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Associations between Oxytocin Receptor Gene (*OXTR*) Methylation, Plasma Oxytocin, and Attachment Across Adulthood

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Abstract

The neuropeptide oxytocin (OT) has been implicated in a wide range of affiliative processes. OT exerts its functions via OT receptors, which are encoded by the oxytocin receptor gene (*OXTR*). Epigenetic modification of *OXTR* through the process of DNA methylation has been associated with individual differences in behavioral phenotypes. Specifically, lower levels of *OXTR* methylation have been linked to better social and affective functioning. However, research on epigenetic mechanisms of *OXTR* is scarce in non-clinical populations, and even less is known about epigenetic variability across adulthood. The present study assessed methylation levels at *OXTR* CpG site -934 and plasma OT levels in 22 young (20–31 years, $M = 23.6$) and 34 older (63–80 years, $M = 71.4$) participants. Lower levels of *OXTR* methylation and higher plasma OT levels were associated with less self-reported attachment anxiety in young but not older participants, with largely independent contributions of *OXTR* methylation and plasma OT levels. In contrast, in the overall sample, lower levels of *OXTR* methylation were associated with higher self-reported attachment avoidance. Age analysis suggested that these results were largely driven by young adults. Plasma OT levels were unrelated to attachment avoidance. Taken together, these findings support the emerging notion in the literature that epigenetic properties of *OXTR*, in

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addition to endogenous OT levels, are related to adult attachment. Further, the age effects observed in the associations between *OXTR* methylation, plasma OT, and adult attachment emphasize the importance of adopting a developmental perspective when studying properties of the OT system and their relation to affiliative processes. Findings contribute to growing evidence suggesting that epigenetic modification of genes regulating OT pathways and endogenous OT levels are associated with the way people form and maintain intimate social relationships.

Keywords

Aging; *OXTR*; DNA Methylation; Plasma OT; Attachment

1. Introduction

1.1 Attachment in Adulthood

Attachment models, such as the style in which individuals approach a caregiver or a partner, aim to understand relationships among people. Adult attachment has been shown to play a key role in mental health and well-being (Karreman & Vingerhoets, 2012). Compared to individuals with secure attachment (i.e., a stable and responsive approach to other people; Bowlby, 1980), individuals with insecure attachment (i.e., a lack of security in their approach to other people) scored lower on various measures of well-being and showed associations with depression, anxiety, and worse physical health (Fagundes, Jaremka, Malarkey, & Kiecolt-Glaser, 2014; Marganska, Gallagher, & Miranda, 2013; McWilliams & Bailey, 2010; Meredith, Strong, Ford, & Branjerdporn, 2015; Palitsky, Mota Afifi, Downs, & Sareen 2013).

A common understanding of adult attachment concerns the dimensions of anxiety and avoidance. These two dimensions are commonly measured in self-report, for example with the Experiences in Close Relationship Scale (ECR; Brennan, Clark, & Shaver, 1998) or with its more recent shortened version, the Experiences in Close Relationship Scale-Short Form (ECR-S; Wei, Russell, Mallinckrodt, & Vogel, 2007). *Attachment anxiety* is the vigilance of social rejection and distress when a partner is unavailable. For example, adults with attachment anxiety tend to respond to separation periods from their partner with more feelings of insecurity than securely attached adults (Feeney, 2008). *Attachment avoidance* is the discomfort with closeness, usually a fear of intimacy and disclosure (Brennan, Clark, & Shaver, 1998). Attachment avoidance not only can lead to a self-fulfilling prophecy of expecting relationships to fail (Birnie, McClure, Lydon, & Holmberg, 2009), but also to beliefs that partners cannot be relied upon or trusted (Shaver & Mikulincer, 2005).

Beyond early life experiences, a number of factors have been associated with attachment orientations in adulthood. For example, adults in romantic relationships typically report less attachment anxiety and avoidance than single individuals (Edelstein & Gillath, 2008). Further, sociodemographic factors such as race, age, and economic status have been shown to be associated with adult attachment. For example, individuals classified as securely attached were more likely to be white, older, and financially well-off (Mickelson, Kessler, & Shaver, 1997).

Regarding adult age differences, previous studies showed that attachment anxiety was lower among older than young adults (Chopik, Edelstein, & Fraley, 2013; but see Kafetsios & Sideridis, 2006). These findings are consistent with general developmental theories of older adults as seeking relatively more positive, closer relationships and being more adept socially (Carstensen, 2006; Luong, Charles, & Fingerman, 2011). Also, relatively higher attachment anxiety in young adults may be due to the ongoing changes in social relationships in that stage of life as young adults shift attachment from parents to peers (Fraley & Davis, 1997; Fraley, Roisman, Booth-LaForce, Owen, & Holland, 2013; Nickerson & Nagle, 2005).

Further evidence suggests that biological factors (e.g., neuropeptide or gonadal hormone levels) contribute to attachment in adulthood. In particular, as summarized next, the neuropeptide oxytocin (OT) has been discussed in the context of secure attachment in adulthood characterized by low anxiety and low avoidance (Buchheim et al., 2009; Costa et al., 2009; Donaldson & Young, 2008; Gordon et al., 2008; Macdonald, 2013; Samuel et al., 2015; Uvnäs-Moberg, 1998).

1.2 Role of Oxytocin in Adult Attachment

OT is a nine-amino acid neuropeptide that has been implicated in a number of physiological functions such as childbirth and lactation (for reviews, see Carter, 2014; Gimpl & Fahrenholz, 2001; Meyer-Lindenberg, Domes, Kirsch & Heinrichs, 2011). In addition, a growing body of evidence supports a role of OT in affiliative processes, both in humans and non-human animals (Buchheim et al., 2009; Carter, 2014; Insel & Shapiro, 1992; Macdonald, 2013; Samuel et al., 2015; Young, Lim, Gingrich, & Insel, 2001; Young & Wang, 2004). This includes anxiety-related behavior (Blume et al., 2008; Ring et al., 2006; Windle, Shanks, Lightman, & Ingram, 1997), maternal care (Pedersen & Prange, 1979), sexual behavior (Argiolas & Melis, 2004; Carmichael, Humbert, Dixen, Palmisano, Greenleaf, & Davidson, 1987), pair bonding (Insel & Shapiro, 1992), and social cognition (Dluzen, Muraoka, Engelmann, & Landgraf, 1998; Kirsch, et al., 2005; for a review see Neumann, 2008).

