Intranasal Vasopressin Affects Pair-Bonding And Peripheral Gene Expression In Male *Callicebus Cupreus*

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Abstract

Arginine vasopressin (AVP) is a neuropeptide hormone and neurotransmitter that has peripheral functions in water regulation, and central functions in the stress response and social bonding in male rodents. In this study, we investigated the role of AVP in partner preference behavior in a monogamous primate, the coppery titi monkey (*Callicebus cupreus*). Seven titi males each received three intranasal treatments: saline, low AVP (40 IU), and high AVP (80 IU) in random order, one week apart. They experienced a series of stimulus exposures to their female partner, a female stranger, and an empty cage. Males were more likely to contact the stimulus and do so faster when either female stimulus was present. When pretreated with saline, males contacted the stranger more frequently than their partner; when pretreated with the high dosage of AVP, males contacted their partner more frequently than the stranger. We used microarray to measure peripheral changes in gene expression associated with intranasal AVP and found reduced expression of several genes coding for proinflammatory cytokines. The data presented here suggest that intranasally administered AVP has both central influences on social behavior and peripheral influences on inflammation in a non-human primate.

Keywords

Callicebus cupreus; arginine vasopressin; partner preference; inflammation; intranasal

Introduction

Arginine vasopressin (AVP) is a neuropeptide hormone with both central and peripheral functions (Shalts *et al.* 1992; Engelmann *et al.* 1996; Chickanza & Grossman 1998; Baker *et al.* 2003; Bielsky & Young 2004; Lim *et al.* 2004; Marler *et al.* 2005; Chassin *et al.* 2007; Knepper & Star 2008). Centrally, AVP plays a role in social behaviors such as initiating pair-bonding in male prairie voles (*Microtus ochrogaster*). AVP increases the tendency of male prairie voles to form a selective partner preference following cohabitation (Winslow *et al.* 1993a; Insel & Hulihan 1995; Insel *et al.* 1997; Lim & Young 2004). Additionally, AVP has been associated with social memory and social recognition (Bielsky *et al.* 2005; Dantzer *et al.* 1988; Engelmann & Landgraf 1994; Engelmann *et al.* 1996), affiliative behaviors (Young 2002), paternal behavior (Bamshad *et al.* 1994; Wang *et al.* 2000), and aggression (Bester-Meredith *et al.* 1999; Marler *et al.* 2005).

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While the prairie vole has provided an excellent model to study the formation of rodent social bonds, we do not know whether the mechanisms for these bonds differ in primates. Coppery titi monkeys (*Callicebus cupreus*) are socially monogamous New World monkeys that show preferences for a specific partner, distress upon separation from that partner, and biparental care of offspring (Mason 1966). As such, they provide an ideal model for investigating the affects of AVP on social behavior and peripheral physiological changes in male primates. In order to assess the behavioral effects of centrally administered exogenous AVP, AVP was administered intranasally in order to bypass the blood brain barrier and ensure central uptake noninvasively. Intranasal administration has been shown to result in efficient absorption by the nasal epithelium reaching maximum uptake in approximately 60 minutes (Born *et al.* 2002) and has been used previously in non-human primate studies with the closely related peptide oxytocin (OT; Parker *et al.* 2005; Smith *et al.* 2010). We predicted that exogenous AVP would, in titi monkeys as in rodents, increase the preference a male had for his pair-mate relative to a female stranger.

Central AVP is also involved in the hypothalamic-pituitary-adrenal (HPA) axis stress response. HPA activation in primates begins with perception of a stressor, which results in the release of corticotropin releasing hormone (CRH) from the parvocellular neurons of the paraventricular nucleus (PVN) of the hypothalamus. These same neurons produce and secrete AVP, resulting in simultaneous release of both AVP and CRH. Activation of receptors in the anterior pituitary results in the release of adrenocorticotropic hormone (ACTH) into peripheral circulation. While CRH is capable of stimulating ACTH release independently, it is well established that maximal ACTH release is achieved following coactivation of pituitary receptors by both CRH and AVP (Rabadan-Diehl & Aguilera 1998; Tanoue *et al.* 2004; Lolait *et al.* 2007). Increasing doses of exogenous AVP result in correspondingly increasing ACTH responses (Gillies *et al.* 1982; Turkelson *et al.* 1982). ACTH activates receptors on the adrenal cortices resulting in the release of cortisol into circulation. A greater ACTH response has the potential for a more pronounced cortisol response. Therefore, central AVP concentrations can affect peripheral cortisol concentrations.

Glucocorticoids (GCs) have strong anti-inflammatory properties, and the cytokines that initiate the inflammatory response are influenced by GCs (Elenkov *et al.* 1996; Blotta *et al.* 1997; Dekruyff *et al.* 1998; reviewed in Sapolsky *et al.* 2000 and Calcagni & Elenkov 2006). Central administration of AVP may be expected to cause an anti-inflammatory state via amplified secretion of cortisol. CRH and GCs also modulate pair bonding in prairie voles. In male prairie voles, treatment with CRH or corticosterone facilitates pair bond formation (DeVries *et al.* 1996; DeVries *et al.* 2002). In female prairie voles the opposite pattern has been observed, with lower levels of corticosterone being associated with faster pair bond formation (DeVries *et al.* 1995). The sexually dimorphic nature of the findings in prairie voles may be linked to the neuropeptides associated with pair bonding in male and female voles: AVP and OT, respectively (Young & Wang 2004). As previously mentioned, AVP generally has a stimulatory effect on HPA activity, and OT has a suppressive effect (Gibbs 1986).

In an attempt to capture peripheral changes associated with changes in central AVP, we also investigated peripheral blood mononuclear cell (PBMC) gene expression profiles with and without AVP administration. We predicted that pharmacologically increasing central AVP would result in increased expression of the AVP receptor genes, and the OT receptor gene, which have been implicated in the social behavior of monogamous males (Winslow *et al.* 1993b). We further predicted that direct or indirect (e.g. via cortisol or ACTH activation) peripheral actions of AVP would change gene expression of stress-related loci.

