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Arginine vasotocin regulates social ascent in the African cichlid fish *Astatotilapia burtoni*

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ABSTRACT

Neuropeptides modulate many aspects of behavior and physiology in a broad range of animals. Arginine vasotocin (AVT) is implicated in mediating social behavior in teleost fish, although its specific role varies between species, sexes, life stages, and social context. To investigate whether the effects of AVT on behavior depend on social context, we used the African cichlid fish *Astatotilapia burtoni*, which is well-known for its remarkable behavioral plasticity. We pharmacologically manipulated the AVT system in established socially dominant and subordinate *A. burtoni* males, as well as in males ascending to dominance status in a socially unstable environment. Our results show that exogenous AVT causes a stress response, as evidenced by reduced behavioral activity and increased circulating levels of cortisol in established dominant and subordinate males. Administration of the AVT antagonist Manning compound, on the other hand, did not affect established subordinate or dominant males. However, AVT antagonist-treated males ascending from subordinate to dominant status exhibited reduced aggressive and increased courtship behavior compared to vehicle-treated animals. Finally, we measured circulating cortisol levels and brain gene expression levels of AVT and its behaviorally relevant V1a2 receptor in all three social phenotypes and found that plasma cortisol and mRNA levels of both genes were increased in ascending males compared to dominant and subordinate males. Our results provide a more detailed understanding of the role of the AVT system in the regulation of complex behavior in a dynamically changing social environment.

1. Introduction

Neuropeptide systems such as the nonapeptide arginine vasotocin (AVT) and its mammalian homolog arginine vasopressin (AVP) are present in diverse taxa, but their functional roles in behavior can vary widely. In addition to being involved in the regulation of a variety of social behaviors in all vertebrate species studied thus far (reproduction, Salek et al., 2002; pair-bonding, Winslow et al., 1993; Oldfield and Hofmann, 2011; parental care, Wang et al., 1994; Kleszczyńska et al., 2012; affiliation, Landgraf et al., 2003; Young and Wang, 2004; social approach, Thompson and Walton, 2004; Braida et al., 2012), the AVT/AVP system also regulates central and peripheral stress responses (Engelmann et al., 2004), although its specific role appears to differ between species, sexes, life stages, and social contexts (for review, see Goodson (2008), Godwin (2010)). This neuropeptide system has also been associated with social status (Ferris et al., 1989; Godwin et al., 2000; Goodson and Bass, 2001; Aubin-Horth et al., 2007; Greenwood et al., 2008; Almeida et al., 2011; Lema et al., 2012) and with aggressive behavior in males (Ferris et al., 1997; Goodson, 1998; Delville et al., 2000). However, the involvement of AVT in male aggression can depend on social context (Semsar et al., 2001; Greenwood et al., 2008; Filby et al., 2010), although a detailed understanding is still lacking.

Even though the role of AVT in behavioral regulation appears to be conserved in some manner across vertebrates, it is clear that AVT might play different roles in different social contexts, and it has been suggested that this may be observed even within a single species (Goodson, 2008; Ophir, 2011). To gain a better understanding of these processes we need a model system in which differences in social context are associated with differences in behaviors and AVT levels, and in which we can experimentally test the behavioral role of AVT in these different social contexts.

The African cichlid fish *Astatotilapia burtoni* is an established model system in social neuroscience and is well-suited to examine AVT function due to its remarkable behavioral plasticity and ease of experimentation (Hofmann, 2003; Fernald and Maruska,

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2012). In this species, males are either dominant (DOM) or subordinate (SUB). DOM males establish and aggressively defend display territories, are brightly colored, and attract females for reproduction. Non-territorial SUB males are non-reproductive and non-aggressive, cryptically colored, and shoal with females and juveniles. Males transition between social states every 4–7 weeks on average depending on social and environmental conditions, likely as a consequence of the high energetic cost associated with being dominant (Hofmann et al., 1999): DOMs grow at a slower rate than SUBs, which allows the latter to eventually gain a size advantage and overtake occupied territories (Hofmann et al., 1999; Hofmann, 2003). This remarkable plasticity has been well characterized at the behavioral, hormonal, and genomic levels for the established DOM and SUB phenotypes (Hofmann et al., 1999; Hofmann and Fernald, 2000; Parikh et al., 2006; Trainor and Hofmann, 2006, 2007; Renn et al., 2008; O'Connell and Hofmann, 2012), including detailed examinations of the phenotypes of males ascending from SUB to DOM (Hofmann and Fernald, 2000; Burmeister et al., 2007; Maruska and Fernald, 2010, 2011; Maruska et al., 2011, 2012; Huffman et al., 2012a). These studies suggest that social status and experience greatly influence how animals respond to the social environment. Interestingly, AVT expression in the brain varies with social status, with DOMs having higher brain expression levels than SUBs (Renn et al., 2008). More specifically, *A. burtoni* DOM males have higher expression in the gigantocellular nucleus of the POA, and SUB males have higher expression in the parvocellular nucleus of the POA, while expression in the magnocellular nucleus does not differ between phenotypes (Greenwood et al., 2008). The expression of AVT has not yet been examined in males ascending from SUB to DOM. Furthermore, brain expression levels of the V1a2 receptor are not known for any of the male social phenotypes. This receptor is the behaviorally relevant AVT receptor based on studies that have shown expression in brain regions associated with behavior and reproduction (Kline, 2010). While these studies clearly suggest that AVT plays a role in the regulation of social status and aggression in *A. burtoni*, no functional tests have been carried out to dissect the role of AVT, yet this species provides an exceptional opportunity to do so in males of the same species that experience dramatically different social environments.

