Impact of early life adversity on DNA methylation of the oxytocin receptor gene (*OXTR*)

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ABSTRACT

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Qian Qian Zhou

Early life adversity (ELA) is a risk factor for the development of mental and physical disorders later in life (McEwen, 2003). Recent models propose DNA methylation as an underlying molecular mechanism responsible to dynamically translate and imprint these negative environmental experiences in different biological pathways (Szyf & Bick, 2013). This study focuses on the oxytocin receptor gene, *OXTR*, given the role of the oxytocinergic system in the modulation of social behavior, anxiety, and depression. The goal of this study is to examine the effect of ELA on CpG methylation frequency in distinct regions of the *OXTR* gene, and to test whether these changes in *OXTR* methylation are related to childhood anxiousness and disruptiveness trajectories rated from age 6 to 12. Drawing from a 27-year longitudinal cohort, we compared 46 adults with high or low early life adversity with regards to their *OXTR* DNA methylation frequency and their associations with teacher-rated childhood anxiousness and disruptiveness trajectories. The main findings are that in females, we observed significantly higher DNA methylation at Intron 1 CpG 4, Intron 1 CpG 5, Promoter CpG 3, Promoter CpG 7, and significantly lower DNA methylation at Enhancer 1 CpG 2 in the high ELA group, compared to the low ELA group. No significant ELA-related differences were found among males. In addition, teacher-rated childhood anxiousness trajectory was significantly associated with methylation frequency in Intron 1 CpG 5, Promoter CpG 3, Promoter CpG 7, and Enhancer 1 CpG 2 among females, but not among males. Furthermore, teacher-rated childhood anxiousness trajectory mediated the impact of ELA on Promoter CpG 7 methylation in females.

This study suggests that early social experiences impact methylation frequency of different regulatory regions within the *OXTR* gene among females and that these differential methylation patterns are related to phenotypic differences in anxiousness during childhood.

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INTRODUCTION

Negative events experienced in early life can have a lifelong impact on one's health and well-being. Childhood adversities are associated with increased risk for psychopathology as well as inflammatory, cardiovascular and metabolic disorders later in life (Nusslock & Miller, 2015). Recent models propose that deoxyribonucleic acid (DNA) methylation of specific neuro-regulatory genes as an underlying molecular mechanism responsible for the increased health risk associated with exposure to adverse early environmental events (Weaver et al., 2004). This thesis examines the impact of early life adversity (ELA) on *OXTR* DNA methylation, a key gene involved in social bonding processes, anxiety, and depression.

Peptide & Receptor Structure and Function

Oxytocin (*OXT*) is a neuropeptide with both physiological and behavioural functions. As a neuropeptide hormone, it is produced in the magnocellular neurosecretory cells of the supraoptic (SON) and paraventricular (PVN) nuclei within the hypothalamus (Barberis, Mouillac, & Durroux, 1998). Oxytocin neurons project to multiple areas of the brain including the amygdala and brain stem; specific neurons project to the posterior pituitary where it is stored and released into the circulation in a pulsatile manner (Gimpl & Fahrenholz, 2001; Rhodes, Morrell, & Pfaff, 1981). Peripherally, *OXT* is involved in the induction of uterine contraction during childbirth as well as the stimulation of milk letdown during lactation (Macdonald & Macdonald, 2010). Centrally, oxytocin is thought to play an important role in promoting and enabling social behaviours. Current models suggests that both central and peripheral *OXT* release contribute to mediate behavioural effects independently and synergistically (Neumann & Landgraf).

Currently, only one oxytocin receptor is known. The oxytocin receptor (*OXTR*) belongs to the G protein coupled receptor family. Oxytocin exerts its effect by binding to high affinity oxytocin receptors in specific areas of the brain or in the periphery (Gimpl & Fahrenholz, 2001). Without the oxytocin receptor, the effects that this peptide exerts physiologically and behaviourally are greatly impaired. Indeed, *OXTR* knockout animals exhibit multiple deficits in social behaviours such as disturbed frontal approach and huddling, impaired social memory, and increased anxiety-like behaviours (Pobbe et al, 2012; Lee, Caldwell, Macbeth, Tolu, & Young, 2008).

Oxytocin plays an important role in a number of social behaviours. A seminal study in the 1990s explored the effects of oxytocin on proceptive and receptive maternal behaviour in sheep (Keverne & Kendrick, 1992). In ewes, the onset of maternal behaviour occurs only after parturition in a time dependent manner. Central administration of oxytocin increased the frequency of receptive maternal behaviour in sexually-naive sheep, indicating a role for this peptide in the initiation of maternal behaviours (Keverne & Kendrick, 1992).

Oxytocin has also been associated with social bonding. The prairie vole is a highly social rodent species that display monogamous behaviours (Carter, DeVries, & Getz, 1995). Early field studies found that prairie voles form long-term bonds, co-occupy nests, travel with their partners in the wild. In addition, the prairie voles display alloparental care whereby both parents take care of the offspring (Young, Gobrogge, Liu, & Wang, 2011). Sexually naïve prairie voles are highly social and display non-selective partner preference (Shapiro & Dewsbury, 1990). Selective partner preference is developed after extended cohabitation and/or mating with a partner. Moreover, this preference behaviour is accompanied by selective aggression towards unfamiliar prairie voles (Gobrogge, Liu, Young, & Wang, 2009).

Direct evidence of role of oxytocin in social bonding behaviour originates from pharmacological studies with prairie voles. Central administration of *OXT* facilitates pair bonding in the absence of mating (Insel, Winslow, Wang, Young, & Hulihan, 1995). In addition, the use of an oxytocin antagonist into the NAcc blocks all expression of maternal-like behaviour towards pups in adult females (Insel, 1992). Moreover, Montane voles, a species that is closely related to the Prairie voles, but does not display partner preference and alloparental care, have lower oxytocin receptors density in brain regions involved with social bonding than prairie voles (Insel & Shapiro, 1992). Further, within species variations in oxytocin receptor density in the nucleus accumbens (NAcc) have been related to display of alloparental behaviour in sexually naïve females (Olazabal & Young, 2006); adult females that display alloparental behaviour have higher densities of oxytocin receptor in the nucleus accumbens compared to those who either attack or ignore their offspring (Ross & Young, 2009). These data provide strong evidence of the implication of *OXT* in social bonding processes.

Recent research using intranasal oxytocin treatments have shown that oxytocin can modulate social cognition and behaviour in humans (Rocchetti et al., 2014). In studies of normal and autistic populations, intranasal oxytocin administration improved emotional and facial recognition (Guastella et al., 2010; LoParo & Waldman, 2015; Rimmele, Hediger, Heinrichs, & Klaver, 2009). In addition, intranasal oxytocin administration also increased trust among humans in an experimental economics game (Kosfeld, Heinrichs, Zak, Fischbacher, & Fehr, 2005). While individual differences and context matters in the impact of intra-nasal administration (Bartz, Zaki, Bolger, & Ochsner, 2011), these findings indicate that oxytocin is also involved in the regulation of social behavior in humans.

There is a growing body of evidence highlighting the importance of the oxytocinergic system in the modulation of stress and anxiety. In animal models, mice that receive intracerebroventricular (ICV) oxytocin administration exhibit antidepressant like effects in the forced swim test (Meisenberg, 1982). Conversely, an oxytocin agonist, carbetocin, produced anti-depressant like effects in a modified forced-swim test as well (Chaviaras, Mak, Ralph, Krishnan, & Broadbear, 2010). In rat models, oxytocin administration reduced the frequency of distress calls emitted by rat pups in social isolation environment, an effect often promoted by anxiolytic drugs (McQuaid, McInnis, Abizaid, & Anisman, 2014).

The literature on the role of OT in anxiety and depression in humans is more mixed. When comparing plasma *OXT* levels between MDD patients and controls, some studies have found lower, higher, or no differences in plasma *OXT* between depressed patients and health controls (Parker et al., 2010; Yuen et al., 2014; Zetzsche, Frasch, Jirikowski, Murck, and Steiger 1996) Further, cerebral spinal fluid (CSF) oxytocin level did not differ in male patients with major depressive disorder compared to healthy controls in a separate study (Sasayama et al., 2012). However, variation in oxytocin measurements across studies might explain some of these discrepant findings in humans (McQuaid et al., 2014; Striepens et al., 2013).

Oxytocin exhibits sexual dimorphic effects. In animal models, sexually naïve male voles treated with oxytocin show more aggression and less social behavior, compared to *OXT* treated female voles (Bales & Carter, 2003; Insel & Hulihan, 1995). Moreover, pair bonding behavior is significantly more pronounced in female voles compared to male voles (Bales & Carter, 2003). In humans, plasma oxytocin levels tend to be higher in females compared to males (Kramer, Cushing, Carter, Wu, & Ottinger, 2004). Furthermore, gender moderates the effects of intranasal *OXT* administration in a social perception task where females rated faces more positively than

males (Hoge et al., 2014). Similarly, men exhibited impaired ability to categorize facial emotions such as anger post *OXT* treatment compared to females, whose ability to categorize angry facial emotions were not affected by *OXT* treatment (Lynn, Hoge, Fischer, Barrett, & Simon, 2014). Published studies to date suggest that the prosocial impact of *OXT* might be stronger in females than in males.