Methods

Subjects

Seven captive-born male coppery titi monkeys (*C. cupreus*) housed at the California National Primate Research Center (CNPRC) were used in this study. All males were sexually mature and had been paired with a sexually mature female for at least one year. None of the males were caring for dependent offspring during the study. Males were 5.5 years of age on average (3.3 to 10.3 years) and were housed in cages (2.13m high × 1.27m wide × 1.27m deep) with their female pair-mate, identical to the housing described by Mendoza (1999). Twice daily (at 0830 and 1300 hrs) animals were fed a diet consisting of monkey chow, cottage cheese, marmoset jelly, apples, raisins, bananas, baby carrots, and vitamins.

AVP Treatment

Each animal received each of three treatments: saline (vehicle control, 300 μl), low dose AVP (40 IU) in 300 μl of saline, and high dose AVP (80 IU) in 300 μl of saline. Doses were based on work by Born and collaborators (2002) indicating what doses would lead to rises in AVP in cerebrospinal fluid. While being manually restrained, treatment was administered intranasally. Treatment solutions were dripped in 50 μl increments into alternate nostrils, and the nostril was then covered immediately to prevent the monkey from expelling the solution. Animals received treatment and were tested once per week for three consecutive weeks, with a single treatment administered on a given test day. Two animals were tested per day, and all seven animals experienced one test session per week. The order of treatments administered was approximately balanced across weeks and subjects.

Behavioral Assessment

Following AVP administration, males were returned to their home-cage with their mate for 20 minutes to allow sufficient uptake prior to beginning observation. During this uptake period animals were video recorded and behaviors involving social interaction with the mate were scored (Table 1a).

Approximately 30 minutes post-administration stimulus presentations began. The partner and female stranger were placed in transport cages and served as the social stimuli, and an empty transport cage was used as a non-social control stimulus. The transport cage dimensions were approximately 0.3m high × 0.3m wide × 0.6m long, constructed of wire mesh that allowed visual, olfactory, auditory, and physical (e.g. fingers through the mesh) contact. Stimuli were presented into the home-cage consecutively for five minutes each, during which time behavioral scans were made every 15 sec. During pilot testing, inter-observer reliability was found to be 0.90. Duration of proximity was measured by the amount of time the male spent within arm's reach of the stimulus cage. Contact was scored as any time the male came into physical contact with the transport cage. All behaviors (Table 1b) were scored using Behavior Tracker 1.5 behavioral analysis software. Throughout the 60-min testing period, each of the three stimuli were presented three times, in random order with a given presentation lasting 5 min.

Blood Collection and Processing

A 3-mL blood sample was collected from the femoral vein after completion of stimuli presentations and two hours after treatment. Whole blood was collected into sterile vacuum-sealed tubes, and was immediately centrifuged at 4°C to separate and extract plasma. Plasma was stored at -80°C until assay. Prior to assay, samples were diluted 1:4 in PBS gel buffer. Plasma concentrations of cortisol were estimated in duplicate using commercial radioimmunoassay kits (Siemens Medical Solutions Diagnostics, Los Angeles, CA). Assay

procedures were modified with the addition of 0.5 and 2.5 µg/dl concentrations of standards along with the provided range of 1.0 to 50 µg/dl. Assay sensitivity has been determined to be 0.26069 µg/dl. Intra- and inter-assay coefficients of variation were 4.85% and 6.11%, respectively. OT and AVP were analyzed by enzyme immunoassay (Assay Designs, Ann Arbor, MI) in assays previously validated for titi monkeys (Bales *et al.* 2005). Intra-assay CV was 2.10% for OT and 3.23% for AVP.

Buffy layer was extracted to isolate PBMC, which were then lysed and RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA). RNA extraction followed protocols and procedures described in the Qiagen RNeasy Mini Handbook (www.qiagen.com).

Gene Expression

Two blood samples per male were used for microarray analysis on a subset of males (n = 4). From each of the four males two samples were assayed, one following saline treatment, and the second following high dose AVP treatment. Microarray analysis was performed with Rhesus Macaque Genome Array (Affymetrix, Santa Clara, CA). Isolated RNA was prepared and labelled following the protocols and procedures described in the Affymetrix Expression Analysis Technical Manual (www.affymetrix.com). Isolated RNA was used to create cDNA through reverse transcription. The cDNA was then used to synthesize biotin-labelled cRNA by *in vitro* transcription (IVT) using the GeneChip array IVT Labelling kit (Affymetrix, Santa Clara, CA). Amplified cRNA was fragmented and hybridized to the arrays according to the manufacturer's procedures (Affymetrix, Santa Clara, CA). The fluorescent signal from each probe on the GeneChip was read by Affymetrix GC3000 Scanner (Affymetrix, Santa Clara, CA). Microarray results were analyzed by robust multiarray analysis (RMA) using ArrayAssist (Iobion Informatics, La Jolla, CA). This software calculates a fold change value, which is a measure of the expression level of the treatment relative to that of the control. In addition, the expression levels of control and treatment were compared by *t* test.

Following microarray analysis several genes were confirmed in their expression levels by using quantitative polymerase chain reaction (qPCR). The genes that were investigated with qPCR are shown in Fig. 1. Manufacturer protocols were followed for qPCR technique (Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide). The TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) contain forward and reverse primers, as well as probes, which are marked with a reporter and quencher dye. An increase in fluorescence signal is detected only if the target sequence is complementary to the probe and is amplified during PCR, thus minimizing nonspecific amplification to non-detectable levels. Cycle thresholds were determined and treatment samples were compared to the individual control sample to which they corresponded. Each additional cycle in the PCR reaction corresponds to a two-fold decrease in starting RNA quantity, so a treatment threshold cycle that is greater than the corresponding control by one cycle represents a two-fold down-regulation of the target gene. Microarray and qPCR expression profiles were significantly correlated across the five genes that were investigated with qPCR ($r_4 = 0.921$, $p = 0.026$; Fig. 1).

Statistical Analysis

Male behavior during the AVP uptake period was analyzed with mixed models (PROC MIXED) using SAS v. 9.2 (SAS Institute, Cary, NC). The mixed effects model was chosen because the data set contains repeated measures for each animal, which violates the default assumption of identical and independent distribution inherent to traditional regression models (i.e. fixed effects models). By using mixed effects models we can describe the relationship between an outcome measure and treatment condition (i.e. fixed-effects) and variation between individuals (i.e. random effects), which is used to adjust the degrees of

freedom. For example, we can determine what differences in tail twining are results of the treatments that animals received as opposed to differences that are a result of variation between individual titi males. The effect of AVP treatment during stimulus presentations was analyzed for two behavioral categories: proximity (latency and duration) and contact (latency, duration, frequency and likelihood (see below for description)).