Here, we examined the functional role of the AVT system in *A. burtoni* males depending on social context. Specifically, we measured neural mRNA levels of AVT and its receptor in the DOM and SUB males that live in a stable social context and ascending males that face an unstable context. We also tested whether the effects of AVT on behavior depend on social context by pharmacologically manipulating the AVT system using an agonist (AVT) and an antagonist to the V1a receptor (Manning compound, MC). We predicted that AVT and V1a2 gene expression would be highest in DOMs and ascending males. Furthermore, since ascending males are exposed to social instability compared to established DOM and SUB males, we predicted that ascending males would be more sensitive to AVT manipulation.

2. Materials and methods

2.1. Animals

All animals used in this study were adult *A. burtoni* males from a laboratory stock originally derived from a wild population in Lake Tanganyika, Africa (Fernald and Hirata, 1977). Fish were maintained at 28 °C on a 12:12 h light/dark cycle with 10 min dawn and dusk periods to mimic their native tropical environment in 110 L aquaria that were integrated into a re-circulating life support system. All tanks contained gravel substrate to facilitate digging behavior and terra cotta pot shards, which served as territorial shelters. Communities were allowed to settle for approximately 1 week before experiments began. All procedures were in accordance with and approved by Institutional Animal Care and Use Committees at The University of Texas and Harvard University.

2.2. Pharmacological manipulations in established DOM and SUB males

To investigate the role of AVT in established males, we tested the effects of AVT and a V1a receptor antagonist (Manning compound, MC) on social status and behavior. We set up communities of ten males and ten females with five terra cotta pots as shelters and allowed them to settle for 5–7 days. One DOM male per community was chosen as the focal male, and after being weighed and measured for standard length on Day 1, he was observed for 10 min on Days 1–3 between the hours of 11:00 and 13:00 to establish a baseline of behavior. Aggressive, reproductive, and neutral behaviors were recorded as described previously (Fernald and Hirata, 1977) as well as any changes in social status. For each observation, aggressive behaviors (chasing, lateral threat displays, border threats) were summed to comprise an aggression score; reproductive behaviors (courting, quivering, digging) were summed to comprise a reproduction score. On Days 4–6, all males from the tank were removed to standardize netting stress and each focal male received an intraperitoneal saline injection ca. 60 min prior to observation, to establish any injection effect on behavior. On Days 7–9, again 60 min prior to observation, each focal male received an injection of either saline ($n = 11$) or AVT (Sigma, 1 μg/gbw; $n = 10$) or MC (Sigma, 3.2 μg/gbw; $n = 6$) dissolved in saline such that each male received only one treatment, for three consecutive days. Doses were based on previous work in bluehead wrasse (Semsar and Godwin, 2004). We also tested a range of doses below those previously used (0.5–0.008 μg/gbw, $n = 6$–14). Following observation on Day 9, their plasma was collected from the dorsal aorta using heparinized 26G butterfly infusion sets (Surflo) and kept on ice until processing. Following blood collection, the animals were killed, and the brains and testes were collected for analysis (see next section).

2.3. Pharmacological manipulations in ascending males

To test the role of AVT in SUBs and ascending males, we set up communities as described previously and chose one SUB male per tank as the focal male. After being weighed and measured for standard length on Day 1, the focal male was observed for 10 min on Days 1–3 at 10:00 h to establish a baseline of subordinate behavior. On Day 4, we again netted all of the males in the tank to inject 60 min before observations. For the ascending males, we did not return the DOM males to their tanks when we removed all of the males, to provide SUB males with an opportunity to compete for dominance. This provided open territories for the SUB males to compete for on Days 4–6. Focal males were injected with either saline ($n = 10$ SUB, $n = 24$ ascending), AVT ($n = 10$ SUB, $n = 8$ ascending), or MC ($n = 9$ SUB, $n = 10$ ascending), 60 min prior to observation at 10:00 h, such that each male received one treatment, for three consecutive days. Following observation on Day 6, males were weighed, their blood was drawn for cortisol measurement, and they were killed for tissue collection.

2.4. Hormone measurements and tissue processing

To separate the plasma from the serum, blood samples were centrifuged at 4000 rpm for 10 min, and the plasma was stored at −80 °C until analysis. Cortisol was measured from plasma samples using ELISA (Assay Designs). Plasma samples were thawed on

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ice and diluted by a factor of 30 using diluted assay buffer according to Kidd et al. (2010) and manufacturer's instructions before being run in duplicate. The coefficient of variation of the assay was 1.94%. After blood collection, animals were killed by rapid cervical transsection. Their brains were removed and stored in RNAlater (Ambion) at −20 °C for qPCR. The testes were removed and weighed to calculate gonadosomatic index (100 × gonad mass/body mass).