Genetics and Epigenetic Influences on Behaviours

The field of behavioural genetics has shown that most behavioural phenotypes are at least partially heritable (Plomin, 1990). Behavioural genetic studies utilize twin studies to quantify genetic and environmental influences on a given trait or disorder. Twin study design compares monozygotic (MZ) or dizygotic (DZ) twin pairs. Since monozygotic twins share almost 100% of their genetic makeup (with exception of de novo and somatic mutations) and dizygotic twins share about 50% of their genes, differences in concordance between MZ and DZ pairs in behavioural phenotype can be attributed to their difference in genetic relatedness. For example, it is estimated that the heritability of major depressive disorder (MDD) is between 40% to 50%, and there is a two to three fold increase in lifetime risk of developing MDD among direct relatives of individuals diagnosed with MDD (Lohoff, 2010). While twin studies allow researchers to assess the heritability of a given trait, they do not provide insight into the specific genes involved in a given phenotype.

Association studies allow researchers to identify specific genes involved in a given phenotypic trait. Association studies probe susceptibility to disease as a function of specific genotypic differences like gene polymorphisms, which are single nucleotide variations within the human genome. Although SNPs are only a single difference among the vast genome, they can have important ramifications with regards to behaviour and disease; they can alter protein

sequence, splicing of RNA, promoter activity, distal regulatory regions of the genes such as enhancers or insulators, stability of mRNA, and localization of protein (Shastry, 2009). In genome-wide association studies (GWAS), genetic markers across the whole genome are scanned to identify genetic variations associated with a particular disease. While GWAS studies have yielded significant discoveries with disorders such as schizophrenia and bipolar disorder, it had limited success with regards to MDD. To date, there is no replicated gene variants associated with MDD identified from GWAS studies (Lohoff, 2010; Major Depressive Disorder Working Group of the Psychiatric et al., 2013; Wray et al., 2012).

In contrast to the atheoretical GWAS approach, in the candidate gene approach, association studies test whether theoretically relevant candidate genes are associated with specific behavioural phenotypes. Using a candidate gene approach, recent association studies examined the association between *OXTR* polymorphisms and different behavioural phenotypes (Voisey et al., 2009). A specific *OXTR* SNP (rs53576) (GG vs. AG, AA) (lack of G in the SNP) was associated with sensitivity to stress and mental health issues (Rodrigues, Saslow, Garcia, John, & Keltner, 2009). The lack of G in the rs53576 genotype has been associated with lower empathy (Rodrigues et al., 2009), lower levels of optimism and self-esteem (Saphire-Bernstein, Way, Kim, Sherman, & Taylor, 2011), and higher physiological stress reactivity (Rodrigues et al., 2009). Similarly, various other SNPs have been found in the *OXTR* to be associated with recognition memory, social integration and mesolimbic reward circuitry (Chang et al., 2014; Skuse et al., 2013).

Recent association studies have also reported associations between *OXTR* SNPs and depression. Specific *OXTR* single nucleotide polymorphisms (SNPs) have been associated with higher depressive symptoms in undergraduate students (Kawamura et al., 2010). In addition,

both the rs53576 (G>A) and rs2254298 (G>A) SNP polymorphisms related to risk for unipolar depression (Costa et al., 2009; McInnis, McQuaid, Matheson, & Anisman, 2015). Together, these evidence suggest an association between specific *OXTR* polymorphisms and depression.

Both genetics and the social and physical environment contribute to behavioural and disease phenotypes but their relative contribution varies for different disorders or context. Gene by environment (GxE) interactions represents situations where a given phenotype is modified by the combination of a given genetic liability with a specific environment. Currently, two major theories account for such interactions between the environment and the genome. The first theory, dubbed the stress-diathesis model, maintains that disorders are expressed when genetic vulnerability interacts with subsequent negative stressful experiences (Booij, Wang, Levesque, Tremblay, & Szyf, 2013). Although childhood adversities increases risk for psychiatric disorders, not everyone exposed to negative childhood experiences will develop psychopathology in adulthood (Luthar, Cicchetti, & Becker, 2000)**.** Indeed, there is evidence showing GxE interactions with the rs53576 *OXTR* polymorphism interacting with childhood maltreatment to increase risk for emotional dysregulation and disorganized adult attachment style (Bradley et al., 2011). Among individuals homozygous to the G allele of the rs53576 polymorphism, there was a dose-response relationship between early adversity and emotional dysregulation, while there was no relationship between early adversity and emotional dysregulation among individuals with the heterozygous genotype (Bradley et al., 2011). Similarly, individuals who experienced significant early life adversity exhibited increased depressive symptomology when the GG/GA rs53576 phenotype was present, compared to individuals with the AA genotype (McQuaid, McInnis, Stead, Matheson, & Anisman, 2013).

Alternatively, the differential susceptibility theory suggests that more "plastic" individuals may be more sensitive to both positive and negative environment (Belsky, 1997). One study supporting this model examined the interaction between the serotonin transporter polymorphism and early family environment on depressive symptomology. It was found that individuals who possessed the 5-HTTLPR risk subtype exhibited greater depressive symptomology when they experienced early adversity and less depressive symptomology when they experienced a supportive early environment (Taylor et al., 2006). This indicates a "for better or for worse" type of effect where positive experiences help alleviate predisposed risk whereas negative experiences reinforce negative predisposition.

While genetic factors are fixed at conception, recent studies have highlighted that environmental factors can influence gene expression through epigenetic processes. Epigenetics is the study of biological mechanisms that influence gene expression without altering the actual nucleotides sequence. Epigenetics comprises three processes: 1) chromatin organization/histone remodeling system, 2) non-coding RNA (ncRNA and 3) DNA methylation and hydroxymethylation. The chromatin remodeling system includes a network of histones proteins tightly wrap around the DNA that inhibit its access and subsequent activation. The system includes a number of enzymes that are able to modify histones in order to open or close access to specific region of the DNA. This system allows the control of gene expression at a macro cellular level (Clapier & Cairns, 2009). Non-coding RNAs are precursor protein sequences that are not translated into actual proteins but are functionally important in gene expression which include different classes of small RNAs such as microRNA and piRNA as well as long noncoding (antisense) RNAs such as Xist (X-inactive specific transcript), Air (Antisense Igf2r RNA) and H19 (Espinoza, Allen, Hieb, Kugel, & Goodrich, 2004).

DNA methylation is a biochemical addition of a methyl group onto the Cytosine residue of DNA, which does not alter the actual nucleotide sequence. DNA methylation is unique amongst other epigenetic modifications by being part of the chemical covalent structure of DNA. Thus, the DNA molecule itself bears both genetic and epigenetic information. Typically DNA methylation occurs on Cytosine bases immediately followed by a Guanosine (G) residue; this is dubbed the CpG dinucleotide (CpG). In the human genome, there are abundant stretches of CpG islands (CpGi), which are short segments of DNA highly enriched in CpG dinucleotides. Current research suggests that these CpG islands play important roles in regulating gene expression as they are enriched in genomic regions where an abundance of transcription factors, important for the regulation of transcribing the genetic code into protein sequences (Szyf & Meaney, 2008). Methylation of Cytosine (C) residues prevents binding of transcription factors to their cis-acting elements in gene promoters and enhancers and facilitates the recruitment of proteins that block the nucleotides from the transcriptional machinery or that modify existing chromatin structure, leading to gene inhibition, which, in most cases, suppresses the activity of the affected gene (Figure 1) (Szyf & Meaney, 2008). DNA methylation in the body of the gene is believed to be involved in gene activity. However, the mechanisms involved in gene body methylation are unknown to date while different mechanisms were speculated such as inhibition of spurious firing of gene expression from cryptic promoters.

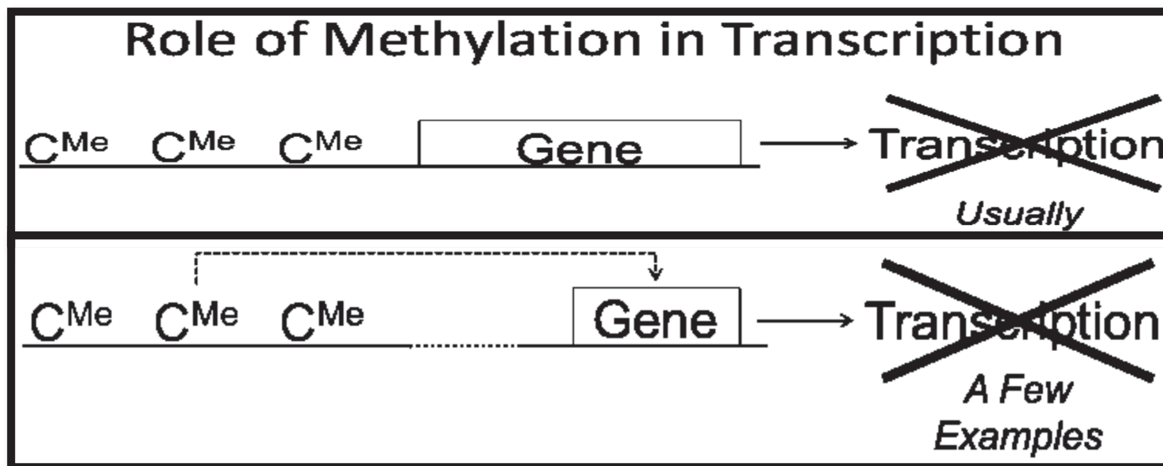


Figure 1. Mechanisms of DNA Methylation. Methylation of CpG dinucleotides is usually associated with inhibition of gene transcription. The first mechanism indicates promoter induced transcription repression via methylation while the second mechanism indicates distal enhancer induced transcription repression via methylation. Adapted from Kaplow et al. (2014).