In particular, single-dose administration of intranasal OT in humans has been shown to enhance perceptions of attachment security in insecurely attached adults (Buchheim et al., 2009). There is also evidence that intranasal OT increased cooperative behavior (Kollock, 1998) in intimacy-seekers (who are anxiously attached, but low in avoidance), while it hindered cooperative behavior in “fearful” individuals (who are anxiously attached, but high in avoidance; Bartz et al., 2011). This supports the notion that individuals’ response to intranasal OT depends, at least to some degree, on their attachment level. It also suggests differential effects of OT for attachment anxiety vs. attachment avoidance.

Using fMRI techniques, a study found that men who received intranasal OT rated their female partner as more attractive than other women, and this enhanced positive partner bias was corroborated with increased neural activation in the nucleus accumbens (Scheele et al., 2013). A recent study further examined chronic (two-week) administration of intranasal OT on attachment in young healthy men and found reduced self-reported attachment avoidance and increased attachment toward peers in the OT compared to the placebo group (Bernaerts et al., 2017). These effects were particularly pronounced for individuals who had reported

less secure attachment toward their peers at baseline, further supporting the role of OT on affiliative processes, as a function of previous attachment-related experiences.

OT's functions are mediated through OT receptors (Breton & Zingg, 1997), which are located peripherally and centrally (Bethlehem et al., 2013; Gimpl & Fahrenholz, 2001). OT receptors are encoded by the oxytocin receptor gene (*OXTR*; Kimura, Tanizawa, Mori, Brownstein, & Okayama, 1992), one of the genes that influences the OT signaling pathway. Research in non-human mammals showed differential patterns of OT receptor expression based on social affiliation. In particular, areas of the brain where OT receptor density was highest in the monogamous prairie vole showed little receptor binding in the polygamous montane vole (Insel & Shapiro, 1992). Further, human brain areas rich in OT receptors (e.g., substantia nigra, globus pallidus, nucleus of Meyner; Loup, Tribollet, Dubois-Dauphin, & Dreifuss, 1991) were activated in response to scenarios of romantic attachment (i.e., viewing the face of a romantic partner vs. an acquaintance; Bartels & Zeki, 2000, 2004), highlighting the brain mechanisms underlying OT's role in affiliative processes.

OXTR undergoes epigenetic modification through the process of DNA methylation. DNA methylation commonly regulates gene expression by influencing the recruitment and binding of regulatory proteins to DNA (i.e., a mechanism in which cells regulate gene transcription). DNA methylation occurs in the context of 5'-Cytosine-phosphate-Guanine-3' (CpG) dinucleotide pairs and is a highly investigated epigenetic modification that may influence behavioral phenotypes. There is evidence that DNA methylation within the promoter region of *OXTR* is variable within the population (Jack, Connelley, & Morris, 2012; Puglia, Lillard, Morris, & Connelly, 2015) and methylation of specific *OXTR* CpG sites reduces transcription of the gene (Kusui et al., 2001).

In particular, *OXTR* methylation at CpG site -934 has been implicated in human qualities, such as social sensitivity (Jack et al., 2012; Puglia et al., 2015), where higher levels of methylation lead to more effortful processing and in some cases association with psychiatric disorders (Bell et al., 2014; Dadds et al., 2014; Gregory et al., 2009). There is evidence that methylation represses expression of *OXTR* (Gregory et al., 2009; Kusui et al., 2001), which may underlie psychopathology and aberrant affiliative functioning, in particular disorders of social behavior (Cappi et al., 2016; Cecil et al., 2014; Dadds et al., 2014; Gregory et al., 2009; Kim, Kim, Kim, & Treasure, 2014; Rubin et al., 2016; Ziegler et al., 2015) and emotion regulation, like depression (Bell et al., 2014; Chagnon, Potvin, Hudon, & Prévile, 2015; Kimmel et al., 2016; Reiner et al., 2015; Simons et al., 2016). In *OXTR*, a region of the gene called 'MT2' that falls within the promoter has been shown to regulate expression of the gene in a DNA methylation dependent manner (Kusui et al., 2001). In particular, several studies implicate CpG site -934 in the MT2 region and support the hypothesis that epigenetic variation that decreases expression of *OXTR* may increase individual susceptibility to affective disorders (Bell et al., 2014; Cecil et al., 2014; Dadds et al., 2014; Gregory et al., 2009; Kim et al., 2014; Rubin et al., 2016; Slattery & Neumann, 2010).

Specifically comparing individuals with autism to controls showed elevated levels of *OXTR* methylation in autistic individuals (Gregory et al., 2009), supporting a role of higher *OXTR* methylation levels in social and affective psychopathology. Taking a developmental

approach, Dadds et al. (2014) documented an association between greater *OXTR* methylation in young and older boys with high callous-unemotional traits (lack of remorse, disregard for others' feelings) compared with low callous-unemotional traits, with this pattern of findings only present in the older but not in the younger boys. They further found that higher *OXTR* methylation levels were associated with lower plasma OT levels in boys aged 9–16 years (i.e., the older boys). When looking at their total sample of young and older boys, however, they did not find an association between plasma OT levels and *OXTR* methylation levels (see also Rubin et al., 2016, for evidence of no association between *OXTR* methylation and plasma OT levels).