Statistical analyses of stimulus testing were complicated by the fact that in addition to the treatment parameters and individual differences accounted for in the AVP uptake period models, these models had to incorporate variability due to the different stimuli, each of which were presented three times in random order. Therefore, for these analyses, the model incorporates seven animals, three treatment conditions, and 3 stimuli (presented 3 times each), resulting in 189 data points, or 189 total degrees of freedom. After subtracting the 11 degrees used on estimating fixed parameters, there are 178 degrees of freedom in the F-test. Post-hoc comparisons were made using t-tests and we used the Bonferonni correction to account for multiple comparisons.

To compare latency variables between treatment/stimulus conditions, we used proportional hazards mixed models for which we report a Chi-square and a hazard ratio (Xu 2004). The 'coxme' procedure of the Kinship library within R (R Foundation for Statistical Computing, Vienna, Austria) was used to fit the models. Since latency is defined as the amount of time from the beginning of a given trial to the occurrence of a behavior for our purposes, it is very similar to a survival time measuring the time to "death." Because there were observations censored by the 5-minute trial time (i.e. behavior of interest was not observed before the end of the trial), standard linear regression modelling is difficult to apply. Therefore, a proportional hazard model for "survival times" was applied. Since multiple latency measures were collected on each animal, we added a random effect reflecting the variation between animals, and the proportional hazard model with mixed effects was used.

Proximity duration showed a normal distribution, and was therefore analyzed using a linear mixed model (PROC MIXED in SAS v. 9.2; SAS Institute, Cary, NC) to handle within-individual correlations (Littell *et al.* 1996).

Contact frequency had a zero-inflated distribution (Rodrigues 2006). That is, several of our trials ended without the male contacting the stimulus cage within the 5-minute time period. The possibility exists that had the trial been longer, fewer trials would have ended without contact. Therefore, these data were analyzed using a two-step model created and analyzed in R (R Foundation for Statistical Computing, Vienna, Austria). The first step determined whether or not the male contacted the stimulus. This binary variable was termed "contact likelihood" and is reported as the odds ratio ± 95% confidence interval. When the confidence interval does not include 1.0, it suggests that the estimate is significantly different from what would be expected by chance. A confidence interval less than 1.0 represents a reduction in likelihood, and a confidence interval greater than 1.0 represents an increase in likelihood. The second step of the model included only the subset of trials in which contact was made, and determined whether the contact frequency was predicted by the stimulus presented and/or the treatment administered to the male. This variable was termed "contact frequency" and is reported as median ± 95% confidence interval.

Results

AVP Uptake Observations

The only behavior that showed a significant response to AVP treatment during the uptake period (first 20 min post treatment) was rubbing the face ($F_{2,12} = 4.19$, $p = 0.04$), which increased when males received AVP (Mean ± SEM Saline: 2.86 ± 1.12 seconds (s); Low

AVP: 8.14 ± 2.63 s; High AVP: 6.86 ± 1.04 s). Males administered the high dose of AVP showed a non-significant trend towards more time with their tails twined with their pair-mate ($F_{2,12} = 3.04$, p = 0.09; Mean $\pm$ SEM Saline: 108.29 ± 54.65 s; Low AVP: 112.14 ± 111.88 s; High AVP: 259.57 ± 133.08 s). Contact duration with partner, male and female approach and leave during the uptake period were not significantly affected by AVP treatment (all p > 0.25).

Stimulus Testing – Latency of proximity to stimulus cage

Female behavior in the stimulus cages was taken into account by recording the number of times she grabbed at the male when he approached. Females showed no trend for grabbing, either more or less, with respect to the treatment the males received or to the stimulus type they represented (partner or stranger).

The latency to approach the stimulus cage was predicted by stimulus, but not by treatment (Fig. 2; stimulus $X^2=41.18$, df=2, p<0.0001; treatment $X^2=3.00$, df=2, p=0.22), and the interaction was not significant. Post-hoc analysis of fixed effects of stimulus revealed that males were faster to establish proximity with the stimulus cage when either the female partner or the stranger female were present than they were when the stimulus cage was empty (partner $X^2=9.10$, df=1, p=0.0026; stranger $X^2 =38.96$, df=1, p<0.0001).

Stimulus Testing – Duration of proximity

Test of fixed effects showed both treatment and stimulus to be significant predictors of the duration of proximity to the stimulus cage (Fig. 3; treatment $F_{2,178} = 6.74$, p = 0.0015; stimulus $F_{2,178} = 7.74$, p = 0.0006). Post-hoc analysis of fixed effects of treatment showed that proximity duration was not significantly different when animals were given high dose AVP as compared to saline ($t_{178} = -0.59$, p > 0.5); however, proximity duration was significantly lower when males were administered low dose AVP (Fig. 3a; $t_{178} = -3.43$, p = 0.0007). Post-hoc analysis of fixed effects of stimulus showed that proximity duration was significantly lower when the empty cage was presented than when either the stranger or partner female was presented (Fig. 3b; $t_{178} = -3.24$, p = 0.0014). There was no difference in proximity duration between the partner and stranger female presentations ($t_{178} = 0.31$, p > 0.5).

Stimulus Testing – Latency of contact with the stimulus

Contact latency was predicted by stimulus, but not by treatment (Fig. 4; stimulus $X^2=40.52$, df=2, p<0.0001; treatment $X^2=2.57$, df=2, p=0.28). Post-hoc analysis of fixed effects of stimulus showed that both partner and stranger presence significantly reduced the latency to contact the stimulus cage, when compared to the empty cage (partner $X^2=9.04$, df=1, p=0.0026; stranger $X^2=38.39$, df=1, p<0.0001).

Stimulus Testing – Likelihood to contact the stimulus cage

Likelihood to contact the stimulus cage was greater when the stranger female (Fig. 5a; OR=4.90, 95%CI: [1.57, 19.41]) was presented, as compared to an empty cage. There was no difference in contact likelihood between the partner and the empty cage (OR=1.10, 95%CI: [0.40, 3.09]). Treatment did not significantly affect contact likelihood (Fig. 5b).