2.5. Quantitative real-time PCR

Whole brain expression of AVT and the V1a2 receptor was measured in established DOM and SUB males as well as in ascending males using quantitative real time PCR, with 18S used as a reference gene. In teleosts, three AVT receptor subtypes have been identified and classified as V1a1, V1a2, and V2 receptors, respectively, based on amino acid homologies (Lema, 2010). Compared with the V1a1 subtype the V1a2 subtype is more widely distributed in the brain and more closely associated with sex, reproduction, and behavior (Kline, 2010; Kline et al., 2011), and its distribution has been mapped throughout the *A. burtoni* brain (Huffman et al., 2012b). Primers were designed using Primer3 (Rozen and Skaletsky, 2000) and Amplify 3 (Engels, 2005), based on the sequences for AVT mRNA (NCBI accession number AF517935, forward: ACTGTGTGGAGGAGAACTACC; reverse: CTCTGTTGG TGAGCTTCTTG, amplicon of 191 bp), AVT V1a2 receptor mRNA (AF517936, forward: GAAAGAAGACTCAGACAGTAGCC; reverse ACCATCACTACACACATCTCG, amplicon of 209 bp) and 18S *rRNA* (U67333, forward: GAGAACCTCGACCGAAAG; reverse: ATTCGTAGACGGGTAAGAGG, amplicon of 183 bp). Only saline-treated individuals from each pharmacological manipulation experiments were used for the gene expression analysis (SUBs: $n = 8$, ascending males: $n = 5$; DOMs: $n = 8$). Total RNA was extracted using Trizol (Invitrogen) and frozen at −80 °C. RNA was treated with DNase Amplification Grade I (Invitrogen). RNA content of the DNAse-treated sample was quantified using Ribogreen quantification (Invitrogen) (Hashimoto et al., 2004). A total of 660 ng of RNA was reverse transcribed in duplicate using Superscript II (Invitrogen) and then pooled for a total of 1320 ng of RNA per fish.

The efficiency of PCR was verified using a quantitative real time PCR experiment in a RealPlex2 instrument (Eppendorf) for each gene, using a cDNA standard curve made of 7 serial dilutions. The efficiency was calculated using this standard curve with the formula $E = 10^{[-1/\text{slope}]} - 1$, (Pfaffl, 2001), where the slope is calculated from the relationship between the log cDNA quantity of a sample and its quantification cycle (Cq). Gene expression was measured by qPCR using a scaled-down version of the manufacturer's protocol: 2 µl of cDNA (diluted 1:10), 12.5 µl of SYBR Green PCR Master Mix (Qiagen), 9.5 µl of Nuclease Free water (Ambion) and 1 µl of primer pairs (10 µM, Integrated DNA Technologies, Coralville, IO) in a total volume of 25 µl, in a 96-well plate. All fish were assayed in triplicate on a single plate for a given gene.

2.6. Statistical analysis

All statistical tests were run in SPSS software, version 19. We tested for an injection effect on various behaviors in DOMs by comparing Days 4–6 (saline injection) to Days 1–3 (no injection) using *t*-tests. No significant effect of injection was found in any behavior ($p > 0.05$), so for all analyses on DOMs and SUBs we compared Day 3 (last day of no injection) to Day 9 (last day of drug treatment). Similarly, for SUB and ascending males, we compared Day 3 (last day of no injection) to Day 6 (last day of drug treatment).

Categorical variables such as social status were examined using Fisher's exact test, which accounts for small sample sizes. Continuous variables were tested for normality using the Shapiro–Wilk test; non-normal variables were subsequently log-transformed and retested. Differences in non-repeated normal variables were tested using 1-way ANOVA and subsequent Tukey's HSD post-hoc tests; non-normal variables were tested using Kruskal–Wallis and subsequent Mann–Whitney *U* pairwise comparisons. To investigate effects of drug treatments on repeated measures (such as behavior), we used a Generalized Estimating Equations (GEE) model and looked for a day-by-treatment interaction effect. Correlations on normal and non-normal variables were tested using Pearson's and Spearman's correlation coefficients, respectively, and categories such as social status were controlled for by using partial correlation coefficients.

3. Results

3.1. AVT system manipulation in established DOM and SUB males

Most of the DOM males treated with AVT (9 of 10) lost their social dominance status, whereas only 2 of 11 saline-treated DOMs lost their social status (Fisher's exact test: $p = 0.002$). However, blocking the V1a receptor with MC had no effect on social status relative to saline controls in DOM males (1/6 vs. 2/11; $p = 0.75$; Fig. 1A). We next examined aggressive behavior, which is characteristic of the DOM phenotype, and found a decrease in DOM male aggression after being treated with AVT (GEE day*treatment effect, $p < 0.001$; Fig. 1C), but no effect after MC treatment. The status of SUB males was unaffected by either treatment (Fisher's 1-sided exact test: $p = 0.500$, 0.526 for AVT and MC, respectively; Fig. 1B). There were no significant effects of manipulation on aggressive behavior in SUB males, as it was extremely low in all treatment groups (GEE day*treatment effect, $p = 0.525$; Fig. 1D).