Of note, the majority of current research involving *OXTR* methylation in humans has focused on clinical populations (Cappi et al., 2016; Gregory et al., 2009; Kim et al., 2014; Rubin et al., 2016). The little evidence that exists on the relationship between *OXTR* methylation and behavioral phenotypes in healthy samples parallels findings in clinical groups. In particular, higher levels of *OXTR* methylation were associated with increased activity in the dorsal anterior cingulate cortex (dACC) and temporal parietal junction (TPJ) as participants engaged in watching animation in which geometric figures showed a pattern of socially meaningful vs. random motion (Jack, Connelly, & Morris, 2012). Greater activation in response to socially relevant information observed in these regions from individuals with higher levels of *OXTR* methylation may reflect greater difficulty in processing this information. Further, individuals with lower levels of *OXTR* methylation showed enhanced emotion-regulation ability as measured by decreased left amygdala activity while viewing face stimuli with negative expressions (Puglia et al., 2015). These studies underscore the idea that epigenetic regulation of *OXTR* may play an important and dynamic role in social behavior, both in healthy subjects and clinical groups, with the previous literature generally suggesting low *OXTR* methylation levels being associated with better social outcomes and high *OXTR* methylation levels with worse social outcomes. However, specific associations between *OXTR* methylation and adult attachment have not been determined yet. Further, currently still close to nothing is known about adult age differences in these associations. Finally, determination of respective contributions of *OXTR* methylation and endogenous OT levels in their impact on adult attachment has not been undertaken yet. The present study set out to fill these gaps.

1.3 Age Variations in the Associations between *OXTR* Methylation and Plasma OT Levels and Attachment

There is a growing body of evidence suggesting developmental change and effects of aging on the OT system (Arsenijevic, Dreifuss, Vallet, Marguerat, & Tribollet, 1995; Barraza et al., 2013; Campbell, Ruffman, Murray, & Glue, 2014; Ebner et al., 2013, 2015, 2016; Keck et al., 2000; Melis, Mauri, & Argiolas, 1995; Parker, Hoffman, Hyde, Cummings, & Maestripieri, 2010). In particular, age-by-sex interactions in level and function of the OT system have been observed. For example, OT relative to placebo improved emotion recognition in older males, but not in older females or young adults (Campbell et al., 2014). Furthermore, OT enhanced attention to feelings in older men and young women (Ebner et al., 2015) and enhanced resting-state connectivity between amygdala and medial prefrontal cortex (mPFC) in young women and trendwise in older men (Ebner et al., 2016). These

findings support the idea that age may play an important role in level and function of the OT system. No previous study, however, has examined adult age variability in the association between levels of *OXTR* methylation and attachment, thereby also considering variations in plasma OT levels, towards improved understanding of adult developmental mechanisms in affiliative processes.

Evidence of effects of aging on epigenetic mechanisms comes from research with monozygotic twins, who are epigenetically identical early on, but demonstrate significant differences in DNA methylation patterns as they become older (Fraga et al., 2005). Early studies examining the relationship between aging and epigenetic mechanisms found decreases in global DNA methylation in aged mammalian tissue (humans, mice, hamsters; Wilson & Jones, 1983). A more recent longitudinal study demonstrated increased and decreased global methylation levels in individuals measured by peripheral blood cell DNA (Bjornsson et al., 2008). Similarly, aging was associated with both increase and decrease in DNA methylation, depending on gene and tissue (Richardson, 2003). Important in the present context, however, currently, no aging data exists pertaining to DNA methylation of *OXTR* and plasma OT levels in their effects on affiliative processes, which is the research focus our study took.

1.4 Purpose of the Present Study

The two central aims of the present study were to determine the associations between (i) *OXTR* methylation levels and (ii) endogenous OT plasma levels, respectively, and adult attachment among young and older adults. We predicted that individuals with lower *OXTR* methylation levels and higher plasma OT levels would self-report more secure attachment, operationalized by less attachment anxiety (*Hypothesis 1&2*) and less attachment avoidance (*Hypothesis 3&4*).

Given mixed evidence of a functional link between *OXTR* methylation and OT plasma levels (Dadds et al., 2014), we explored unique vs. shared contributions of *OXTR* methylation and OT plasma on attachment anxiety and avoidance respectively. Based on growing evidence of age variations in OT level and function (Campbell et al., 2014; Ebner et al., 2015, 2016; see also Huffmeijer, van Ijzendoorn, & Bakermans-Kranenburg, 2013; Ebner et al., 2013 for reviews), we also explored age as moderator in all analyses. Of note, the previously outlined literature largely refers to young adults and suggests that a relationship between DNA methylation of *OXTR*, plasma OT, and adult attachment exists. However, some studies suggest that *OXTR* methylation and plasma OT levels change over the course of the adult lifespan (Huffmeijer et al., 2013; Ebner et al., 2013 for reviews), and it is possible that the respective associations between *OXTR* methylation and OT plasma with attachment anxiety and avoidance are less prevalent with advanced age.

There is evidence in the literature of sex differences in adult attachment (Chopik et al., 2013; Del Giudice, 2011) and in *OXTR* methylation levels (Carter et al., 2007; Fuke et al. 2004; Puglia et al., 2015; Rubin et al., 2016) and sex-differential function of OT (Macdonald, 2013; Rilling et al., 2014) while possibly sex-comparable plasma OT levels (Feldman, Gordon, Schneiderman, Weisamn & Zagoory-Sharon, 2010). Given the small sample size for the age-by-sex subgroups in the current study, providing us with reduced power to detect

significant interaction effects, we did not consider participant sex as factor in the design. However, we explored sex differences in adult attachment, *OXTR* methylation levels, and plasma OT levels as reported in Table A in the Supplementary Material.

2. Methods

2.1 Participants

The present data report is part of a larger project, which comprised 105 (51 young, $M = 22.4$ years, $SD = 2.99$, 18–31, 47% female, and 54 older $M = 71.2$ years, $SD = 4.92$, 63–81, 56% female) adult volunteers (see Ebner et al., 2015, 2016). Both young and older participants were recruited via the community outreach program HealthStreet and handouts and flyers throughout the community (including campus). Additionally, older participants were recruited through two university participant registries particularly geared toward older adults, as well as flyers and handouts in senior citizen homes and community centers. Only Caucasian individuals were eligible for study participation based on evidence that DNA methylation varies across race/ethnicity (Bakermans-Kranenburg, & Van IJezdoorn, 2014; Heyn, et al., 2013; Liu et al., 2010). All participants were in good health, with no history of heart disease, stroke, or primary degenerative neurological disorders.