Stimulus Testing – Frequency of contact with the stimulus

Males showed increased contact frequency toward either the partner (Fig. 5c; median difference=2.27, 95%CI: [1.39, 3.66]) or the female stranger (median difference=1.82, 95% CI: [1.15, 2.86]) as compared to an empty cage when all treatment conditions were combined. There was no overall effect of treatment (Fig. 5d). However, when saline was

administered, males showed higher contact frequency towards the stranger as compared to the partner (Fig. 6; OR=0.221, 95%CI: [0.044, 0.889]), while with low dose AVP there was no difference in the frequency of contact between the partner or stranger (OR=0.331, 95%CI: [0.080, 0.889]), and with high dose AVP, increased contact frequency was directed toward the partner over the female stranger ([OR=15.625, 95%CI: [1.887, 468.604]).

Hormone Analysis

Cortisol did not show a differential response to treatment. Plasma cortisol concentrations when given saline, low AVP, and high AVP were 25.9 ± 3.7 µg/dl, 25.3 ± 2.1 µg/dl, and 30.8 ± 8.0 µg/dl, respectively (mean $\pm$ SE). Neither plasma OT nor AVP showed a response to AVP treatment at the time of blood collection. Plasma OT concentrations were 82.6 ± 42.4 pg/ml, 100.9 ± 59.2 pg/ml, and 94.6 ± 54.6 pg/ml when given saline, low AVP, and high AVP, respectively. Plasma AVP concentrations were 200.2 ± 54.7 pg/ml, 134.2 ± 23.7 pg/ml, and 162.9 ± 22.3 pg/ml when given saline, low AVP, and high AVP, respectively.

Gene Expression Analysis

Gene expression was analyzed first by pooling the results from all four individuals and analyzing by treatment (Table 2a). 372 out of 40,000 genes were identified as showing significantly different expression levels after the AVP treatment relative to the controls. Criteria for being significantly different due to treatment included a fold change of at least 1.5 (50% change in expression level), and fulfilling the 0.05 alpha level. Out of the 372 genes showing a change in expression levels, 267 were down-regulated and 105 were up-regulated. We did not see significant changes in any of the genes that we predicted to be affected by AVP administration; neither the AVP receptor genes nor the OT receptor gene showed changes in expression levels. Among the genes that showed significant changes in expression were 24 inflammatory response relevant genes (Table 2a). Inflammatory genes were significantly more likely to be down-regulated than up-regulated (X^2=13.5, df=23, p<0.001).

Gene expression was then analyzed for consistency across individuals. Genes that showed consistent significant changes in expression in at least 3 of the 4 males are shown in Table 2b. Eight inflammatory response relevant genes showed consistent differences in regulation across individuals and were all down-regulated when AVP was administered (X^2=8.00, df=7, p<0.005).

Discussion

Male titi monkeys showed an ability to distinguish between social and non-social stimuli in all of the behavioral measures we recorded: they contacted the transport cage more frequently, spent more time in proximity to the cage, and took less time to approach and contact the cage when social stimuli were present. Furthermore, they showed significantly different behavioral responses to the two social stimuli. When given saline, males contacted the stranger female more frequently than the partner, but when given high dose AVP they contacted their partner more frequently than the stranger, thus supporting our prediction that AVP would increase affiliative behavior directed to the partner. It is very common for monogamous male mammals (Williams *et al.* 1992; Smith *et al.* 2010), as it is for male mammals in general, to investigate an unfamiliar female for a longer period or prior to a familiar female (Bermant *et al.* 1968; Crawley 2007); so this result was not surprising. While we believe that central AVP is directly responsible for the observed behavioral changes, there remains the possibility that AVP augmented the peripheral stress response (although we did not observe a change at the time-point we chose, see below). This physiological response may have then altered brain functioning and behavior. Previous work

in prairie voles has shown that acute stress, including administration of peripheral corticosterone, results in altered social bonding (DeVries *et al.* 1996). Future work will investigate the mechanisms driving the behavioral changes observed in this study.

An unexpected finding is that low dose (but not high dose) AVP resulted in decreased proximity to all stimuli, both social and non-social. While this contradicts our predictions, previous work has suggested that at some dosages AVP may be anxiogenic (Landgraf *et al.* 1995; Griebel *et al.* 2002; Bielsky *et al.* 2004). Some peptides exert their behavioral effects in an inverted u-shaped dose response fashion, and it is possible that AVP could result in increased anxiety and decreased willingness to approach the stimulus at one dose and not another.

The hormone analyses showed no difference in response to treatment. It is possible the stress response was activated by the handling and administration of AVP but returned to baseline by the time the blood samples were taken (2 hours post-administration). The lack of significant differences in OT and AVP two hours following administration is not surprising considering the short half-life of these peptide hormones (Share 1962; Rydén & Sjöholm 1969; Janáky *et al.* 1982). While cortisol has a longer half-life than OT and AVP (Kerrigan *et al.* 1993), it too would be expected to return to basal levels within two hours. The gene expression data provide evidence of a possible change in peripheral hormone concentrations that occurred before the blood samples were collected. The down-regulation of pro-inflammatory cytokines suggests a change in the inflammatory state. Cortisol is a known mediator of cytokine expression, however, the mechanism for the observed changes in gene expression in this study remains unclear.

While the blood collection time was not ideal for hormone analyses, two hours following a stressor is sufficient time to observe changes in inflammatory gene expression (Breen *et al.* 2000; Louis *et al.* 2007), and we found changes in the expression of several inflammatory genes following AVP administration. The proteins corresponding to the down-regulated genes include interleukin (IL)-1β, IL-7, IL-13, IL-15, and IL-16. IL-1β is well known for its role in inflammatory processes, and is suppressed by glucocorticoids (reviewed in Sapolsky *et al.* 2000). IL-7 is capable of expanding lymphocyte populations, leading to increases in both the B and T cell populations (Hofmeister *et al.* 1999), leading to humoral and cell-mediated immune response, respectively. IL-13 is secreted primarily by Th2 cells, and is an important mediator of allergic inflammation (Wills-Karp *et al.* 1998). IL-15 and IL-16 are critical to the induction of inflammatory processes (Center *et al.* 2000), and IL-15 is elevated during chronic inflammation. Taken together, reduced activity of these genes suggests a suppression of inflammatory processes. Therefore, manipulation of central AVP appears to have affected peripheral inflammatory state. One possible explanation is that intranasally administered exogenous AVP could have co-activated pituitary receptors with CRH and resulted in an increased adrenocortical response. Acutely increased peripheral cortisol concentrations would exert anti-inflammatory effects, resulting in decreased gene expression of genes coding for pro-inflammatory cytokines in any tissues containing glucocorticoid receptors. PBMC have glucocorticoid receptors (Lippman & Barr 1977), and are responsive to changes in glucocorticoid levels (Adcock *et al.* 1995).