DNA methylation is a critical developmental process that permanently alters the expression of genes during cellular differentiation, repressing or activating critical genes during development. This is important for stem cells developing into specific tissues during embryonic development (Iqbal, Jin, Pfeifer, & Szabó, 2011). DNA methylation patterns that lead to such cell differentiation are usually permanent, thus preventing differentiated cells from reverting into stem cells. Environmental factors can also influence methylation patterns during development such that the same genotype can lead to a range of phenotypic manifestations as a function of epigenetic modifications.

Early Life Adversity and DNA Methylation

Early life adversity (ELA) increased risk for poor mental and physical health in adulthood. Early life adversities encompass physical abuse, sexual and emotional abuse and other negative experiences in childhood such as parental neglect or loss. Early life adversity is associated with altered stress sensitivity and structural and functional changes in several neurodevelopmental pathways regulating stress responses (Fan et al., 2015; Grimm et al., 2014).

Current evidence from epidemiological studies indicates ELA as a risk factor for onset and development of psychopathology (Agnew-Blais & Danese, 2016; Li, D'Arcy, & Meng, 2016).

DNA methylation is one molecular mechanism through which these social experiences can be embedded into the epigenome to affect broad biological pathways and systems (Szyf, McGowan, & Meaney, 2008). It has been demonstrated that DNA methylation of the glucocorticoid receptor (*GR*) varies as a response to maternal care in rats (Weaver et al., 2004). This study examined the DNA methylation status of *GR* exon1_7 promoter region of the offspring from mothers who exhibited either low or high maternal care behavior (licking and grooming). It was found that offspring who received high levels of maternal care had significantly different DNA methylation across multiple CpG dinucleotides of the *GR* promoter region than offspring who received less maternal care. Importantly, this observed methylation pattern was reversed when offspring from high licking and grooming mothers were cross-fostered at birth to low licking and grooming mothers and vice versa. Furthermore, this methylation change was associated with changes in hippocampal *GR* expression, where pups that received low maternal care had lower *GR* expression (Weaver et al., 2004). The changes in *GR* methylation in pups emerged soon after the initiation of maternal behavior in mothers and persisted in adulthood, which in turn led to a heightened physiological stress response in adult pups that received low licking and grooming maternal care (Weaver et al., 2004). This seminal study provided strong evidence of the impact of the early social environment on DNA methylation, the stability of the methylation signature, and its impact on subsequent physiological consequences.

Early life adversity has been associated with genome-wide epigenetic differences in animal and human studies (McGowan et al., 2011). Provencal et al. (2012) showed that in rhesus macaques differential rearing conditions (maternal vs. surrogate peer rearing) led to global

methylation differences in as much as ~1300 distinct gene promoters in the prefrontal cortex (PFC). Similarly, postmortem studies of hippocampal tissues of suicide victims examining epigenetic changes as a response to early-life trauma yielded more than 300 differentially methylated promoters among individuals who experienced high early-life trauma compared to control individuals not exposed to ELA (Labonte et al., 2012). Another study examining differential methylation patterns between institutionalized children and children raised by their biological parents yielded differences in more than 800 gene promoters in whole blood (Naumova et al., 2012). These epigenome wide association studies highlight the impact of ELA on numerous genes involved in neural development, brain functioning, receptor signaling and immune function. This suggests that ELA exposure leads to a system wide epigenetic change spanning multiple different functional systems within different tissues (Figure 2) (McGowan et al., 2009; Naumova et al., 2012).

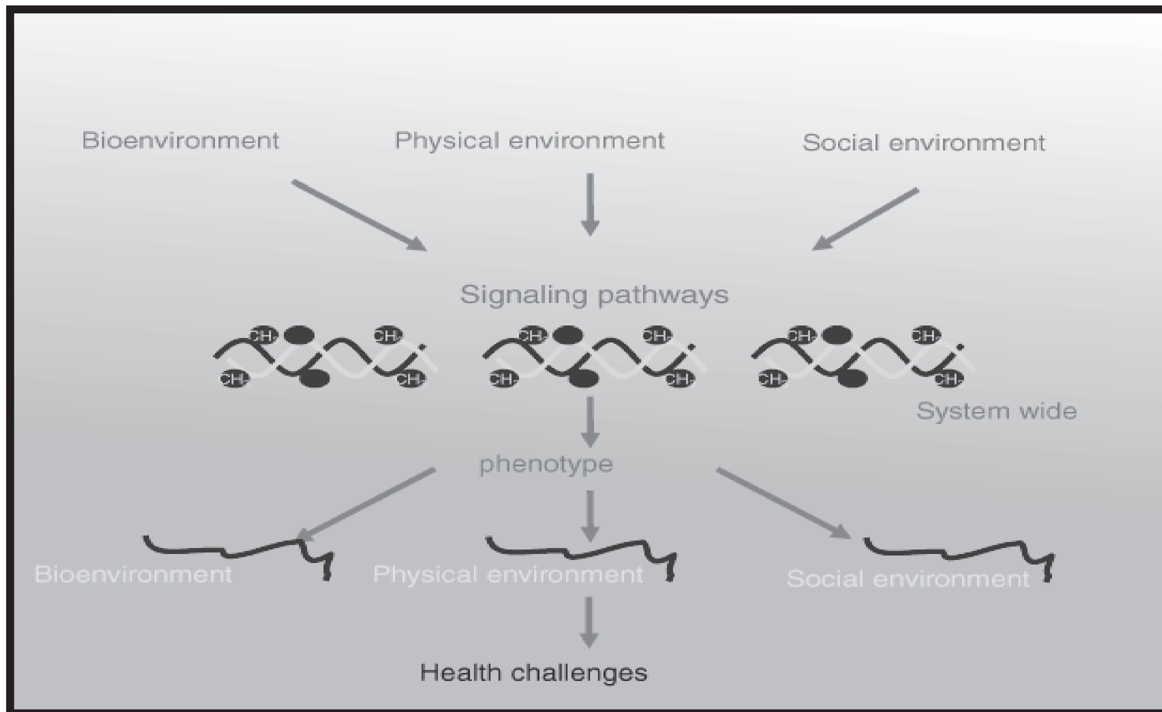


Figure 2. DNA methylation is hypothesized to be a system-wide genome adaptation mechanism. Methylation acts as mediation between environmental cues and behavioural phenotypes. Adapted from Szyf (2012).

Candidate gene studies provided evidence of the impact of early life adversity and DNA methylation of key neuroregulatory pathways. As stated previously, early life environment influences DNA methylation of the gene encoding the glucocorticoid receptor, playing an important role in the modulation of neuroendocrine stress related response (Bick et al., 2012). In rats, exposure to early life trauma induced lasting increases BDNF methylation, which subsequently led to decreases in BDNF expression in the prefrontal cortex, compared to control rats not exposed to early life trauma (Roth, Lubin, Funk, & Sweatt, 2009). Moreover, this methylation-induced gene expression difference was associated with changes in maternal behaviours of maltreated female rats; they displayed significantly more abusive behaviours towards their offsprings, compared to non-maltreated mothers (Roth et al., 2009). Similarly, Murgatroyd et al. (2009) reported that early life stress was associated with hypermethylation of a

regulatory region of *AVP*, a gene implicated in depression for its role in potentiating the HPA-axis. Furthermore, increased promoter methylation of the serotonin transporter (SLC6A4) is associated with early life adversity and worse clinical presentations in MDD patients (Kang et al., 2013). Moreover, promoter methylation of the SLC6A4 gene is increased in individuals with childhood aggression and associated with *in vivo* measures of serotonin synthesis in peripheral blood monocytes (PBMCs) (Booij, Tremblay, Szyf, & Benkelfat, 2015; Wang et al., 2012). These studies suggest that epigenetic changes likely mediate the long-lasting effects of early life adversity by impairing normal receptor signaling by inhibiting gene expression in relevant biological pathways (Lutz & Turecki, 2014). This study provided evidence for correspondence of brain and blood DNA methylation, which is critical for pursuing epigenetic studies of *OXTR* in humans.