The present analysis focused on a subsample consisting of 56 adult volunteers for which we had collected blood samples to measure *OXTR* methylation, resulting in 22 young (20–31 years, $M = 23.6$, $SD = 2.95$, 40.9% female) and 34 older (63–80 years, $M = 71.4$, $SD = 4.82$, 55.9% female) participants.¹ Table 1 presents information regarding demographics, physical and cognitive functioning, and personality of the young and older participants in the subsample used for the present data analysis, for sample descriptive purposes, to allow evaluation of the representativeness of the present sample with other samples reported in the literature. For older participants, only those who scored 30 or higher on the Telephone Interview for Cognitive Status (TICS; $M = 35.32$, $SD = 2.53$, range = 29–40; Brandt, Spencer, & Folstein, 1988) were recruited into the study².

2.2 Procedures

Prior to study enrollment, interested individuals underwent a phone screening to determine study eligibility (e.g., fell in the targeted age range, not pregnant or breastfeeding; see also Ebner 2015, 2016). Eligible participants first attended a screening visit followed by a second visit (which included administration of intranasal OT or placebo and an fMRI protocol, discussed elsewhere; Ebner et al., 2016). All data reported in this paper were collected during the screening visit, which started with written informed consent. Participants also completed an intake interview to determine number of hours slept the night before, current stress level ($1 = not at all stressed$ to $10 = extremely stressed$), food intake (*yes* vs. *no*),

¹Collection of *OXTR* methylation data was not part of the original aim of the larger project that this study was embedded in. The idea for the current study's research question emerged at a collaborative visit from Dr. Connelly at University of Florida, at a point where data collection was already underway. This resulted in only a subsample of 56 participants with *OXTR* methylation data out of the 105 participants in the larger project. The subsamples of young and older participants in this study were not significantly different from the total sample of young and older participants recruited into the larger project regarding chronological age, years of education, physical health, mental health, processing speed, episodic memory, personality, and cognitive status (see Tables 1&2).

²One older adult scored 29 on the TICS, but was not excluded from participation due to the fact that the individual missed the questions about address given a very recent move. Exclusion of this participant did not change the results.

caffeine intake (*yes* vs. *no*), engagement in exercise (*yes* vs. *no*), engagement in sex (*yes* vs. *no*), smoking (*yes* vs. *no*), alcohol intake (*yes* vs. *no*), physical contact (*yes* vs. *no*), and experience of fever (*yes* vs. *no*), factors that have potential to influence plasma OT levels³. A health review followed, in which participants self-reported any issues with any major bodily system. They then met with a licensed clinician to verify eligibility for continuation in the study and completed the blood draw. Two self-report questionnaires followed: the Ten-Item Personality Inventory (TIPI; Gosling, Rentfrow, & Swann, 2003), a brief measure of the Big Five personality dimensions, and the ECR-S (Wei, Russell, Mallinckrodt, & Vogel, 2007) to assess self-reported adult attachment. In addition, participants completed the Digit Symbol Substitution Task from the WAIS (DSST; Wechsler, 1981), as measure of sensorimotor processing speed, and the Rey Auditory Verbal Learning Test (RAVLT; Rey, 1964), as a measure of short-term verbal memory. Upon study completion, participants were debriefed about the study goals and reimbursed for study participation. The university's Institutional Review Board approved the protocol.

2.3 Measures

2.3.1 Adult Attachment—The ECR-S assesses individual differences in adults regarding their general attachment by presenting twelve rating statements that fall into two subscales, attachment anxiety (e.g., “*I need a lot of reassurance that I am loved by my partner*”) and attachment avoidance (e.g., “*I want to get close to my partner, but I keep pulling back*”). Participants indicated their agreement with each statement on a 7-point Likert scale (1 = *Disagree strongly* to 7 = *Agree strongly*). Higher scores indicated more insecure attachment. In line with previous studies that have confirmed reliability and validity of this measure (Lopez & Gormley, 2002; Vogel & Wei et. al, 2005; Wei et al., 2007), Cronbach's alpha in the total sample for attachment anxiety was $\alpha = 0.79$ (Young participants: $\alpha = 0.91$; Older participants: $\alpha = 0.77$) and for attachment avoidance $\alpha = 0.87$ (Young participants: $\alpha = 0.88$; Older participants: $\alpha = 0.87$). The intercorrelation between the two subscales was neither significant in the total sample, ($r = .093$, $p = .495$), nor among young ($r = -.047$, $p = .835$) or older ($r = .206$, $p = .244$) participants separately.

2.3.2 Blood Collection, DNA Extraction, and Epigenotyping—Eight cc of blood were collected in mononuclear cell separation tubes (BD Vacutainer CPT with sodium citrate, BDBiosciences, Franklin Lanes, NJ). Upon collection, blood samples were spun at 1800 RCF for 20 minutes to separate the mononuclear cell fraction per product protocol. The mononuclear cells were lysed and stored at -20°C and shipped to the Connelly Lab on dry ice. DNA was extracted using the reagents supplied in the Gentra Puregene Blood Kit (Qiagen) per product protocol. Prior to epigenotyping, 200 nanograms of DNA were bisulfite converted for each participant (Kit MECOV50; Invitrogen), which allows for detection of methylated cytosines by converting all non-methylated cytosines to uracil. *OXTR* methylation levels were examined at CpG site -934 (hg38, chr3:8,769,121). We focused on CpG site -934 based on supporting evidence of a functional role of this site in social perception and disorders of social relevance (e.g., autism; Bell et al., 2015; Dadds et

³The pattern of results for all reported findings was comparable when controlling for these variables.

al., 2014; Gregory et al., 2009; Jack et al., 2012; Kim et al., 2014; Rubin et al., 2016; Puglia et al., 2015).

Twenty nanograms of bisulfite-treated DNA were used as template for PCR amplification, performed in triplicate on three identical machines (C1000 Thermal Cycler; Biorad; see also Jack et al., 2012). Successful amplification was confirmed with agarose gel electrophoresis. Pyrosequencing was subsequently performed using Qiagen PyroMark Q24 Classic and Pyromark reagents per the manufacturer's protocol; samples during this procedure were randomized to account for plate/run variability. Site-specific primer pairs and PCR/pyrosequencing conditions are given below. All epigenotypes reported were an average of the three replicates.