An alternative explanation for the changes in gene expression is that they were caused by the altered social behavior. Growing evidence supports a role for social condition affecting inflammatory state (reviewed in Uchino *et al.* 1996; Kiecolt-Glaser 1999; Klein & Nelson 1999; DeVries *et al.* 2003). This idea predicts that individuals with better social support experience an anti-inflammatory state, while those without social support experience chronic inflammation. Chronic inflammation is currently known to be associated with several chronic health problems (e.g. cardiovascular disease, stroke, viral-mediated cancer). The

results of this study, therefore, warrant further investigation of AVP as a possible mechanism mediating chronic inflammation and its downstream consequences.

There are a number of ways in which future studies, with different design parameters, could add to the information gained from this study. For example, in this study we administered treatments once per week. We chose this timing because we believed that it would allow more than enough time for the subjects to return to pre-treatment conditions. Based on the clearance rate of AVP, it is safe to assume that no exogenous AVP remained from a given treatment at the time of the second treatment a week later (Share 1962; Rydén & Sjöholm 1969; Janáky *et al.* 1982). However, we can not rule out the possibility that the physiological cascade of effects initiated by exogenous AVP administration remained beyond one week. A second choice we made with our behavioral assessment was to present stimuli sequentially rather than simultaneously. This allowed us to assess behavior in the subjects' home cages rather than in a novel apparatus, which had advantages because these animals are strongly neophobic (Fragaszy & Mason 1978; Hennessy *et al.* 1995). However, a simultaneous choice would have been closer to the model used in rodent research. A third design choice was that all three stimuli were presented during the same 60-minute period. While this was based on designs used in previous titi monkey studies (Mendoza & Mason 1986; Fernandez-Duque *et al.* 1997), it is possible that this testing method yielded different results than would be seen had each stimulus been presented on separate days.

In summary, intranasal AVP appeared to alter both central and peripheral physiology, resulting in both changed behavior and peripheral gene expression. Male titi social behavior changed in the predicted direction, as males contacted their female pair-mates more frequently when given high dose AVP. Pro-inflammatory cytokine gene expression was reduced in the periphery two hours after treatment with high dose AVP. Future work will further investigate the directionality of relationships between AVP, social behavior, and gene expression of pro-inflammatory cytokines.