OXTR and Early Life Adversity

The oxytocinergic system is also sensitive to early experiences. In monkeys, exposure to early life abuse led to lower cerebro-spinal fluid (CSF) *OXT* levels and was associated with more disturbed and asocial behaviours such as avoidance of physical contact and attachment to inanimate objects (Winslow, 2005). In rats, female offspring of high maternal care (licking and grooming) mothers exhibited increased *OXTR* binding in the central amygdala (CeA), lateral sepum (LS), medial pre-optic area (MPOA) compared to offspring from low licking and grooming mother (Francis, Champagne, & Meaney, 2000). In addition, decreased *OXTR* mRNA expression was observed in female adolescent rats exposed to early adversity (Hill, Warren, & Roth, 2014). The findings have been replicated in humans where higher levels of early adversity was associated with lower circulating CSF *OXT* level in women and related to altered stress reactivity and limbic deactivation in response to *OXT* administration, compared to subjects

without a history of early life adversity (Grimm et al., 2014; Heim et al., 2009). Collectively, these results suggest that early live adversity might have an impact on *OXTR* methylation.

Early epigenetic studies of *OXTR* gene using gene segments cloning led to the identification of a specific sequence in the 2nd intron of the gene that regulates gene expression in myometrium tissue (Mizumoto, Kimura, & Ivell, 1997). Subsequent research showed that an area adjacent to the first exon also displayed regulatory activities, which showed differential methylation pattern in different cellular tissues (Kusui et al., 2001**.** This specific region was dubbed the MT2 region. In a seminal paper, five CpG dinucleotides within the MT2 region had significantly higher methylation in autism patients, compared to healthy control subjects (Gregory et al., 2009). The difference was found to be not only in blood DNA from blood, but also in with DNA extracted from brain tissues (Gregory et al., 2009). This study provided evidence for correspondence of brain and blood DNA methylation, which is critical for pursuing epigenetic studies of *OXTR* in human.

Most of the human research to date has focused on the MT2 region (Figure 3). Individual differences in *OXTR* methylation in PBMCs have been related to social cognition and behavior as well as psychopathological symptoms. To date, there is evidence linking *OXTR* DNA methylation with anorexia nervosa symptoms, child conduct problems, acute psychosocial stress and perception of ambiguous social stimuli (Dadds, Moul, Cauchi, Dobson-Stone, Hawes, Brennan, Urwin, et al., 2014; Jack, Connelly, & Morris, 2012; Kim, Kim, Kim, & Treasure, 2014; Mizumoto et al., 1997; Unternaehrer et al., 2012; Unternaehrer et al., 2015). Altered *OXTR* DNA methylation levels across specific gene regions has been found in patients with social anxiety disorder and major depression, compared to healthy group and sex matched controls (Reiner et al., 2015; Ziegler et al., 2015).

A few studies suggest that ELA impacts *OXTR* methylation. Greater *OXTR* methylation in *OXTR* exon 3 was associated with low maternal care in childhood in peripheral blood cells (PBMC) (Unternaehrer et al., 2015). A recent epidemiological study of more than 1000 participants examining 18 key stress and inflammation related genes, reported that low childhood socioeconomic status (SES) was associated with increased *OXTR* DNA methylation (Needham et al., 2015). Moreover, a recent study by Smearman et al. (2016), found that early child abuse was associated with higher *OXTR* DNA methylation of two particular CpG sites, cg04523291 and cg02192228, located in exon 3 in whole blood from a low SES African-American sample.

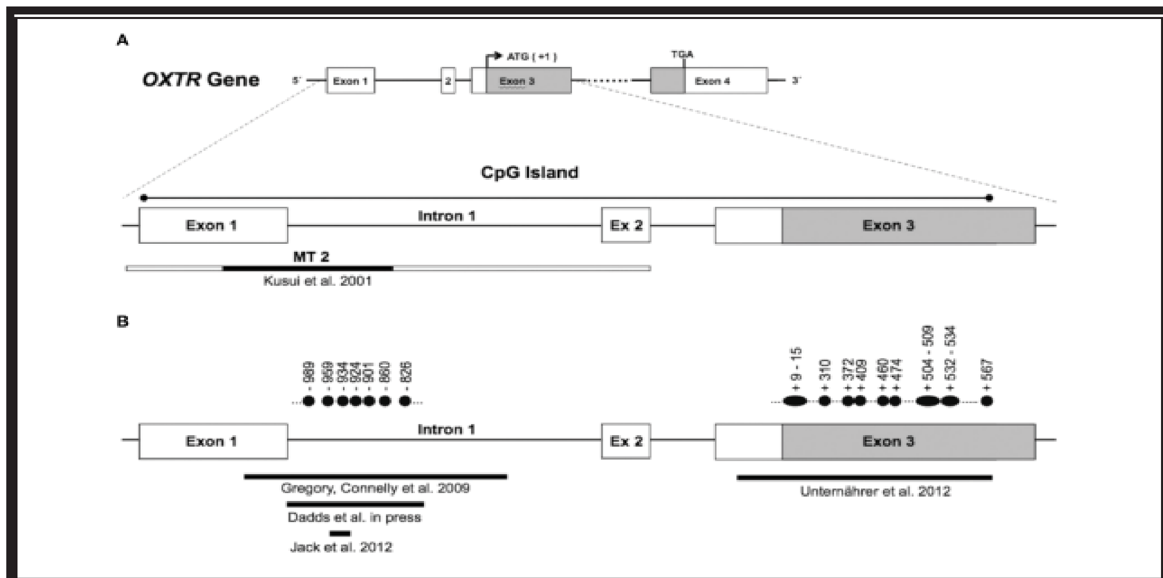


Figure 3. An overview of previously investigated regions in *OXTR*, adapted from Kumsta et al. (2013)

Past studies on *OXTR* focused on a limited subset of genomic regions, which do not encompass all the important regulatory regions of the gene. An area of particular interest is the promoter region of the *OXTR*. Promoter regions are interesting targets because they are enriched in both CpG dinucleotides and binding sites for transcriptional activators and repressors. Methylation of such regions can hinder the proper interaction between DNA and transcription factors. Disruptions of such interactions usually bring about inhibition of downstream gene

expression (Saxonov, Berg, & Brutlag, 2006). Furthermore, chromatin immunoprecipation sequencing (CHIP-sequencing) indicates that there are two sites within the enhancer element, one of which falls into the regulatory region previously characterized by Mizumoto et al. (1997), that have not been examined in prior research. Enhancer elements are short distal regions involved in the co-regulation of gene transcription, usually acting cis to the promoter elements (Blackwood & Kadonaga, 1998).

The Present Study

To date, the extant literature is consistent with the hypothesis that ELA increases risk for psychopathology, implicating oxytocinergic system is involved in social behavior, anxiety, and depression, and that DNA methylation is a potential biochemical mechanism mediating the impact of early environmental experiences on gene expression. With regards to *OXTR*, there is evidence suggesting the effects of ELA on the oxytocinergic system and the role of oxytocinergic system in modulating social behaviour in animal and human models. Recent studies of *OXTR* implicate DNA methylation as a mechanism for imprinting negative early experiences. However, many questions remain as to the identity of the specific *OXTR* genomic regions affected, the functional relevance of the affected CpG sites, as well as the relationship between such DNA methylation changes and childhood trajectories of anxiousness and disruptiveness. Moreover, sex differences in *OXTR* DNA methylation are often overlooked, and sample heterogeneity may confound previous findings. The aims of present study are to address these unanswered critical questions:

A) What is the impact of childhood adversities on *OXTR* DNA methylation difference in a longitudinal human cohort?

B) What are the DNA methylation differences in *OXTR* gene regulatory regions in human longitudinal cohorts with different ELA?

C) What is the relationship between *OXTR* methylation and childhood trajectories of anxiousness and disruptiveness?

D) What is the impact of sex on the impact of ELA on *OXTR* methylation?

The main hypothesis of the proposed study is that exposure to early life adversity will be associated with greater methylation in different regulatory regions of the oxytocin receptor gene in adulthood and these differences will be affected by sex.

MATERIALS AND METHODS

Participants

The current study is based on participants recruited from l'Étude longitudinale des enfants de maternelle au Québec (ÉLEMQ), a longitudinal study of 3785 children recruited during their time in kindergarten in francophone schools in Québec. Of this broad sample, a randomly selected, representative group of 2000 boys and girls was followed longitudinally. The cohort was followed yearly from age 6-12, at mid-adolescence (mean age = 15), in emerging adulthood (mean age = 21) and in adulthood (mean age = 27). At the age 27 follow-up assessment, participants provided a blood sample for epigenetic analysis. A subset of these participants was selected for exposure to high or low levels of early adversity, as described below. To reduce genetic admixture, only Caucasian individuals of Western European ancestry were included in the study.

Psychosocial Measures

The Early Family Adversity Index: Exposure to early adversity has been well

characterized in this cohort using seven socioeconomic indices collected prospectively during childhood. These indices are parental occupational prestige, age at birth of their first child, education level and familial composition. The first six indices are given a score of 1 if they are below or at the 30th percentile, or a score of 0 if they are above the 30th cohort percentile. For familial composition, a score of 1 is given if the participant was not living with his two biological parents in childhood before age 6. This index prospectively predicted a range of behavioural and cognitive outcomes in 3 independent, population-based samples, providing strong evidence for the predictive validity of this measure (Brezo et al., 2010; Haapasalo, Tremblay, Boulerice, & Vitaro, 2000). Given that the index has high test-retest reliability during childhood (r=0.85), the score when the child was 6 years of age was used.