Although the DOM males treated with AVT lost their dominant status, it was clear from our behavioral observations that they did not become SUB. In fact, the treatment left the animals lethargic and unresponsive for up to several hours. In addition, they displayed dark vertical bars along their bodies, which is a typical stress response in *A. burtoni* and other haplochromine cichlids (Baerends and Baerends-van Roon, 1950). After AVT treatment, DOMs also spent significantly less time feeding than saline-treated SUBs (Mann–Whitney *U* test: $p = 0.040$). This behavioral response was also observed following a range of lower doses, with even the lowest dose (0.008 µg/gbw) causing the majority of males (6/9) to lose their territories. To investigate this apparent stress response further, we measured plasma cortisol levels and found that AVT-treated DOMs had significantly higher cortisol than either saline- or MC-treated males (Tukey's HSD, $p < 0.001$ for both comparisons; Fig. 2A). Circulating cortisol levels of SUB males were also significantly increased following AVT treatment relative to SUB controls (Mann–Whitney *U*, $p = 0.004$; Fig. 2B).

3.2. AVT system manipulations in ascending males

Next, we investigated the role of AVT in SUB males given an opportunity to ascend to DOM status. As expected, ascending saline-treated males significantly increased aggression during the three days of social instability (GEE day effect, $p < 0.001$), although they did not become as aggressive as established DOM control males (Mann–Whitney *U*, $p = 0.012$). As in DOM males, ascending males appeared to have a stress response to AVT treatment, as none of them successfully ascended to DOM ($n = 8$; Fig. 3A), and they also showed the characteristic stress coloration and were behaviorally non-responsive. Finally, MC treatment did not have any effect on the likelihood of successfully ascending: 3 out of 10 MC-treated males and 7 out of 24 saline-treated males became DOM (Fisher's exact test: $p = 0.633$; Fig. 3A). However, when we examined the amount of aggressive behavior displayed by these

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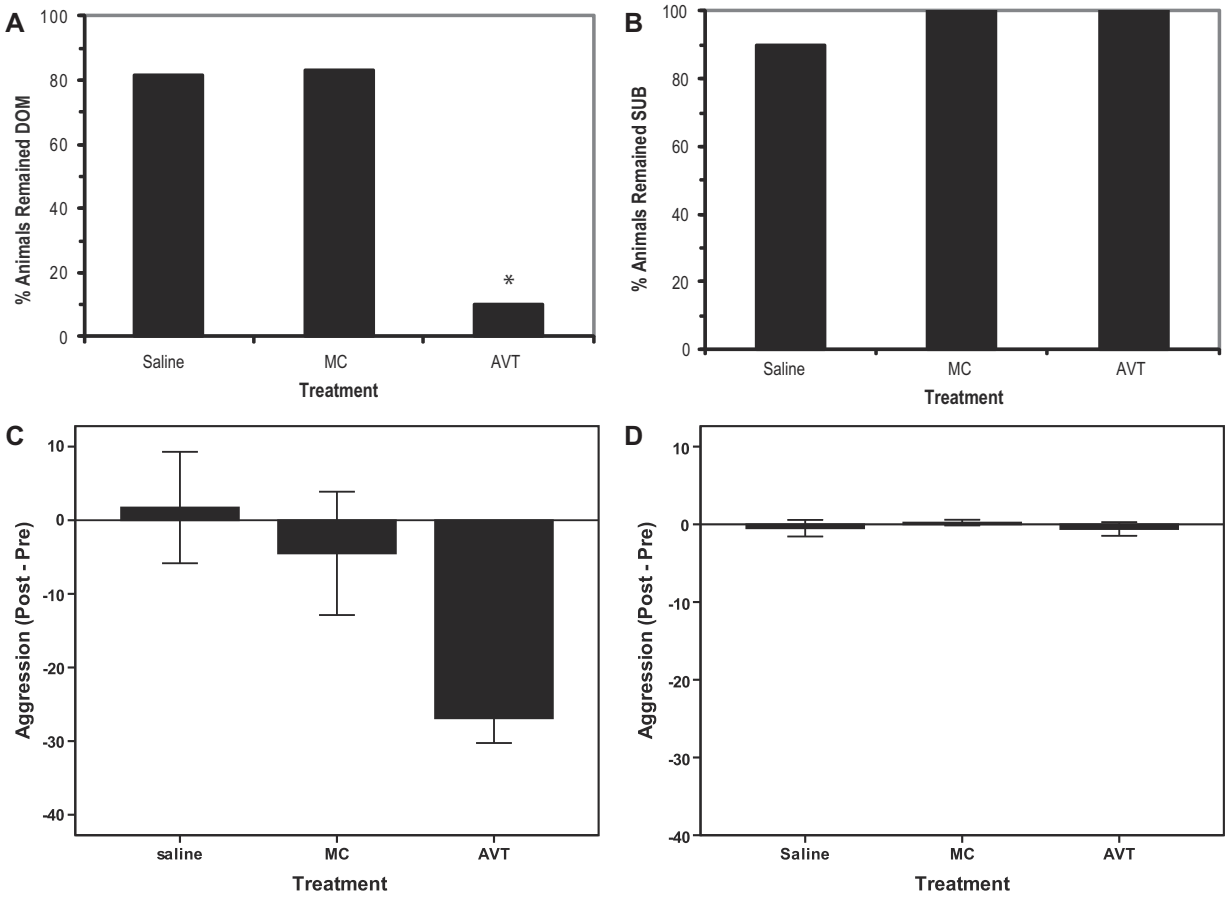

Fig. 1. Effects of AVT on social status and behavior in DOM and SUB males. Fraction of (A) DOM males that remained dominant and (B) SUB males that became dominant following treatment with saline, MC, or AVT. (C) Change in aggressive behavior in DOM males following treatment of saline, MC, or AVT. (D) Change in aggressive behavior in SUB males following treatment of saline, MC, or AVT. Asterisks denote statistically significant differences.