Acknowledgments

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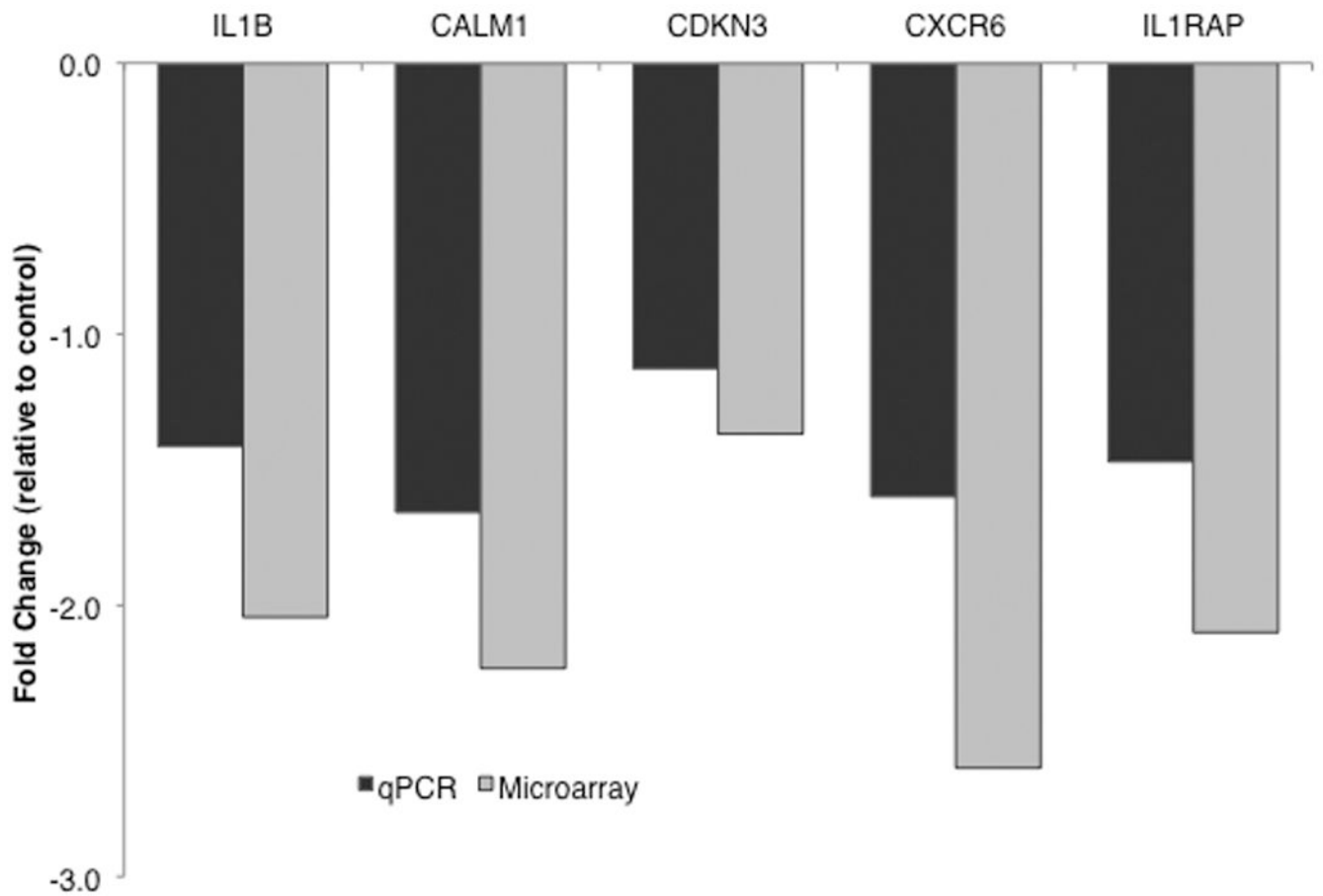
FIGURE 1.
Gene expression as evaluated by microarray and qPCR. Fold change values represent difference in gene expression with AVP treatment compared to expression with saline treatment (r_4=0.921, p=0.026). Negative values represent down-regulation of gene expression. IL1B = interleukin 1 beta, CALM1 = calmodulin 1, CDKN3 = cyclin-dependent kinase inhibitor 3, CXCR6 = C-X-C chemokine receptor type 6, IL1RAP = interleukin 1 receptor accessory protein.
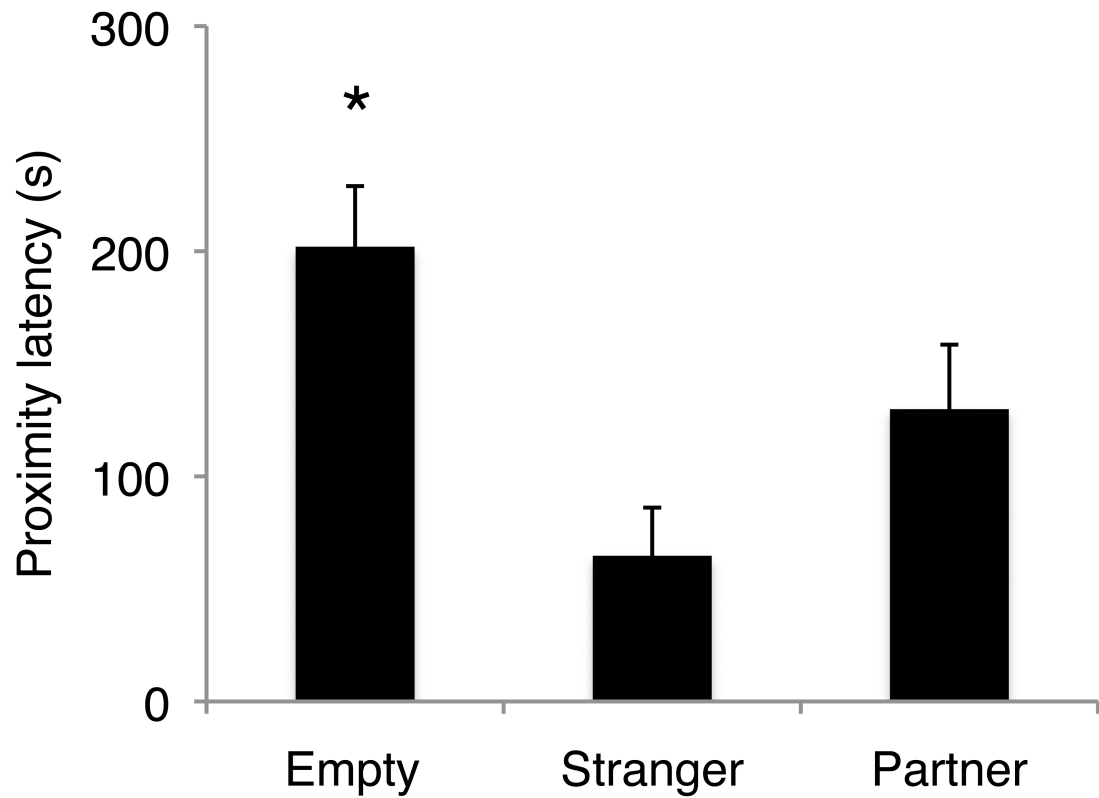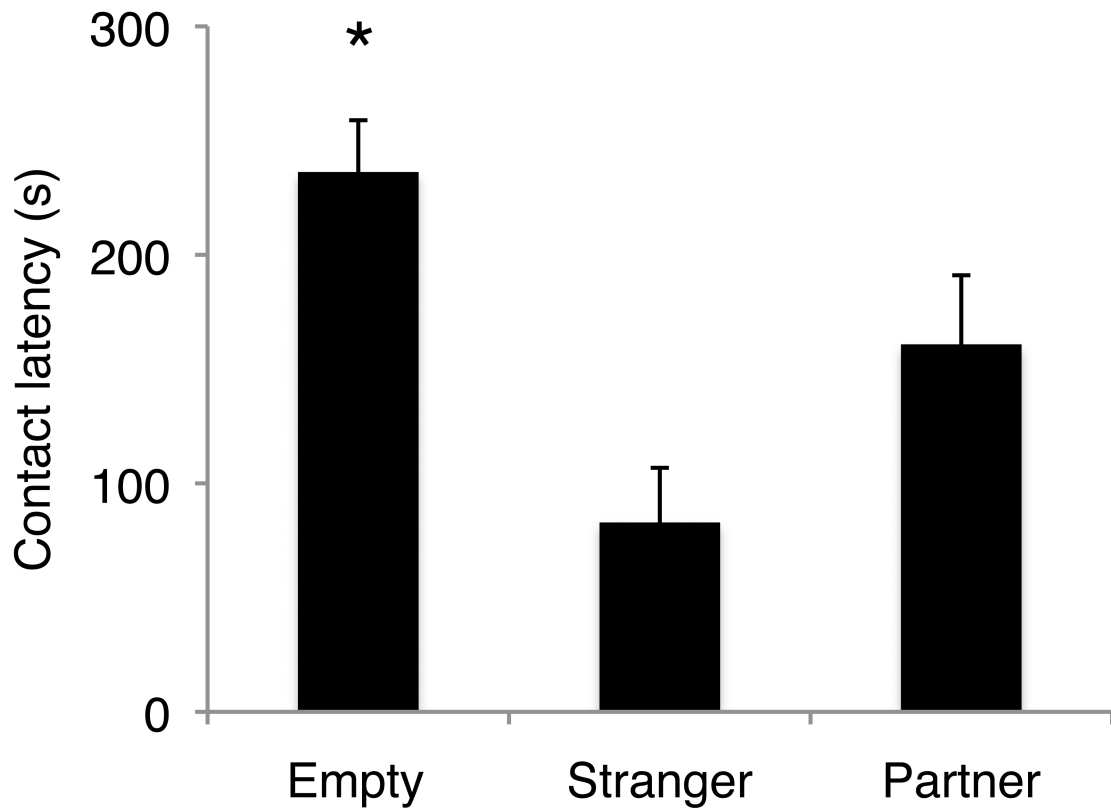
FIGURE 2.
Stimulus type influences latency to approach in male titis. Stimulus $X^2=41.18$, df=2, p<0.0001. Males approached their partner ($X^2=9.10$, df=1, p=0.0026) and the stranger female ($X^2=38.96$, df=1, p<0.0001) faster than the empty cage. Bars represent mean latency to approach ± SEM.