The Sexual and Physical Abuse Index is a combination of two separate surveys at age 15 and 21 aimed at assessing the total amount of abuse experienced before adulthood. *The Adverse Childhood Experiences Study Questionnaire* focuses on assessing total amount of sexual violence experienced before age 18 via self-reported measures including identity of perpetrator, type of abuse, and severity evaluation (Felitti et al., 1998). *The Revised Conflict Tactics Scales* is a 32-item scale that is aimed at evaluating self-reported childhood incidents of physical assaults, psychological aggression (Straus, Hamby, Finkelhor, Moore, & Runyan, 1998). The scores from these scales were combined using z-scores to form an overall Abuse index.

In order to create two groups with differential exposure to early life adversity, 46 participants with available DNA were selected based on their scores on the early family adversity index and the abuse index. The z-scores calculated on the entire cohort for both surveys were used to create extreme groups in term of exposure to early adversity. Participants from high ELA group (n=24) have low scores on the Early Family Adversity Index (indicating low SES) and

high scores on the Abuse index, while participants from the low ELA group (n=22) all have high scores on the Early Family Adversity Index and low scores on the Abuse index. An equal number of males and females were selected in each group.

Childhood trajectories of anxiousness and disruptiveness were determined using items from the Social Behavior Questionnaire, an instrument assessing childhood traits using teacher reports. The Social Behavior Questionnaire was administered yearly from age 6 to 12. The Disruptiveness subscales (Cronbach's alpha, 0.90) encompasses the following items: fighting with others, disobedience, lie, mistreat, peer intimidation, peer sharing (reverse coded), agitation, restlessness. The Anxiousness subscale (Cronbach's alpha, 0.74) encompasses the following items: fearful or afraid of new things or situations, worry, worry about many things, cries easily, tendency to work alone, sad and unhappy, tearful and easily distracted (Masse & Tremblay, 1997). The trajectories (low, fluctuating and high) were characterized using semi-parametric group-based modeling (Masse & Tremblay, 1997; Nagin & Tremblay, 1999). Children classified in the *low* trajectory were consistently rated as display low levels of anxiousness or disruptiveness, while participants in the *high* trajectory high elevated ratings of anxiousness or disruptiveness throughout childhood. Participants in the *fluctuating* trajectories varied between high and low ratings of anxiousness or disruptiveness.

***OXTR* Target Selection**

To identify potential regulatory region of the gene, chromatin immune-precipitation (ChIP)-sequencing experiments data available in the ENCODE histone project open-access database (ENCODE Project Consortium, 2012) were used. In the present study, both H3K4Me1 and H3K4Me3 signals are obtained through the official ENCODE database and visualized via

UCSC genome browser (Karolchik, Hinrichs, & Kent, 2012). H3K4Me1 and H3K4Me3 are specific histone modifying proteins that are indicative of active promoters and active enhancers respectively. Region of peak signal intensity are regions of DNA sequences that bind specifically to H3K4Me1 and H3K4Me3 antibodies with high signal strength, which indicate methylation activity even after background noise correction (Landt et al., 2012). Local peak regions correspond to active DNA methylation activity after background noise correction. The exact DNA sequences corresponding to a signal region was extracted with full annotation of all CpG dinucleotides. For the promoter, the region length was defined from 700 base pairs upstream of the gene to the transcription start site (TSS) (Figure 4). For enhancers, DNA sequence was extracted 100 base pairs upstream and downstream from the H3K4Me1 signal peak, which corresponds to the region with the highest confidence of protein binding activity (ENCODE Project Consortium, 2012). A total of four regions were selected for the present study (Figure 5):

A. Promoter region (chr3: 8811303-8811915, upstream of transcription start site and 1st exon)
B. Intron 1 region: A prior defined region based on past literature (chr3: 8810699-8810875, within the 1st intron)
C. Enhancer region #1 (chr3: 8806851-8806950, within the 3rd intron)
D. Enhancer region #2 (chr3: 8797101-8797350, within the 3rd intron)

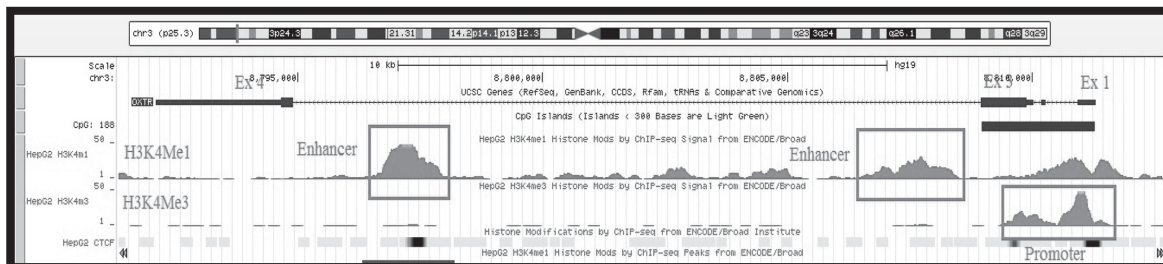


Figure 4. Histone signal example. Target selection in *OXTR* is based on histone signals. Rectangular boxes highlight *OXTR* regions of highest DNA methylation activity and are selected for the purpose of this study.

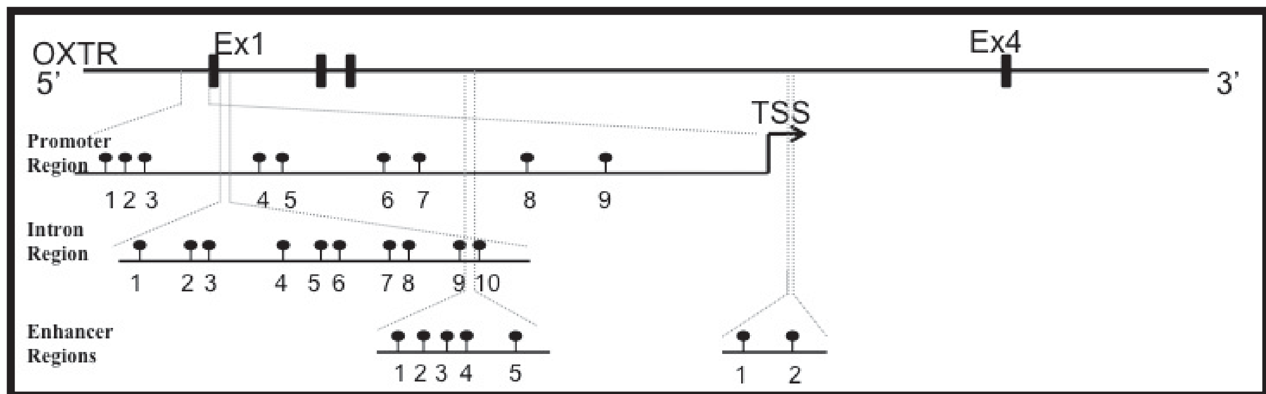


Figure 5. Final selected regions for *OXTR* investigation. Black circles indicate CpG dinucleotides within the particular region. 3 regions (Promoter, Enhancer 1 and Enhancer 2) and 1 from previous studies (Intron 1).

Sample Preparation

Peripheral blood samples were collected from participants and stored in EDTA coated tubes at 4°C before extraction. DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen, #51304) according to the manufacturer's instructions and stored at -20°C. Extracted DNA was stored in an -80°C freezer until the pyrosequencing assay was performed.

Pyrosequencing

To investigate the DNA methylation pattern, a total of four sets of outside primers and five sets of inside primers were developed to probe all CpG sites within the target regions (promoter, intron 1, enhancers). The nested reverse primers were biotinylated for pyrosequencing (IDT Technologies). 500 ng of DNA was treated with sodium bisulphite (EZ Methylation Gold, Zymo Research) and underwent two rounds of PCR amplification (#1, 95C x 15 min, [94 x 1 min, Primer TM * 1 min, 72C * 1 min] for 35 cycles, 72C * 10 min; #2 95C x 15 min, [94 x 1 min, Primer TM * 1 min, 72C * 1 min] for 40 cycles, 72C * 10 min). The subsequent PCR product then undergoes gel electrophoresis to confirm the purity and the success of the amplification protocol. 20 ul of the PCR product is then used to perform pyrosequencing

using PyroMarkQ24 (Qiagen) according to the manufacturer protocol. The complete PCR primers used is listed as in Supplementary Table 1. The methylation percentage at each individual CpG site was analyzed and exported using PyroMark Q24 software (Qiagen). Triplicate analyses were performed per sample to assure accuracy. Data reported as the average of the triplicates.

We encountered issues in pyrosequencing several target CpG sites because of difficulties in replicating target DNA fragments without contaminations due to co-replication of other unintended DNA fragments. In addition, we also experienced difficulties in assessing Enhancer Region 2 CpG methylation due to difficulties in designing the sequencing primer that adheres to the target Enhancer segment with high affinity. This is caused by the repetitive composition of the Enhancer Region 2 DNA sequence post sodium bisulfite treatment. A list of all reported CpG sites with their respective genomic positions is listed in Table 1. Intron CpG 4 contains 3 missing subject data, promoter CpG 1 and CpG 4 contain 2 missing subject data, promoter CpG 8 contains one missing subject data while enhancer 1 CpG 2, CpG 3, CpG 4 contain 2 missing data points. Missing subject data points are caused by failure to obtain data from these specific subjects.