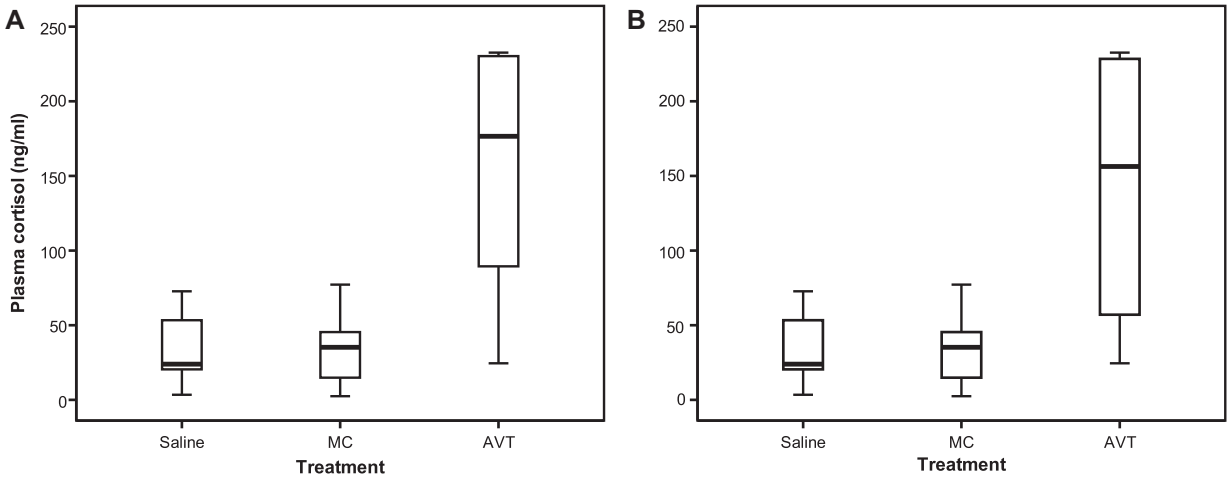

Fig. 2. Plasma cortisol levels in DOM and SUB males following treatment. (A) Cortisol levels in DOM males following treatment of saline, MC, or AVT. Asterisk denotes statistically significant difference. (B) Cortisol levels in SUB males following treatment of saline, MC, or AVT.

ascending males, we found that among individuals that successfully ascended to DOM status, those treated with MC were significantly less aggressive compared with saline-treated males (Mann–Whitney *U* test: $p = 0.05$; Fig. 3B). We also found that among these males, those treated with MC showed more courting behavior (Mann–Whitney *U* test; $p = 0.02$; Fig. 3C). Note that animals who failed to ascend never displayed any courtship behavior.

Because the stress hormone cortisol has been implicated in the regulation of dominance behavior, especially in socially unstable environments (Fox et al., 1997; Sapolsky, 1992), we compared plasma cortisol levels of saline-treated ascending males with those of control SUB and DOM males presented above. We found that cortisol levels varied significantly among these social phenotypes (ANOVA, $p = 0.031$; Fig. 4). Specifically, while cortisol levels did

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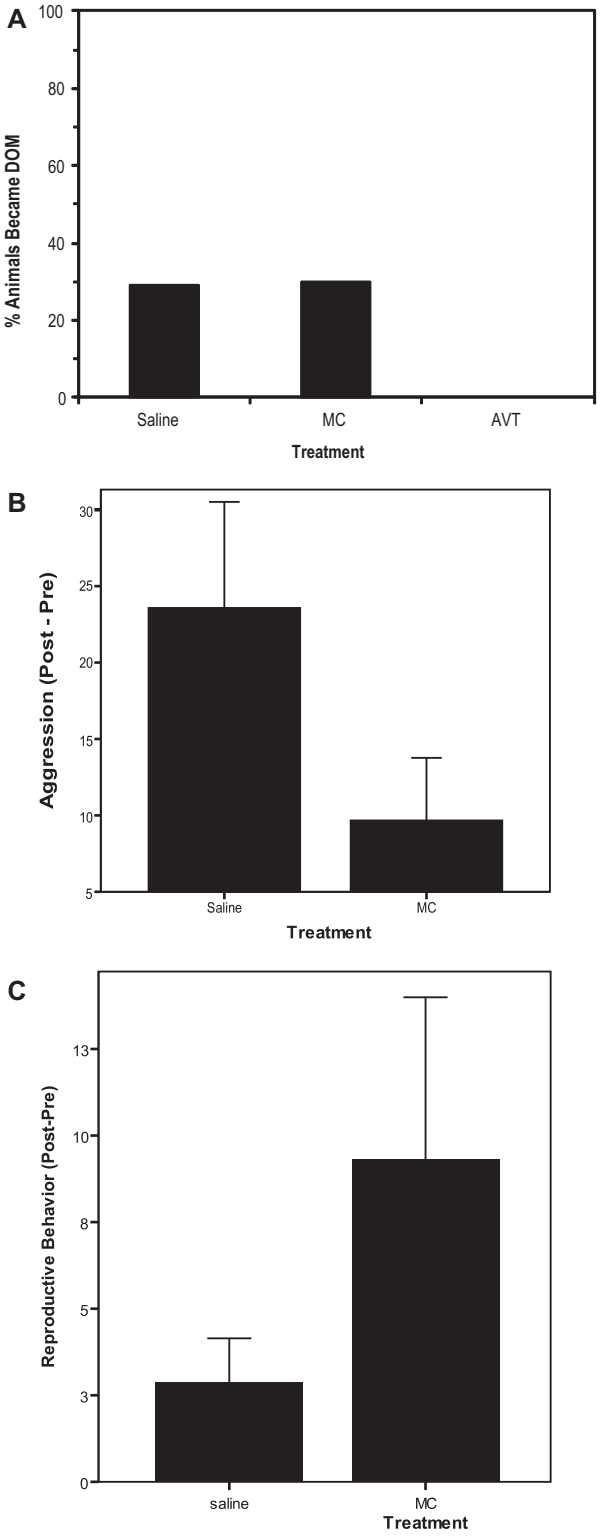