Sample amplification via PCR was performed using 0.20 μ M of primers TSL101F (5'-TTGAGTTTTGGATTAGATAATTAAGGATT-3') and TSL101R (5'-biotin-AATAAAATACCTCCCACTCCTTATTCCTAA-3'). Thermal cycling conditions were: Step 1: (95 °C/15 min)/1 cycle; Step 2: (94 °C/30 s, 56 °C/30 s, 72 °C/30 s)/50 cycles; Step 3: (72 °C/10 min)/1 cycle; Step 4: 4 °C hold. Pyrosequencing was then performed using primer TSL101S (5'-AGAAGTTATTTTATAATTTT-3'). On average, replicate variability deviated from the mean $\pm$ 1.52%.

2.3.3 Plasma OT Collection and Assay—Blood samples for plasma OT were drawn into a six mL EDTA tube. Upon filling, the blood was inverted five times and then chilled until centrifugation. Within thirty minutes of the blood draw, the specimens were centrifuged at 1600 $\times$ g for fifteen minutes at 4 °C. After centrifugation, the EDTA tube was chilled and the plasma was aliquoted into pre-chilled cryovials with O-ring seals. The samples were stored in a freezer at -80°C.

Plasma samples were kept frozen until thawing at room temperature before the assay in the Carter lab. OT levels were measured using an Enzyme Immunoassay (EIA) purchased from Enzo Life Sciences, Inc. (Farmingdale, New York). The assay was performed in accordance with manufacturer instruction. The plasma was diluted in assay buffer at a ratio of 1:8 times to give results reliably within the linear portion of the standard curve. The manufacturer reports the EIA to have a minimal detection rate of 15.6 pg/ml OT and little antibody cross-reactivity for other neuropeptides. All samples were run at once and the inter- and intra-assay coefficients of variation were less than 8%.

We chose not to extract our samples. This decision was based on tests for parallelism, spike-recovery, and cross-reactivity/specificity validating the present study's EIA procedure and suggesting that EIA without extraction may constitute the most accurate assessment of OT in human blood plasma (Carter et al., 2009; MacLean et al., 2017; Lancaster et al., 2017). For example, determining the parallelism of dilutions in non-extracted vs. extracted salivary OT samples MacLean et al. (2017) provide evidence of high correlations of OT concentrations when analyzed in the same samples with and without extraction, with non-extracted samples yielding considerably higher concentrations than extracted samples. EIA without extraction (compared to EIA with extraction) has typically resulted in much higher estimates of OT concentrations (Szeto et al., 2011). Recent work has demonstrated the removal of bound OT

during the extraction step may explain this discrepancy in estimates (Brandtzaeg et al., 2016). That is, large amounts of OT are quickly bound in blood. Thus extraction may eliminate much of the biologically relevant peptide, with the specific mechanisms underlying this process not fully explored yet. In particular, OT has high affinity for binding to proteins in blood plasma through disulfide bonds. Breaking these bonds before OT concentration measurement results in estimates that are comparable to levels stemming from unextracted samples (Whittington et al., 2007). Solid-phase extraction further artificially reduces OT levels in plasma samples (Furman, Chen, & Gotlib, 2011; Kirsch et al., 2005), resulting in low amounts of authentic peptide labelled as OT in extracted samples (Szeto et al., 2011).

3. Results

3.1 Preliminary Analyses

As shown in Table 2, young and older participants differed in their self-reported attachment anxiety, with young participants reporting higher levels than older participants. The age groups did not differ in their self-reported attachment avoidance. The age groups were also comparable in their levels of *OXTR* methylation (in %) and plasma OT (in pg/ml) (Table 2). In addition, there were no sex differences in attachment anxiety, attachment avoidance, *OXTR* methylation level, or plasma OT level (Table A in Supplementary Material). Therefore, sex was not considered as a covariate in the subsequent analyses.

3.2. Association between *OXTR* Methylation, Plasma OT, and Adult Attachment

We estimated the effects of *OXTR* methylation and plasma OT levels on adult attachment in two separate regression models for each of the two attachment dimensions (i.e., anxiety, avoidance; Figure 1). Attachment anxiety and avoidance served as dependent variables, respectively, and *OXTR* methylation, plasma OT, and age as independent variables. Each model also considered the interaction between *OXTR* methylation and age and the interaction between plasma OT and age. We used mean centering for calculation of each interaction product (Aiken, & West, 1991). We conducted bootstrapping with 2,000 samples for the estimation of standard errors (SEs) and report confidence intervals (CIs) based on these bootstrapped SEs for each effect.

3.2.1 Attachment Anxiety—Neither the main effect of *OXTR* methylation ($B = .02$, $\beta = .18$, $t(50) = 1.28$, $p = .21$, Partial $R^2 = .03$, $CI = [-.01, .07]$, see Figure 1A) nor the main effect of plasma OT ($B = -.002$, $\beta = -.18$, $t(50) = -1.34$, $p = .19$, Partial $R^2 = .03$, $CI = [-.005, .001]$) on attachment anxiety were significant. However, both the interaction between *OXTR* methylation and age ($B = -.09$, $\beta = -.31$, $t(50) = -2.24$, $p = .03$, Partial $R^2 = .09$, $CI = [-.20, -.03]$) and the interaction between plasma OT and age ($B = .01$, $\beta = .26$, $t(50) = 1.96$, $p = .05$, Partial $R^2 = .07$, $CI = [.001, .01]$) were significant, indicating that the associations between attachment anxiety and *OXTR* methylation and plasma OT, respectively, differed for young and older participants.

Follow-up regression models estimated the effects of *OXTR* methylation and plasma OT on attachment anxiety in young and older participants separately. In young participants, the

effects of *OXTR* methylation ($B = .08$, $\beta = .44$, $t(19) = 2.46$, $p = .03$, Partial $R^2 = .24$, $CI = [.04, .18]$, Figure 2A) and plasma OT ($B = -.006$, $\beta = -.43$, $t(19) = -2.36$, $p = .045$, Partial $R^2 = .23$, $CI = [-.01, -.001]$, Figure 2B) were significant: lower *OXTR* methylation levels and higher plasma OT levels were associated with less self-reported attachment anxiety in young participants, supporting *Hypothesis 1&2* in young participants. In contrast, neither the effect of *OXTR* methylation ($B = -.01$, $\beta = -.11$, $t(19) = -.60$, $p = .56$, Partial $R^2 = .01$, $CI = [-.06, .03]$) nor the effect of plasma OT ($B = .001$, $\beta = .05$, $t(19) = .28$, $p = .75$, Partial $R^2 = .002$, $CI = [-.003, .004]$) on attachment anxiety was significant in older participants.