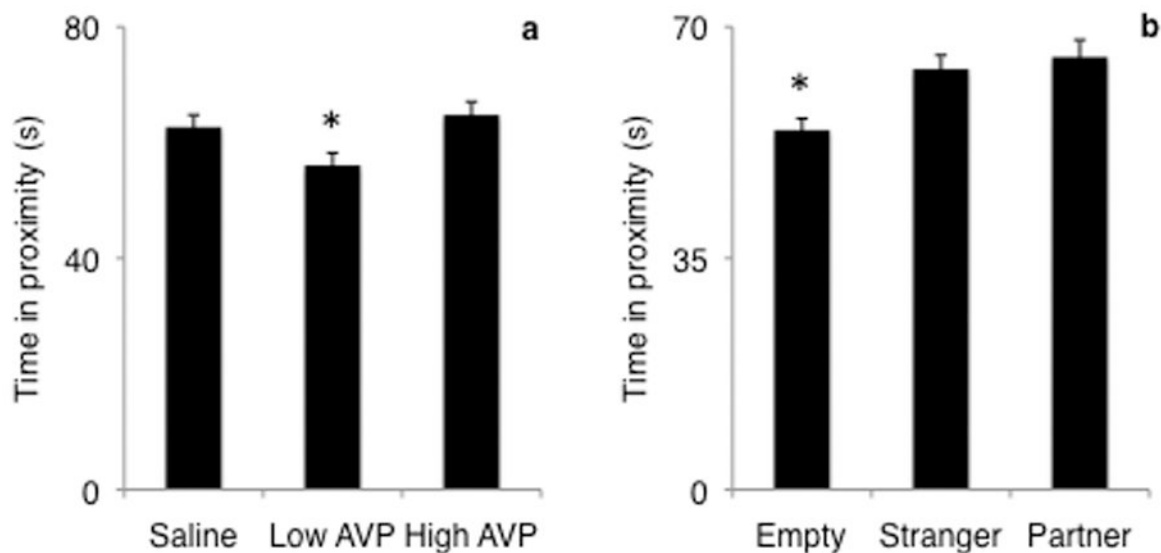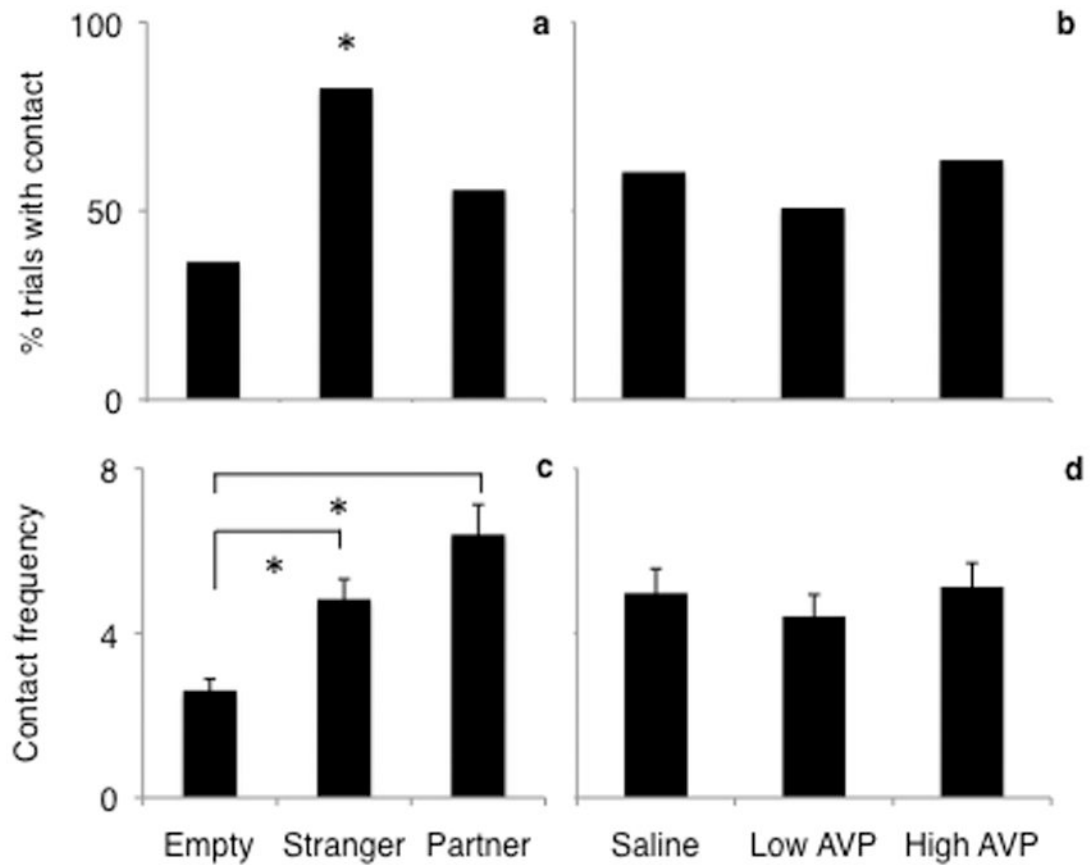
FIGURE 3.
Effect of AVP treatment (a) and stimulus (b) on proximity duration. Male titis were administered varying doses of AVP and the amount of time that they spent in proximity to the stimulus cage was recorded. Both treatment ($F_{2,178}=6.74$, $p=0.0015$) and stimulus ($F_{2,178}=7.74$, $p=0.0006$) predicted proximity duration. Proximity was greater when low dose AVP was administered, but not when high dose AVP was administered. Proximity was greater when either the partner or the stranger female were presented. Bars represent mean proximity duration ± SEM. * = $p < 0.01$.
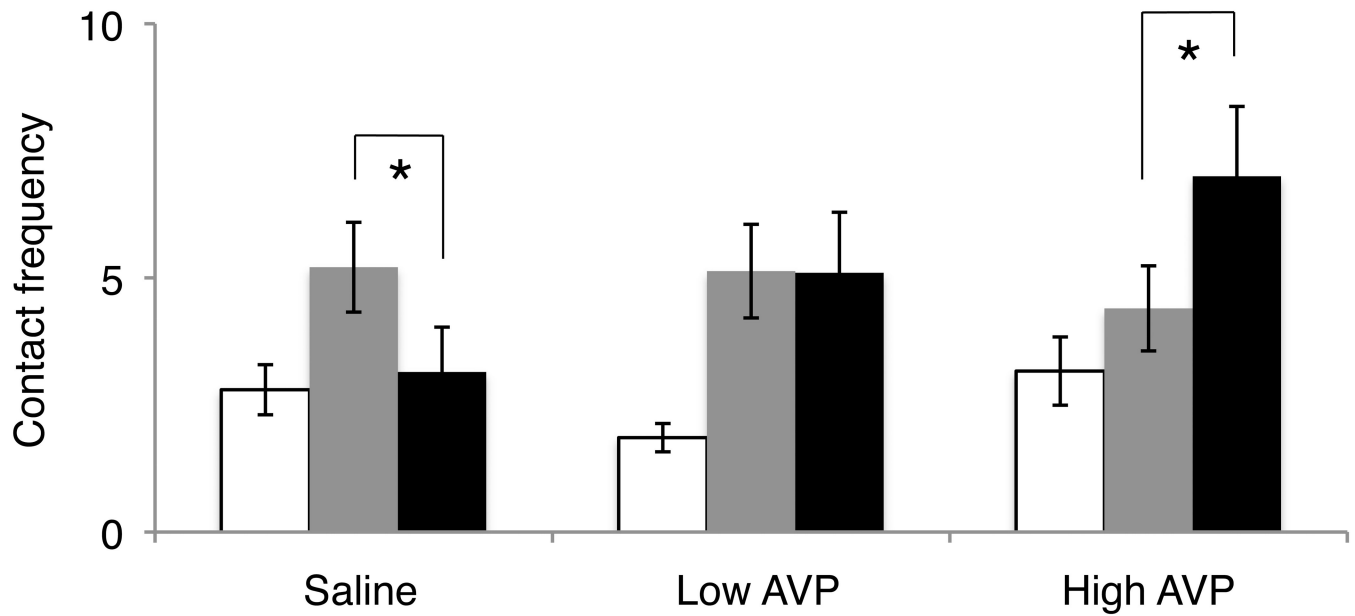
FIGURE 4.
Effect of stimulus on latency to contact the stimulus cage. Males contacted the stimulus cage faster when either their partner or the stranger female were presented as compared to an empty stimulus cage. Bars represent mean ± SEM. * = p < 0.05.