Fig. 3. Effects of AVT on social status and behavior in ascending males. (A) Percentage of SUB males that successfully ascended to DOM following treatment of saline, MC, or AVT. (B) Change in aggressive behavior in SUB males that successfully ascended to DOM following treatment of saline or MC. (C) Change in reproductive behavior in SUB males that successfully ascended to DOM following treatment of saline or MC.

not differ between established DOMs and SUBs (Tukey's HSD, $p = 0.347$), ascending males had significantly higher levels than established DOM males (Tukey's HSD, $p = 0.027$; $p = 0.245$ compared to SUB males). Circulating cortisol levels did not differ (Student *t*-test, $p = 0.563$) between ascending males (which were successful in establishing a territory; $n = 7$) and those that failed to do so ($n = 16$).

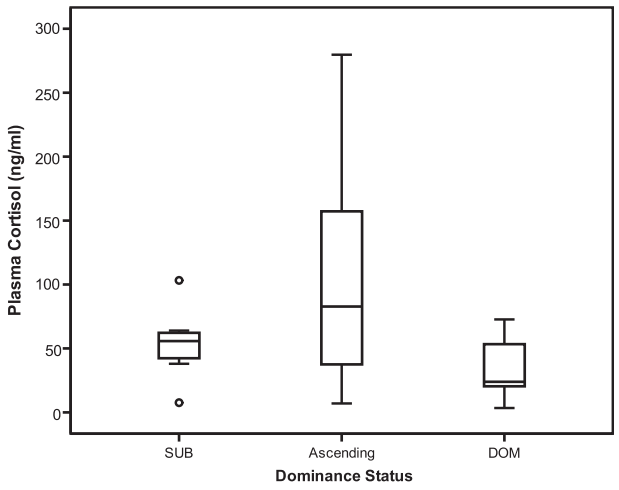

Fig. 4. Plasma cortisol levels by social status. Circulating cortisol levels in SUB, ascending, and established DOM males following saline treatment.

3.3. Neural AVT and V1a2R gene expression

We investigated whole-brain mRNA levels of AVT and the V1a2 receptor in saline treated SUBs, established DOMs, and ascending males, and found that both genes tended to vary in expression between phenotypes (ANOVA: $p = 0.07$ for AVT; $p = 0.004$ for V1a2R). Contrary to our previous findings (Greenwood et al., 2008; Renn et al., 2008), AVT mRNA levels did not differ between established DOMs and SUBs (Tukey's HSD, $p = 0.892$). Gene expression of AVT was, however, increased in the brains of ascending males compared with established DOMs, though the difference was only marginally significant (Tukey's HSD, $p = 0.07$; Fig. 5A). Levels of V1a receptor mRNA were significantly higher in ascending males than established DOMs (Tukey's HSD, $p = 0.003$; Fig. 5B), with intermediate expression levels in SUBs (Tukey's HSD, $p = 0.110$ and $p = 0.134$ compared to established DOM and ascending males, respectively). Finally, we conducted a partial regression analysis, controlling for initial dominance status (DOM, SUB, or ascending), and found that AVT and V1a2R expression correlated strongly with each other (Pearson's partial $r^2 = 0.511$, $p = 0.021$, df = 18; Fig. 5C).

4. Discussion

Here, we investigated the role of the AVT system in males given an opportunity to ascend to dominance in comparison to established DOM and SUB males. We tested the hypothesis that AVT regulates aggressive behavior depending on social context. We have shown that blocking a V1a pathway in male *A. burtoni* decreased aggressive behavior and increased reproductive behavior in the unstable social context faced by males ascending to DOM status but, contrary to our prediction, did not affect males of either the stable DOM or SUB phenotype. We have also shown that circulating cortisol and brain expression levels of both AVT and the V1a2 receptor were increased in ascending males compared to DOMs and SUBs. The gene expression measurements and the effects of the V1a receptor antagonist suggest that ascension to dominance in an inherently unstable social environment is perceived as stressful and that AVT increases aggressive behavior specifically in this context.