To further explore the shared and unique contributions of *OXTR* methylation and plasma OT on attachment anxiety in young participants, we conducted a Pedhazur communality analysis. The results showed that of the 38.1% variance in attachment anxiety among young participants, *OXTR* methylation uniquely accounted for 19.6% and plasma OT for 18.0%. Only 0.5% variance in attachment anxiety among young participants was commonly explained by *OXTR* methylation and plasma OT.

3.2.2 Attachment Avoidance—The main effect of *OXTR* methylation on attachment avoidance was significant ($B = -.05$, $\beta = -.36$, $t(50) = -2.50$, $p = .01$, Partial $R^2 = .11$, $CI = [-.08, -.01]$). However, in contrast to *Hypothesis 3*, lower *OXTR* methylation levels were associated with more self-reported attachment avoidance (Figure 1B). The interaction between *OXTR* methylation and age on attachment avoidance was significant ($B = .08$, $\beta = .24$, $t(50) = 1.73$, $p = .048$, Partial $R^2 = .06$, $CI = [-.03, .14]$; however, note that the estimated CI contained 0). In particular, the effect of *OXTR* methylation on attachment avoidance was largely driven by young participants⁴ (Figure 2C). In addition, neither the main effect of plasma OT ($B = .002$, $\beta = .17$, $t(50) = 1.26$, $p = .20$, Partial $R^2 = .03$, $CI = [-.001, .006]$) nor its interaction with age ($B = .001$, $\beta = .04$, $t(50) = .30$, $p = .77$, Partial $R^2 = .002$, $CI = [-.004, -.008]$) on attachment avoidance was significant. Thus, our data did not support *Hypothesis 4* that plasma OT levels were associated with attachment avoidance in young and older participants (Figure 2D).

4. Discussion

The aims of the present study were to identify the relationships between levels of (i) DNA methylation of *OXTR* and (i) plasma OT with adult attachment in an age-heterogeneous sample. We report several novel findings as discussed next.

4.1 *OXTR* Methylation and Plasma OT Levels Were Associated with Young (but not Older) Adult Attachment

In support of our hypotheses, while the age groups were comparable in their levels of *OXTR* methylation and plasma OT, our data showed that lower levels of *OXTR* methylation and higher levels of plasma OT were uniquely associated with less self-reported attachment anxiety in young but not older adults. In contrast to our predictions, however, lower levels of *OXTR* methylation were associated with greater self-reported attachment avoidance,

⁴A post-hoc power analysis showed that a sample of 125 participants would have been needed for this interaction with the reported effect size and a power of 80% to reach the significance level of $p < .05$.

particularly in young adults. That is, the associations between *OXTR* methylation and plasma OT levels, and attachment-related phenotypes were more pronounced in young than in older adults, while *OXTR* levels and plasma OT levels were comparable across these age groups.

The observed age differences are in line with theoretical suggestions from lifespan theory that the impact of environmental influences may be less pronounced in later years of life (Baltes, 1997). Important environmental processes (e.g., parenting styles, abuse, chronic stress) can lead to methylation of genes critical for neurodevelopment (Meaney & Szyf, 2005; van Ijzendoorn, Bakermans-Kranenburg, & Ebstein, 2011). While the impact of biological effect prevails in older age, such environmental processes may become less salient. It is also possible that in older adults the epigenome has undergone comparably more replication-dependent changes in methylation (e.g., as a result of time) compared to the epigenome of young adults. In this context, consideration of possible reversal of *OXTR* methylation over time, including examination of behaviors and environmental factors contributing to reversing the process, is warranted in future research on a sample spanning adults of varying ages (Wu & Zhang, 2014).

Of note, although not statistically significant, numerically there was greater variability in *OXTR* methylation in older than young adults. This greater within-group variability in the older compared to the young adults, may have resulted in less sensitivity to detecting effects in the older adults, especially given the present study's small sample size, as discussed in more detail below.

Importantly, the adult developmental approach taken in the present study, though highly innovative in this field of study, was cross-sectional in design. Thus, we cannot conclude from the present data that lower levels of *OXTR* methylation and higher levels of plasma OT *cause* reduction in attachment anxiety in young adults—in fact, the reverse might be true. Individual differences in adult attachment, and the behaviors and experiences associated with it, may be driving changes in the OT system (including levels of *OXTR* methylation and plasma OT). Future research that tests *OXTR* and plasma OT associations with adult attachment longitudinally and across developmental transition points (e.g., into emerging adulthood, parenthood, retirement, etc.) is needed to inform causal mechanisms across the adult lifespan. Promising evidence toward a causal developmental model came from a longitudinal study that examined neonatal exposure to maternal stress (e.g., criminal behavior, substance abuse, maternal psychopathology) and showed that these stressors were associated with increased *OXTR* methylation levels at birth (Cecil et al., 2014). While *OXTR* methylation was temporally stable from birth to 9 years, higher *OXTR* methylation at birth was associated with higher callous-unemotional traits at age 13. Further extending the current approach, investigation of a more continuous age sample covering the entire adult lifespan, including midlife, and across time, is warranted to fully unravel the impact of epigenetic and neuropeptide change on affiliative processes throughout adulthood and aging.