CpG Reference	Illumina450k cgID	Genomic Position
I1	n/a	chr3:8810832
I2	n/a	chr3:8810807
I3	n/a	chr3:8810797
I4	n/a	chr3:8810774
I5	n/a	chr3:8810733
I6	n/a	chr3:8810708
P1	n/a	chr3:8811332
P2	n/a	chr3:8811348
P3	n/a	chr3:8811359
P4	n/a	chr3:8811363
P7	cg00247334	chr3:8811543
P8	cg17036624	chr3:8811601
E1	n/a	chr3:8806906
E2	n/a	chr3:8806899
E3	n/a	chr3:8806894
E4	n/a	chr3:8806887
E2_1	n/a	chr3:8797331
E2_2	n/a	chr3:8797259

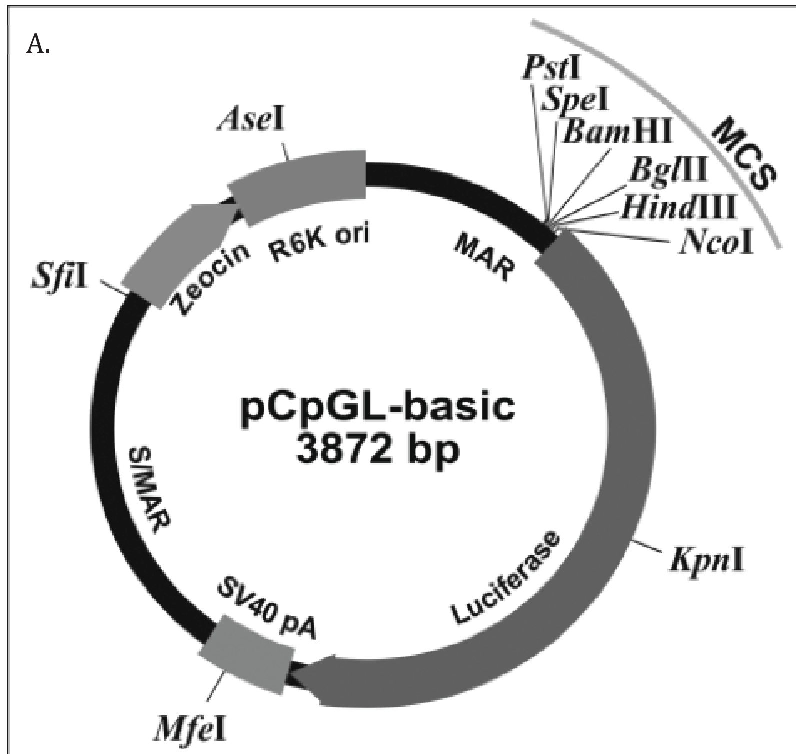
Table 1. Genomic coordinates for investigated CpG sites. Contains reference to exact human hg19 CpG genomic coordinates and Illumina450k methylation array cgID when available. I1, P1, E1, E2 denotes Intron 1, Promoter, Enhancer 1 CpG # and Enhance 2 CpG # respectively

Luciferase Reporter Construct

Three artificial constructs of the *OXTR* promoter was generated (602 base pairs whole promoter sense, 602 bp whole promoter antisense, and 50 base pair promoter CpG 7) using primers with artificially introduced restriction sites to allow for sense and antisense insertion of DNA fragment (Supplementary Table 2**)**. A sense sequence is a DNA sequence in its 5' to 3' direction while an antisense sequence is the complement of the sense sequence in the reverse 3' to 5' direction. Human genomic DNA from whole blood was subject to two rounds of PCR amplification with HotStar Taq DNA Polymerase (Qiagen). The resulting DNA was digested with BamHI and HindIII and subcloned into the CpG-free pCpGL-basic luciferase reporter

plasmid. SssI DNA methyltransferase (New England Biolabs) was then used to methylate the plasmid construct *in vitro.*

The pCpGL-basic plasmid is a construct with no-inherent CpG sites, methylation of such plasmid will occur exclusively on the introduced gene fragment sequence. This strategy ensures no other confounding sites of methylation along the whole plasmid construct. The resultant constructs were then validated via sequencing (Genome Quebec, Montreal). Both the methylated and non-methylated plasmids were then transfected into HEK293 (human embryonic kidney) cells using standard methods. HEK293 cells were cultured in DMEM 1X (GIBCO, Invitrogen) with 10% fetal bovine serum (GIBCO, Invitrogen), plated and co-transfected with 200ng of plasmid DNA. Cells were lysed and harvested 48 hours post transfection, and luciferase activity was measured via the Luciferase Assay System (Promega).



B.

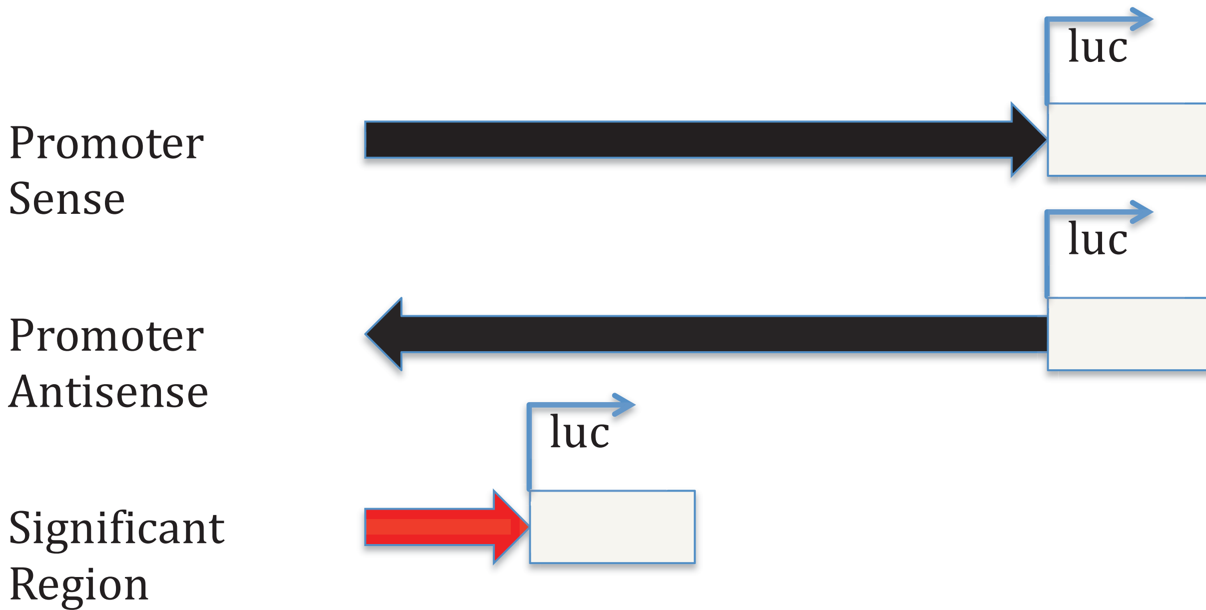


Figure 6A) original pCpGl-basic vector and contents, adapted from Klug & Rehli (2006) **B)** Sequence inserts into the plasmid, block arrow indicates the size of the insert and direction of sequence

Statistical Analysis

A two-tailed t-test with early adversity as the grouping factors and methylation frequencies as the dependent variable was used to test significance of absolute methylation difference between ELA groups. We then performed the same analyses by gender to examine gender-specific effects of *OXTR* DNA methylation. Furthermore, a univariate general linear modle (GLM) with statistically weighted posterior probabilities tested the association between *OXTR* DNA methylation and the childhood trajectories of anxiousness and disruptiveness. A mediation analysis was performed on CpG sites differentially methylated as a function of ELA, where it was hypothesized anxiousness/disruptiveness trajectories Y) acts as a mediator between ELA (X) and in methylation frequency within *OXTR* (Z). The mediation test was performed using the PROCESS module within SPSS (Hayes & Preacher, 2014). For the luciferase assay, a two-tailed t-test was used to test for significance of differences in average signal between control

and test plasmids. The alpha was set at $p < 0.05$. These analyses were not corrected for multiple comparisons given the exploratory nature of the study.

RESULTS

OXTR Methylation and early life adversity (ELA)

DNA methylation frequency of four distinct genomic regions within the oxytocin receptor gene (*OXTR*) was quantified in 46 individuals via pyrosequencing. All participants were 27 years of age and of Western European ancestry, with an equal number of males and females and a similar number of individuals in high and low ELA groups (n=46).

Range of overall methylation

A total of 17 CpG sites were probed. The methylation frequency across all tested CpG sites and ranges from 0% to 95%. The average methylation frequency of all CpG sites is listed in **Figure 7**.