FIGURE 5.
Effect of stimulus (a, c) and AVP treatment (b, d) on contact likelihood (a, b) and contact frequency (c, d). Likelihood to contact the stimulus cage was greater when the stranger female was presented. Males showed increased contact frequency towards either the partner or the female stranger when all treatment conditions were combined. Contact likelihood is presented as the percent of all trials during which contact occurred. Contact frequency is presented as the mean $\pm$ SEM. * = p < 0.05.

FIGURE 6.
The combined effects of AVP treatment and stimulus on contact frequency. When males were treated with saline (control) they contacted the stranger female cage more frequently, however treatment with the high dose AVP was associated with more frequent contact of the partner cage. Bars represent mean ± SEM. * = p < 0.05.

Table 1

TABLE 1a. AVP uptake period ethogram.

Behavior	Description	Measure
Contact	Male in physical contact with female	Duration
Tail twine	Male in contact with female and tails are entwined	Duration
Male approach	Male moves towards female and establishes contact	Frequency
Male leave	Male breaks contact with female	Frequency
Female approach	Female moves towards male and establishes contact	Frequency
Female leave	Female breaks contact with male	Frequency
Vocalize	Male engages in calling behavior, usually with female	Frequency
Chest rub	Male rubs chest or armpits with forearms	Frequency
Face rub/sneeze	Male rubs face with hands or on cage, or sneezes	Frequency
Groom	Male grooms female	Frequency
Autogroom	Male grooms himself	Frequency
Lipsmack	Male smacks lips toward female	Frequency
Arch	Male arches back while walking	Frequency
Tail lash	Male wags tail, usually a sign of agitation	Frequency

TABLE 1b. Stimulus testing behavioral categories

Behavior	Description	Measure
Proximity	Male comes within arm's reach of transport cage	Duration
Contact frequency	Male contacts transport cage	Frequency
Proximity latency	Time between start of trial and time when male comes into proximity with transport cage	Latency
Contact latency	Time between start of trial and time when male contacts transport cage	Latency

Table 2

TABLE 2a. Genes showing significant changes in expression associated with AVP treatment.

Gene	Fold change	Function
CASP1	-3.40	Cleave and activate IL1
CST7	-2.50	Immune response
LILRB1	-2.25	Immune response
SPA17	-1.68	Immune cell migration and metastasis
LTB	1.56	Immune response
IL13	-1.43	Interleukin 13
IL7	-1.42	Interleukin 7
CEACAM8	-1.37	Immune response
DEFA5	-1.36	Antimicrobial humoral response
CXCL16	-1.36	Chemotaxis, immune response
IL10B	-1.31	Interleukin 10 beta
MHC2TA	-1.30	Parasite perception, immune response

TABLE 2b. Genes showing consistent and significant changes in expression associated with AVP treatment in at least 3 of the 4 males.

Gene	Fold change	Function
IGJ	-2.77	Immunoglobulin J chain, activation of B lymphocytes
IGK	-2.66	Immunoglobulin K chain
IGHA1	-2.51	Immunoglobulin heavy constant alpha 1
IL1B	-2.40	Interleukin 1 beta
IL16	-2.20	Interleukin 16, chemoattractant factor
NFIL3	-1.90	Nuclear factor, interleukin 3
CRHR1	-1.86	Corticotropin releasing hormone receptor 1
CD94	-1.86	Antigen preferentially expressed on natural killer cells
TNFα-P6	-1.64	Tumor necrosis factor alpha-induced protein 6
IL10RA	-1.64	Interleukin 10 receptor activity
TNFSF13B	-1.62	Tumor necrosis factor receptor binding, B cell activation and differentiation
IL6ST	-1.60	Interleukin 6 signal transducer
IL15	-1.46	Interleukin 15, T and natural killer cell activation
A2M	-1.44	Cytokine transporter