Another explanatory account for the observed age-differential associations between *OXTR* DNA methylation and plasma OT levels and adult attachment could consider known hormonal differences (e.g., pertaining to gonadal hormones such as testosterone and

estradiol) between young and older adults (Ebner, Kamin, Diaz, Cohen, & MacDonald, 2015, for a recent summary). There is evidence that estrogen upregulates *OXTR* expressivity and OT production (Bale & Dorsa, 1997; Vasudevan et al., 2001). It has been suggested that hormones may act as “internal environmental factors” that impact DNA methylation levels (Fuke et al. 2004), making sex differences likely. Some studies on DNA methylation that did not specifically examine *OXTR* have documented higher levels of methylation in males than females (El-Maarri et al., 2007; Fuke et al., 2004; Sarter et al., 2005) or have found DNA methylation to be relatively stable between the sexes and across age groups (Eckhardt et al., 2006). However, two previous studies that specifically focused on the *OXTR* CpG site -934 have found higher levels in women than men (Puglia et al., 2015; Rubin et al., 2016). Further, adult attachment varies by sex, with mixed evidence regarding the direction of the effect. For example, Chopik et al. (2013) reported that, in young adult populations, women tend to have higher attachment anxiety and avoidance than men, while Del Giudice (2011) found that men reported higher avoidance but lower anxiety than women, with population size and geographical region moderating these effects. While our sample comprised men and women, the sample size was small and thus did not allow us to examine age-by-sex variations on the epigenome and plasma OT levels in their impact on affiliative phenotypes. Our data did not suggest sex differences in attachment anxiety, attachment avoidance, *OXTR* methylation level, or plasma OT level but future studies are warranted to systematically examine the effects of sex as well as culture, geographic region, and race/ethnicity on the associations between *OXTR* methylation, OT plasma, and attachment across the adult lifespan. Being the first adult developmental examination into this topic, we held race/ethnicity constant to only Caucasian participants based on evidence that DNA methylation varies across race/ethnicity (Bakermans-Kranenburg & Van IJzendoorn, 2014; Heyn, et al., 2013; Liu et al., 2010).

Moreover, although we collected *OXTR* polymorphisms in the context of the present study, the small sample size did not allow us to look into interaction effects between *OXTR* polymorphisms and *OXTR* methylation and OT plasma levels, while also considering age or sex. These relationships and moderating processes will be interesting to explore in future studies.

4.2 *OXTR* Methylation and Plasma OT Were Differentially Associated with Attachment Anxiety vs. Attachment Avoidance

Lower levels of *OXTR* methylation and higher levels of plasma OT were associated with lower attachment anxiety while lower levels of *OXTR* methylation were associated with greater attachment avoidance in young participants. There is some suggestion of a positive intercorrelation between both attachment avoidance and anxiety in the literature (Wei et al., 2007). In our study, however, the two subscales were uncorrelated, suggesting that they constituted two independent dimensions of adult attachment.

Our findings regarding attachment anxiety are in line with a recent study showing that individuals with lower DNA methylation levels of the structural gene for oxytocin (*OXT*) have more secure attachment characterized by lower levels of attachment anxiety (Haas et al., 2016). However, like in our study, this pattern of findings was not supported for

attachment avoidance. In fact, Haas et al. found no significant effects for attachment avoidance. Our finding of differential relationships for the two subscales is also in line with work of varying effects of intranasal OT administration on cooperative behavior as a function of individual attachment. In particular, while OT has been shown to increase cooperative behavior (Kollock, 1998) in intimacy-seekers (who are anxiously attached, but low in avoidance), it has been shown to hinder cooperative behavior in “fearful” individuals (who are anxiously attached, but high in avoidance; Bartz et al., 2011). It will be interesting to extend the current examination of associations between *OXTR* and affiliative processes in young and older men and women, to other OT signaling genes such as *OXT* or *CD38*, a transmembrane glycoprotein shown to be critical for social behavior by regulation OT secretion (Jin et al., 2007).

Limiting the scope of our research and warranting future work is that we relied exclusively on self-report indices of adult attachment and thus cannot determine the extent to which *OXTR* methylation and plasma OT predict actual behavioral differences in individuals across adulthood. Behavioral measures might provide more sensitive and predictive results. The self-report adopted in our study is in line with the current standard in the adult attachment literature that largely uses self-report questionnaires or structured interviews. One measure, the Adult Attachment Projective (AAP), consists of a procedure in which adults are shown eight drawings characterizing dimensions of attachment like done in child studies and asked to describe the pictures (George & West, 2001). Although not entirely behavioral in nature, the AAP demonstrates that adult attachment research can extend techniques from child research into the realm of adults. Some research has also used coding techniques to determine specific nonverbal cues in couples and qualities associated with their adult attachment and then has examined correlations between these objective codings with participants’ subjective self-reports (Tucker & Anders, 1998).

4.3 *OXTR* Methylation and Plasma OT Uniquely Contribute to Variance in Adult Attachment

Our exploration of unique vs. shared contributions of *OXTR* methylation and OT plasma levels on attachment anxiety and avoidance suggested that that lower levels of *OXTR* methylation and higher levels of plasma OT were largely uniquely associated with less self-reported attachment anxiety in young adults, thus constituting unique predictors of adult attachment anxiety in young adulthood. Rubin et al. (2016) found no associations between *OXTR* methylation and plasma OT in their sample of psychotic patients and healthy controls. Also, Dadds et al. (2014) found no associations between *OXTR* methylation and plasma OT in their total sample of young and older boys. When looking at only the older boys in their sample, however, they observed a negative relationship (i.e., higher *OXTR* methylation levels were associated with lower plasma OT levels in boys aged 9–16 years). However, there are various methodological differences between Dadds et al. and our study. Puberty, characterized by strong hormonal changes, may be a particularly important developmental period for epigenetic modification of gene expression (Szyf & Bick, 2013). That is, while Dadds et al. examined a sample of pre-pubertal and pubertal males, who are likely undergoing rapid maturational and hormonal changes, our sample comprised young and older adults and included both sexes.

Further, in the current study we assumed that *OXTR* methylation in blood is congruent with *OXTR* methylation in the brain. The extent to which blood-based DNA methylation levels reflect DNA methylation patterns in brain loci specifically, however, is not clear yet (van Ijzendoorn et al., 2011). Thus, our findings do not provide a direct empirical test regarding production, function, or accessibility of OT within the central nervous system. Also, blood samples should be taken more than once in future studies to specifically determine change in *OXTR* and plasma OT levels in response to specific social and affective events, and to account for fluctuations.