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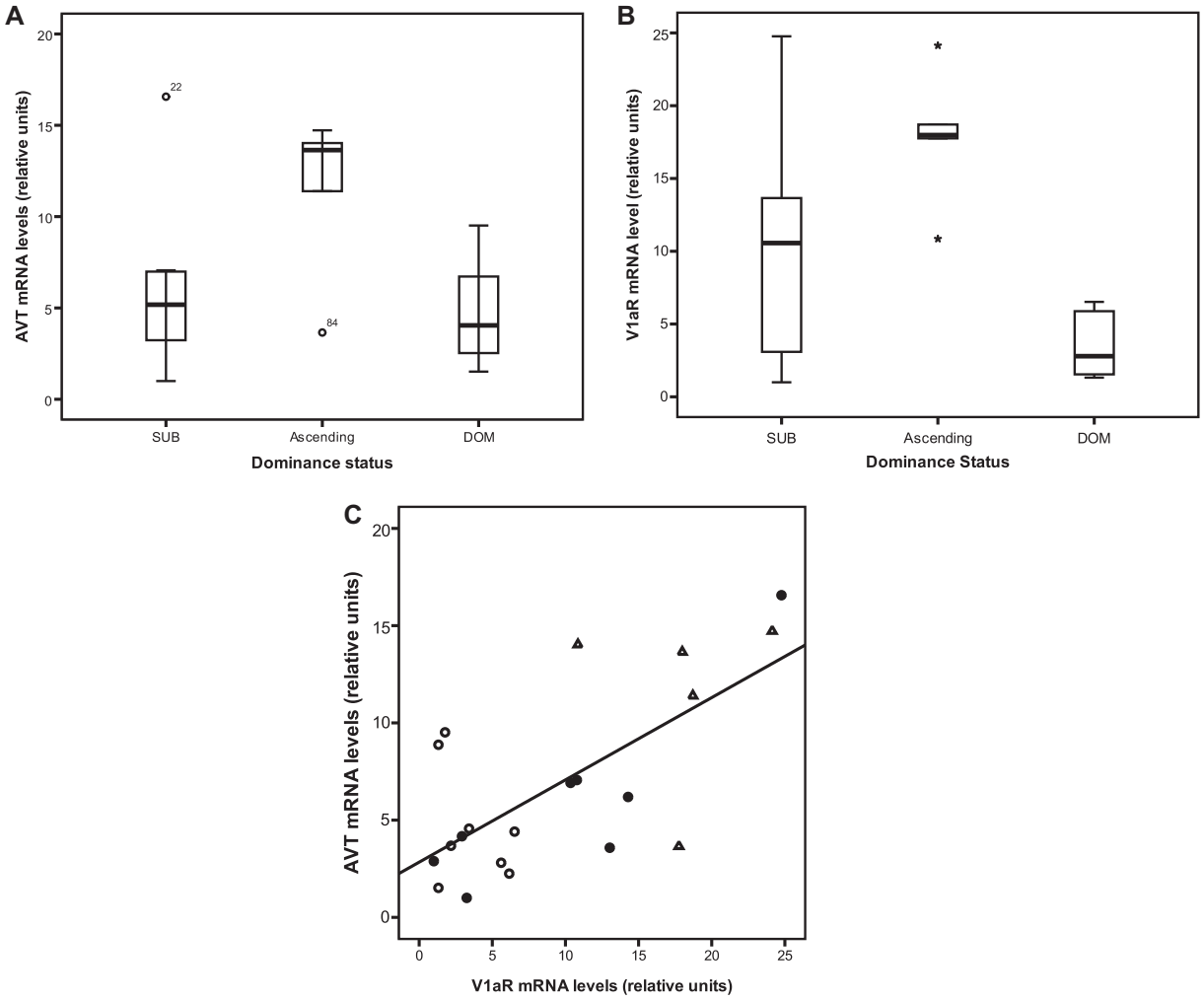

Fig. 5. AVT and V1aR expression by social status. (A) AVT expression in whole brains of SUB, ascending, and established DOM males following saline treatment. (B) V1aR expression in whole brains of SUB, ascending, and established DOM males following saline treatment. (C) Linear regression analysis of AVT and V1aR expression levels. Symbols denote different social states (filled circles: SUBs; open triangles: ascending males; open circles: DOMs).

Even though we did not find a significant difference in whole brain AVT expression between DOMs and SUBs, ascending males did indeed show higher AVT and V1a receptor expression, indicating a role for AVT during the transition to (as opposed to the maintenance of) dominance. Ascending *A. burtoni* males typically engage mostly in aggressive behavior and less in reproductive behavior (Maruska and Fernald, 2010; Huffman et al., 2012a). However, when we blocked the V1a receptor in ascending males, they performed fewer aggressive and more courtship displays than the controls, even though MC did not reduce the overall fraction of males that ascended to dominance successfully. This shift from aggressive to reproductive behavior supports the hypothesis that AVT may affect behavior by regulating specific motivational systems or even specific motor patterns (Thompson and Walton, 2004). Alternatively, AVT might be involved in determining the salience of aggressive and/or sexual stimuli (Goodson, 2008). In either case it seems likely that different AVT sub-systems vary in activity depending on social context and organize different behavioral outputs. Specifically, the fact that the V1a antagonist inhibits aggression and stimulates courtship in ascending males is consistent with the complementary roles of two known AVT sub-systems in teleost fish: on the one hand the gigantococellular pathways, which likely regulate aggression (Greenwood et al., 2008), which might be activated (or disinhibited) by social opportunity; and on the other hand the parvocellular pathways, which appear to control more affiliative behaviors such as schooling and courtship.