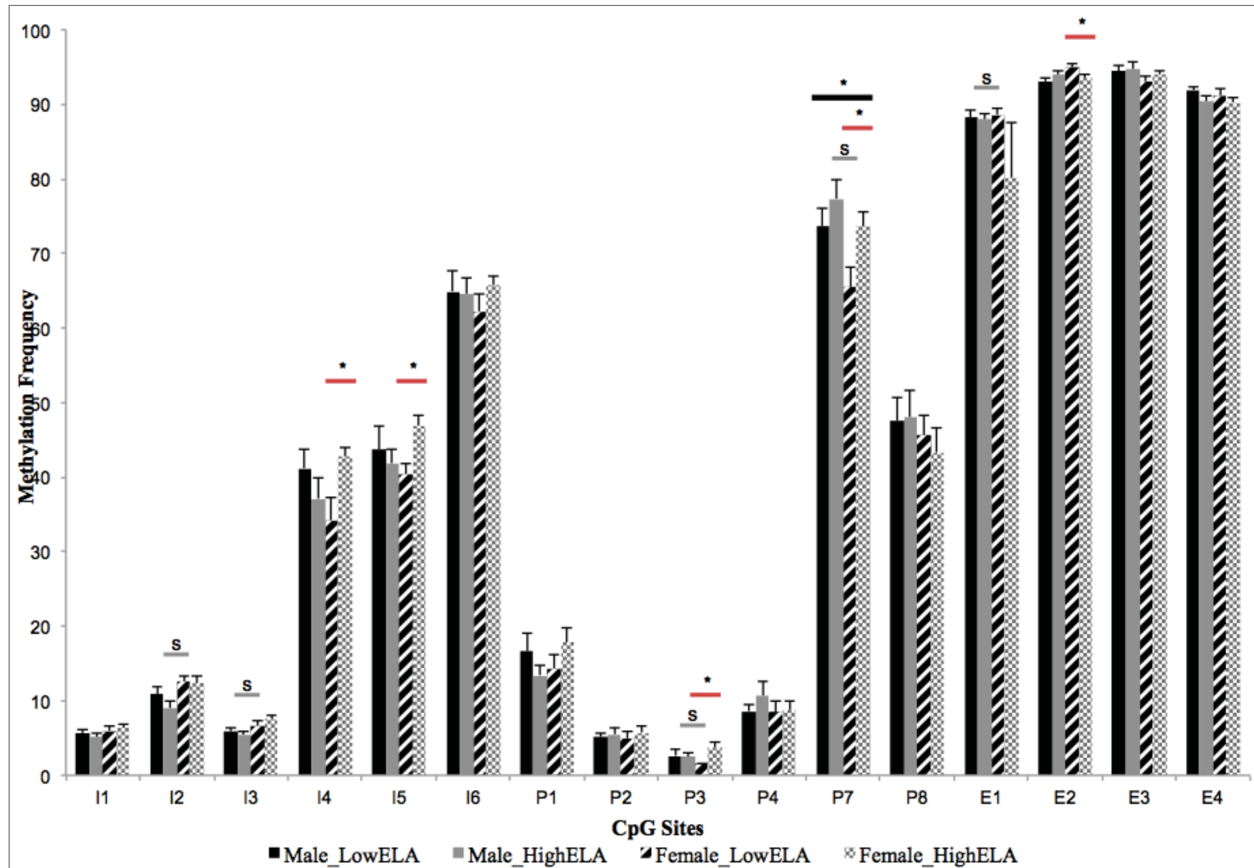


Figure 7. Raw methylation frequency as a function of sex and early life adversity. **S** denotes significant sex differences, ***** Denotes significant overall methylation differences against ELA. Shorter bar length indicates female specific ELA methylation differences. **I1, P1, E1** denotes Intron 1, Promoter, Enhancer 1 CpG # respectively.

ELA-related group differences

Overall, one CpG site was significantly different between groups. Promoter CpG 7 (*t*=-2.29, *p*=0.027, d=0.69, mean difference between high early adversity and low early adversity groups = 5.9%), with individuals exposed to early life adversity presenting with greater methylation than their counterparts with lower ELA exposure. Two other CpG sites exhibited marginally significant differences as a function of ELA, Promoter CpG 3 (*t*=-1.75, d=0.52 *p*=0.09, mean difference =1.1%) and Enhancer 1 CpG 4 (*t*=1.75, d=0.52, *p*=0.09, mean difference = 1.3%).

Sex differences in *OXTR* methylation and ELA

Given the sexually dimorphic effect of *OXT* on social behavior (Uhl-Bronner, Waltisperger, Martinez-Lorenzana, Condes Lara, & Freund-Mercier, 2005), we examined sex differences on *OXTR* methylation. A total of 4 CpG sites exhibited significant sex differences: Intron 1 CpG 2 ($p<0.01$, higher methylation in female), Intron 1 CpG 3 ($p<0.01$, higher methylation in female), Promoter CpG 7 ($p<0.05$, lower methylation in female), and Promoter CpG 9 ($p<0.05$, lower methylation in female).

Given the sex differences in the methylation frequency of some CpG sites, we conducted sex-specific analysis evaluating the impact of ELA on methylation. Two-tailed *t*-tests showed a total of 5 CpG sites significantly different between high and low ELA groups within females. These CpG sites were: Intron 1 CpG 4 (t=-2.33, d=1.04, p=0.04, *mean difference* = 8.5%), Intron 1 CpG 5 (t=-3.51, d=1.57, p=0.002, *mean difference* = 6.5%), Promoter CpG 3 (t=-2.98, d=1.33, p=0.011, *mean difference* = 2.3%), Promoter CpG 7 (t=-2.35, d=1.05, p=0.029, *mean difference* = 8.0%), and Enhancer 1 CpG 2 (t=2.21, d=0.99, p=0.04, *mean difference* = -1.5%). All CpG methylations were lower in low ELA group with the exception of Enhancer 1 CpG 2 where low ELA was associated with higher methylation. The same hypotheses were tested among males. No CpG site was differentially methylated as a function of ELA exposure in males.

***OXTR* methylation and Childhood Trajectories of Anxiousness, and Disruptiveness**

Given that ELA was more strongly associated with *OXTR* methylation among females, the association between *OXTR* methylation and childhood anxiousness and disruptiveness trajectories were performed on females only. Teacher-rated childhood anxiousness trajectory was significantly related to methylation frequency on 4 CpG sites: Intron 1 CpG 5 (R^2=0.18, p=0.043*), Promoter CpG 3 (R^2=0.22, p=0.022*) and Promoter CpG 7 (R^2=0.50, p<0.001**),

and Enhancer 1 CpG 2 (R^2=0.23, p=0.026*). The teacher rated childhood trajectory of disruptiveness was not significantly associated with any CpG sites.

Mediation analysis

Prior analyses indicate that there is a relationship between ELA, childhood trajectories of anxiousness, and *OXTR* DNA methylation. We hypothesize that teacher-rated childhood anxiousness trajectory may act as a mediator between early life adversity and subsequent *OXTR* methylation changes. We observed a significant mediation between ELA, teacher-rated childhood anxiousness trajectory, and Promoter CpG 7 methylation in our model (*ab*= 5.75, p<0.05* [95% CI, 0.057, 0.76]). Please refer to **Figure 8.** For the other models, we observed significant independent effect of ELA on one other CpG (Intron 1 CpG 5,p<0.05*) (**Table 1)**. No other significant independent effects of teacher-rated childhood anxiousness trajectory were observed.

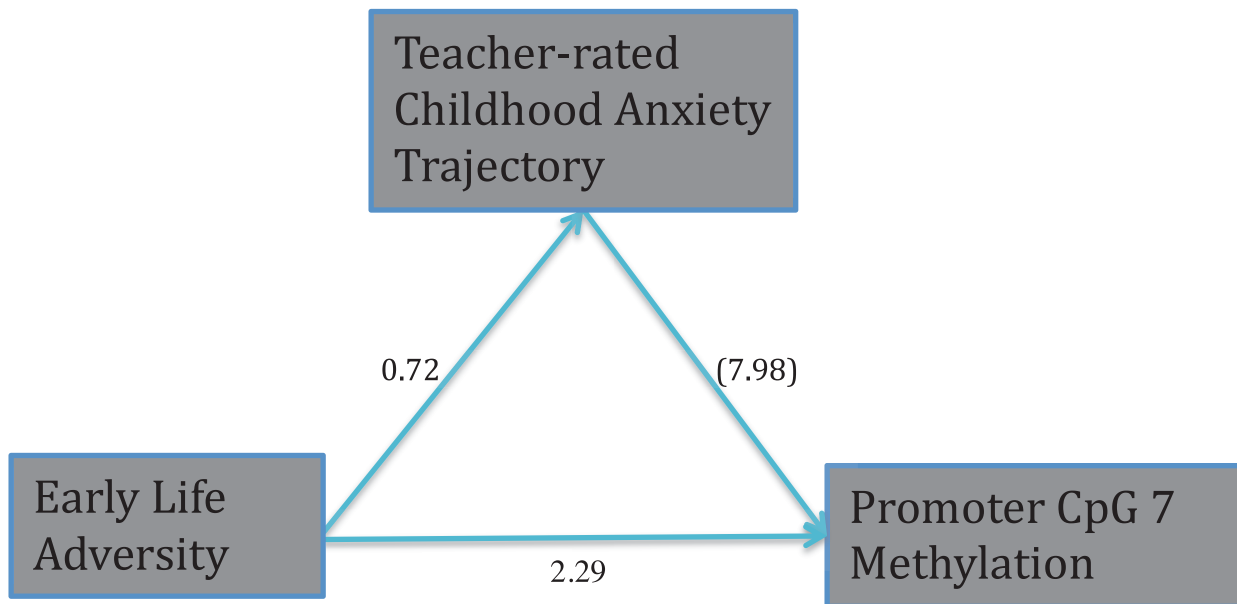


Figure 8. Mediation analysis, where X=ELA, M=Teacher-rated Childhood Anxiousness Trajectory, and Y= Promoter CpG 7 Methylation.

