effect by reorganizing the AVT system itself, but rather by briefly changing what is attended to during development and, thus, what is learned. Of course, these possible explanations are not mutually exclusive, but further research is needed to determine the most likely mechanisms.

Unfortunately, a major challenge for interpreting the present results is the lack of detailed comparative information about where and when nonapeptides and their receptors are expressed in the developing brain. Detailed experiments have outlined the ontogeny of the nonapeptide systems in rats, which demonstrate that the production of AVP by the hypothalamus begins during fetal development. AVP can be labeled in the SON and PVN by fetal day 14 (gestation is 21 days in rats), reaching adult levels by postnatal day 30 (Buijs et al., 1980; Szot and Dorsa, 1993). The production of AVP mRNA in the meAMY and BSTm begins later in development (between postnatal day 3 and day 14) and is highly sexually dimorphic, with production of AVP starting later in females and taking longer to reach adult levels (Szot and Dorsa, 1993). Additionally, binding sites were found in the developing rodent brain in both the amygdala and septum between postnatal days 0 and 8, as well as several brain regions where nonapeptide receptors are not expressed in adulthood, including the hippocampus, dentate gyrus, and caudate nucleus (mice: Hammock et al., 2013; rat: Petracca et al., 1986; multiple vole species: Wang et al., 1997). AVT is produced in the brain at least as early as 4 weeks of age in male canaries, though earlier ages were not assessed (Voorhuis et al., 1991). In zebrafish, both AVT and the two teleost V1a receptor subtypes are expressed together very early in development, implicating AVT in the development of the nervous system or control of early behavior across vertebrates generally (Eaton et al., 2008; Iwasaki et al., 2013).

Additionally, experimental evidence from rodents does provide evidence consistent with the hypothesis that nonapeptides may modulate many brain regions early in development, particularly those relevant for social behavior. In rodents, the beginning of production of AVP/OT from the extrahypothalamic sources and central nonapeptide receptor expression is coincident with important milestones in early social attachment and learning (Blass, 1987; Buijs et al., 1980; Hammock et al., 2013; Petracca et al., 1986; Szot and Dorsa, 1993; Wang & Young, 1997). A limited number of experimental manipulations of nonapeptides during development in rodents provide further evidence for the organizational hypothesis. Vasopressin-deficient Brattleboro rat pups show hyperactivity, reduced huddling and reduced proximity to other pups in the nest compared to wild-type rats (Schank, 2009). Wild-type rat pups treated with a nine-day exposure to AVP showed increased emotionality, activity levels and grooming in an open field test as juveniles, as well as smaller overall brain size (Boer et al., 1994). Acute central administration of AVP in wild-type neonatal rat pups was found to decrease the number of ultrasonic vocalizations and

reduced locomotor activity in a maternal isolation test (Winslow and Insel, 1993). In juvenile male rats, both targeted infusion of AVP into the LS and intracerebroventricular infusion increased preference for investigating novel individuals, whereas a V1aR antagonist increased the preference for investigating familiar individuals (Veenema et al., 2012). In addition, V1aR blockade in the LS increased social play behavior in males and decreased it in females, but only when it tested in a familiar environment (Bredewold et al., 2014; Veenema et al., 2013). Neonatal manipulation of AVT or OT in the socially-monogamous prairie vole, leads to significant changes in nonapeptide binding in several brain regions in adults and alterations to social behaviors (Bales et al., 2007; Bales and Carter, 2003a; Bales and Carter, 2003b; Stribley and Carter, 1999; Yamamoto et al., 2004). Thus, there is evidence from multiple rodent species that nonapeptides are critically involved in the development of species-typical social behaviors.

Our results are consistent with the idea of a similar organizational role for nonapeptides in social development in zebra finches. However, it is important to note that the time between the end of the injections and behavioral testing in the present experiment was as little as 8 days, although it was over 12 weeks by the end of the four-way tests. While the influences of the early injections on affiliative behaviors after sexual maturity are much more likely to be organizational effects, it is possible that the results observed in the SI tests represent direct effects on the activation of the nonapeptide system in juveniles. Furthermore, we do not test the effects of similar manipulations on other time points, so we cannot conclude that these or similar effects would be restricted to this early developmental window. However, Goodson et al. (2004) used similar dosages of AVT and MC in adult birds and found no effects on affiliation in a partner preference test. Additionally, we have not tested for whether the effects are either permanent or reversible, so further research is needed to confirm that these effects can indeed be considered organizational.

A further challenge, which remains a challenge for the study of nonapeptides generally, is the issue of receptor specificity. Because nonapeptide receptor subtypes have high degrees of structural homology with each other and each subtype is highly variable across species, we can infer that both AVT and MC will bind to multiple receptor subtypes in the zebra finch brain with unknown affinities (Leung et al., 2011, 2009). Although MC most potently targets V1aR, it also serves as a mild antagonist for OT-like receptors as well (Kruszynski et al., 1980; Leung et al., 2009; Manning et al., 2012). To complicate matters further, several authors have speculated that AVT (not MT) may in fact be the endogenous ligand of the avian VT3 (OT-like) receptor both inside and outside the brain because AVT may actually have a higher affinity for VT3 receptors than MT (Baeyens and Cornett, 2006; Gubrij et al., 2005). AVT may be the more relevant endogenous ligand for a number of social functions via its action at multiple receptor subtypes, despite evidence that alterations of MT affect behavior (Kelly and Goodson, 2014b; Lowrey and Tomaszycki, 2014). Thus, further research is needed to determine whether the observed effects are a result of the actions of AVT and MC at V1a receptors, VT3 (OT-like) receptors, or, more likely, both.

Conclusion

These results provide evidence that the nonapeptide system, particularly AVT and V1aR, plays an important role in organizing the neural pathways necessary for species-typical affiliative behaviors and attachment in zebra finches. The nonapeptides have been identified as important mediators of individual and species differences in social behaviors more broadly (Goodson, 2005). These results provide support for the idea that changes to the nonapeptide system during development may in fact be an important mechanism underlying the evolution of species-differences in social phenotypes (Syal and Finlay, 2011). Much additional research is needed, but these results suggest that explorations of the evolution and development of the nonapeptide systems will prove fruitful.

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