What might explain the lack of V1a antagonist effects on stable DOMs and SUBs? It is possible that in *A. burtoni* the role of AVT in aggression is specific to unstable social environments, when males have an opportunity to ascend to dominance and when obtaining information on the social environment significantly affects the chances of staying dominant and eventually reproducing. Alternatively, AVT sub-systems might be particularly susceptible to experimental perturbation in times of social instability and/or during phenotypic transitions (as in ascending males). Interestingly, blocking the AVT/AVP system decreases aggression in males of several other species during the formation of social bonds, but does not seem to affect established bonds (prairie vole, *Microtus ochrogaster*: Winslow et al., 1993; zebrafinch, *Taeniopygia guttata*: Kabelik et al., 2009; convict cichlid, *Amatitlania nigrofasciata*: Oldfield and Hofmann, 2011).

Systemic administration of AVT has been used in numerous other species, including fish, to elicit specific changes in social behavior (bluehead wrasse: Semsar et al., 2001; plainfin midshipman: Goodson and Bass, 2000; pupfish: Lema and Nevitt, 2004; damselfish: Santangelo and Bass, 2006; cleaner wrasse: Soares et al., 2012). It is worth noting that the species of fish that have previously been investigated using systemic administration of

exogenous AVT were mostly marine species, with the exception of zebrafish, where exogenous AVT reduces aggression (Filby et al., 2010) and increases social preference (Braida et al., 2012), and Amargosa pupfish (*Cyprindon nevadensis*), which lives in desert pools that vary greatly in salinity. Lema (2006) demonstrated a tight relationship between aggression, salinity, and AVT phenotype for this species, and suggested that the pupfish AVT system adapted to high salinity first and then later was co-opted to regulate social behavior. It is possible that the osmoregulatory system of a freshwater fish such as *A. burtoni* is more sensitive to systemic manipulation of the AVT system, as AVT/AVP, as anti-diuretic hormone, is also essential to the regulation of water and salt balance in the gill and renal systems (Balment et al., 1993; Warne et al., 2002). Since we cannot separate here peripheral from central effects, it is therefore possible that the socially depressing effects we observed after systemic AVT administration were due to effects on osmoregulation. Future studies on *A. burtoni* and other freshwater fish may benefit from more refined methods, such as intracerebroventricular administration (Thompson and Walton, 2004).

We found that DOMs increased circulating levels of cortisol and lost their territories in response to exogenous AVT. It was unclear from these results whether the central stress response caused the loss of territory, or if losing a territory was inherently stressful. Previous work by Fox et al. (1997) suggested that in *A. burtoni*, plasma cortisol levels increase as a consequence of social defeat, a phenomenon observed across vertebrates (Huhman et al., 1991; Overli et al., 1999). When we examined SUBs treated with AVT, we found that they also displayed a behavioral stress response and had higher plasma cortisol levels relative to saline controls, suggesting that the stress response was indeed largely due to exogenous AVT. However, the difference in cortisol levels between controls and AVT treated-fish was larger in DOMs (~6-fold on average) than SUBs (~2-fold), so the experience of territory loss possibly contributed to the stress response. Alternatively, it is possible that DOMs are more sensitive to stressors than SUBs, so the same stimulus (AVT injection) would elicit a stronger response in DOMs relative to SUBs, which is supported by studies in mammals showing a reduced stress response in subordinate individuals (Blanchard et al., 1995). Interestingly, the stress axis is known to be dependent on AVT, as AVT stimulates release of adrenocorticotropic hormone (ACTH), which in turn induces the release of corticosteroid hormones such as cortisol (Antonii, 1986). Previous studies in teleost fishes have demonstrated that various stressors induce both AVT expression (Gilchriest et al., 2000) and ACTH release (Ruane et al., 1999).

5. Conclusion

We have demonstrated that in the model cichlid *A. burtoni* exogenous AVT, even at very low doses, caused a systemic stress response independent of social status, whereas blocking AVT signaling via a V1a-like pathway did not result in any behavior changes in DOMs and SUBs, contrary to our initial hypothesis that aggressiveness would be higher in DOMs following AVT injections. However, blocking AVT reduced aggressive and increased sexual behavior in ascending males. Further, the expression of both AVT and the V1a2 receptor was increased in ascending males. By investigating males in both stable and unstable social contexts and by quantifying behavior, hormones, and neural gene expression in the brain, we have provided a more detailed understanding of the role of this neuropeptide system in the regulation of social behavior. Clearly, more studies will be necessary to untangle these mechanisms. Regardless, the remarkably dynamic social system of *A. burtoni* provides a great opportunity to gain further insights into how the AVT system might modulate behavior in different social contexts.

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