4.4 Conclusion

In sum, our research findings extend the existing literature with important conceptual and empirical implications. The present approach adopts an adult developmental perspective on *OXTR* epigenetics and endogenous OT levels. Thus, it qualifies current evidence regarding the associations between epigenetic modification of *OXTR* through the process of DNA methylation and endogenous OT levels with individual differences in behavioral phenotypes of affiliative processes. This present examination of age-differential associations is particularly relevant, given growing evidence of age effects on the OT system (Campbell et al., 2014; Ebner et al., 2015, 2016). Our findings are in line with emerging evidence (Dadds et al., 2014; Haas et al., 2016) suggesting that epigenetic modification of genes regulating OT pathways and endogenous OT levels are associated with the way people form and maintain intimate social relationships with others. Our results further advance understanding of the genetic and biological mechanisms underlying human affiliation. In line with the previous literature (Bell et al., 2014; Dadds et al., 2014; Gregory et al., 2009; Jack et al., 2012; Puglia et al., 2015), our data suggests that low *OXTR* methylation and high plasma OT levels are associated with better social outcomes, (i.e., lower attachment anxiety) in young adults. Our findings also inform the interplay between *OXTR* methylation and endogenous OT levels on attachment in adulthood and suggest largely unique contributions.

Taken together, the current study supports the link between the OT system and affiliative processes in humans and qualifies existing work by suggesting age-differential variations in these associations. We hope that our findings will spur replication and extension with larger samples sizes. Research into this topic has the potential, in the longer term, to inform ways to use genetic and biological markers toward prevention, diagnosis, and treatment of pathology characterized by impairments in social cognition and affective behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- The neuropeptide oxytocin (OT) has been implicated in a wide range of affiliative processes. Research on epigenetic mechanisms of the oxytocin receptor gene (*OXTR*) is scarce in non-clinical populations, and even less is known about epigenetic variability across adulthood.
- The present study assessed methylation levels at *OXTR* CpG site -934, endogenous plasma OT levels, and self-reports of adult attachment in young and older adults.
- Lower levels of *OXTR* methylation and higher plasma OT levels were associated with less self-reported attachment anxiety in young but not older adults, with largely independent contributions of *OXTR* methylation and plasma OT.
- Findings contribute to growing evidence suggesting that epigenetic modification of genes regulating OT pathways and endogenous OT levels are associated with the way people form and maintain intimate social relationships. The study findings also emphasizes the importance of adopting a developmental perspective when studying properties of the OT system and their relation to affiliative processes.

Figure 1.

Effects (B (SE)) of *OXTR* methylation levels, plasma OT levels, age, and their interactions on (A) attachment anxiety and (B) attachment avoidance. Solid lines with bolded text indicate significance at $p < .05$; Solid lines with non-bolded text indicate $p < .10$; dashed lines indicate non-significant paths.

Figure 2.

(A) Lower *OXTR* methylation levels and (B) higher plasma OT levels were associated with less self-reported attachment anxiety in young but not older participants. (C) Lower *OXTR* methylation levels were associated with more self-reported attachment avoidance in both age groups but particularly in young participants. (D) There were no significant associations between plasma OT levels and self-reported attachment avoidance in young or older participants.

Table 1

Means (Standard Deviations) and Age Differences in Demographics, Physical and Cognitive Functioning, and Personality

Construct	Young Participants	Older Participants	<i>t</i> -value	<i>df</i>
<i>Demographics</i>				
Years of Education	15.91 (1.95)	16.06 (2.99)	-.21	53
<i>Physical, Mental, & Cognitive Health</i>				
Physical Health	8.55 (1.14)	8.41 (1.05)	.45	54
Mental Health	8.41 (1.26)	8.59 (1.35)	-.50	54
Processing Speed	66.64 (10.85)	43.82 (8.10)	8.21	54
Episodic Memory	8.73 (2.16)	7.38 (2.37)	2.14	54
<i>Personality</i>				
Emotional Stability	5.68 (1.01)	5.53 (1.17)	.50	54
Extraversion	4.61 (1.38)	4.47 (1.46)	.37	54
Openness to Experience	5.57 (.81)	5.75 (1.03)	-.70	54
Agreeableness	5.11 (.94)	5.56 (.78)	-1.92	53
Conscientiousness	5.89 (.96)	5.51 (1.23)	1.20	54

Note. Physical Health “Please rate your general physical health” and Mental Health “Please rate your general mental health/mood” were measured by single self-report items on a scale ranging from 1 = *Poor* to 10 = *Excellent*. Processing Speed was measured by the WAIS-R Digital Symbol Substitution Test (Wechsler, 1981), higher scores indicated faster processing speed; Episodic Memory was measured by the Rey Auditory Verbal Learning Test (RAVLT; Rey, 1964), higher scores indicated better episodic memory. Five dimensions of personality were measured by the Ten-Item Personality Inventory (TIPI; Gosling, Rentfrow, & Swann Jr. 2003), on agreement to 10 statements using a rating scale from 1 = *Disagree strongly* to 7 = *Agree strongly*. There were no significant differences between participants with *OXTR* methylation data vs. those without methylation data on any of the measures in Table 1. Bold indicates significant age differences at $p < .05$.

Table 2

Means (Standard Deviations) and Age Differences in Adult Attachment (ECR-S), OXTR Methylation Levels, and Plasma OT Levels

Construct	Young Participants	Older Participants	<i>t</i> -value	<i>df</i>
Attachment Anxiety	3.10 (1.19)	2.55 (1.13)	-1.74	54
Attachment Avoidance	2.10 (1.01)	2.33 (1.33)	0.69	54
<i>OXTR</i> Methylation	42.42 (6.49)	46.36 (9.57)	1.69	54
Plasma OT	795.89 (81.99)	792.28 (109.28)	-0.13	54

Note. ECR-S = *Experiences in Close Relationship-Short Form* (Wei et al., 2007), higher scores indicated greater attachment anxiety and attachment avoidance; response scale ranged from 1 = *Strongly disagree* to 7 = *Strongly agree*. *OXTR* = Oxytocin receptor gene, OT = Oxytocin. There were no significant differences between participants with *OXTR* methylation data vs. those without methylation data on attachment anxiety, attachment avoidance, or plasma OT levels. Young participants with *OXTR* methylation data were significantly older than young participants without *OXTR* methylation data ($t = 2.47$, $p = 0.02$, Cohen's $d = 0.70$). There was no age difference among older participants ($t = 0.51$, $p = 0.61$, Cohen's $d = 0.14$). Bold indicates significant age differences at $p < .05$.