X	ELA				
M	ANXT				
Y	Intron 1 CpG 5				
	coeff	se	t	d	p
ELA	6.030	2.220	2.710		0.0135*
ANXT	0.590	1.650	0.356		0.723
Ab				0.423	ns. [95% CI, -1.46,2.51]
Y	Promoter CpG 3				
	coeff	se	t	d	p
ELA	1.810	0.943	1.920		0.069
ANXT	0.645	0.700	0.921		0.368
Ab				0.460	ns. [95% CI, -0.34,2.67]
Y	Promoter CpG 7				
	coeff	se	t	d	p
ELA	2.300	3.330	0.689		0.498
ANXT	7.980	2.470	3.230		0.004
Ab				5.740	p<0.05* [95% CI, 0.057, 0.76]).
Y	Enhancer 1 CpG 2				
	coeff	se	t	d	p
ELA	-0.935	0.783	-1.190		0.247
ANXT	-0.883	0.572	-1.540		0.140
Ab				-0.610	ns. [95% CI,-2.23, .063]

Table 2. Mediation Table. ab denotes indirect effect of X on Y. X denotes independent variable ELA, Y denotes dependent variable, *OXTR* methylation, and M denotes the mediator, childhood anxiousness.

Functional validation of the differentially methylation promoter region in *OXTR*

While the previous results show that there are significant correlations among DNA methylation, ELA and childhood trajectory of anxiety, it is still unclear whether methylation of these CpG sites does indeed have an effect on transcriptional machinery that may alter *OXTR* expression or other affected downstream genes. To determine the functional activity of Promoter CpG 7, a key CpG impacted by ELA, we introduced the differentially methylated regions in *OXTR* (Ctrl-no insert, Promoter Sense, Promoter Antisense, and Promoter Subregion) to the the

pCpGL-basic CpG-free luciferase reporter plasmids and performed *in vitro* methylation with SSSI methyltransferase. The introduced promoter region contains the Promoter CpG 7 that we found differentially methylated between high and low ELA group in among females. Since the plasmid and the reporter do not contain CpG sequences, SSSI methylats the *OXTR* regions exclusively and we could therefore measure the effects of methylation of the *OXTR* without confounding effects of vector methylation. Introduction of the putative promoter region to the reporter vector induced reporter luciferase activity, confirming that the region is indeed an active promoter. We then compared the luciferase activity driven by the unmethylated promoter with the unmethylated promoter sense plasmid construct, the empty vector and methylated and unmethylated anti-sense constructs (*Promoter sense unmethylated* versus *sense methylated*, average fold change =6.8, t=7.38, p=0.002**. Promoter *sense unmethylated* versus *antisense unmethylated*, average fold change =2.6, t=-4.41, p=0.01*. Promoter *sense methylated* versus *antisense unmethylated*, average fold change = 2.6,t=2.81, p=0.04*) (**Figure 9)**. These data are consistent with the idea that Promoter CpG 7 is important for regulating the expression of the *OXTR* gene.

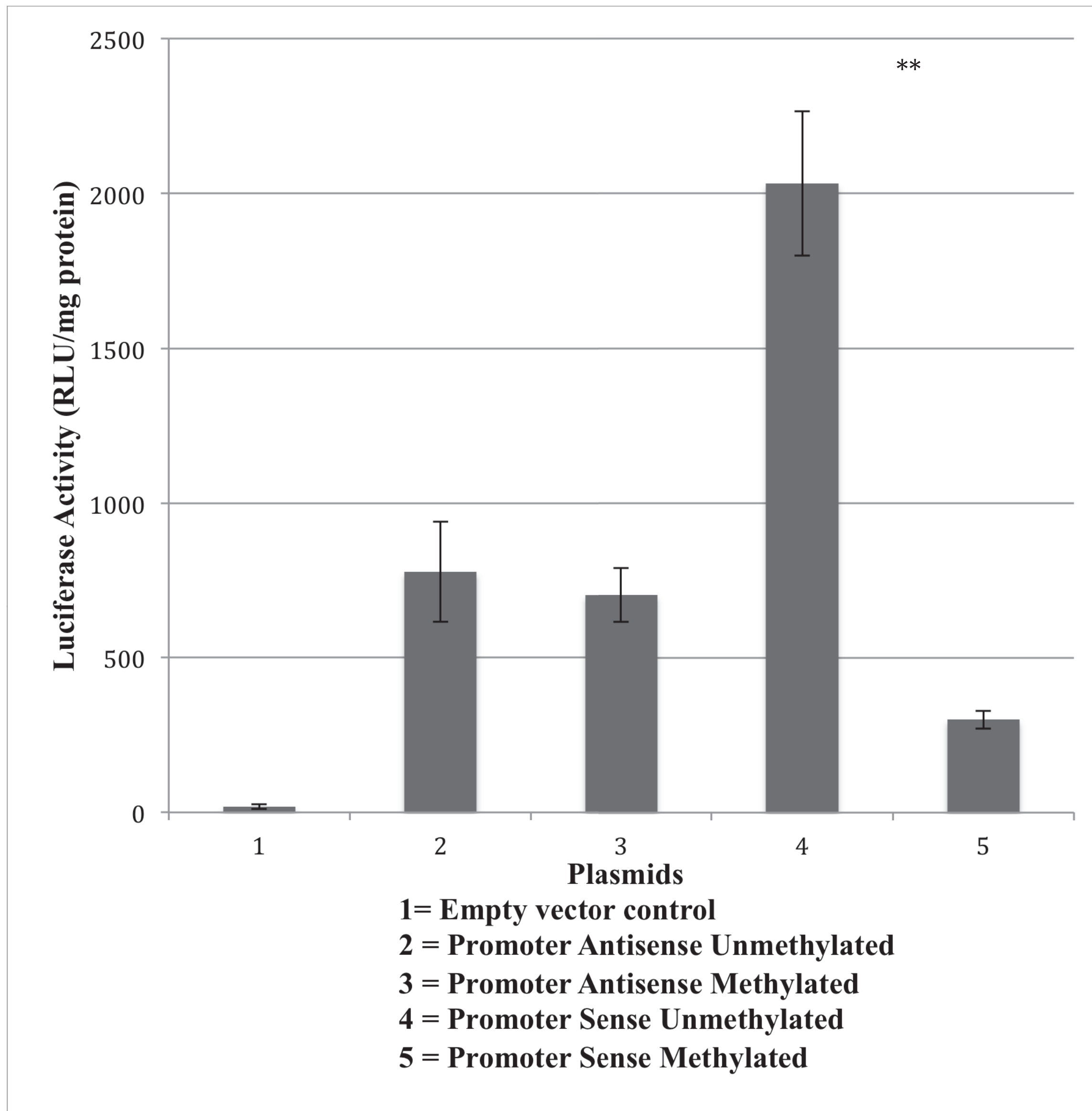


Figure 9. *In vitro* methylation regulates gene expression at *OXTR* promoter. Values are expressed as mean±SEM. Antisense promoter sequence insert exerts some activity as to be expected. Methylation of promoter sense sequence significantly reduced luciferase activity, **=p<0.01

DISCUSSION

We investigated associations among exposure to early life adversity, childhood trajectories of anxiousness and disruptiveness, and *OXTR* DNA methylation in adulthood. Exposure to early life adversity was significantly associated with *OXTR* DNA methylation in females, but not in males. Furthermore, *OXTR* DNA methylation was associated with teacher-

rated childhood anxiousness trajectory. For Promoter CpG 7, teacher-rated childhood anxiety trajectory also mediated the relationship between ELA and methylation changes in adulthood among females. Lastly, the functional significance of this promoter CpG site was validated using an *in vitro* methylation of a plasmid construct with inserted promoter sequence.

The current study investigated 4 distinctive genomic regions within the *OXTR* gene including a promoter region upstream of the transcription start site (TSS; where transcription of DNA to mRNA starts) next to exon 1, a separate distal enhancer element located within intron 3 and an intron 1 region previously associated with autism symptoms, externalized behaviours, and psychological distress (Dadds, Moul, Cauchi, Dobson-Stone, Hawes, Brennan, & Ebstein, 2014; Gregory et al., 2009; Jack et al., 2012; Reiner et al., 2015; Smearman et al., 2016). In the present study, CpG methylation sites across the *OXTR* promoter, Intron 1, and an enhancer element were significantly differentially methylated as a function of ELA exposure among females, with high ELA exposure being associated with greater *OXTR* DNA methylation. In contrast, Needham et al. (2015) found that low childhood SES (a factor within our ELA measure in the present study) was associated with increased DNA methylation at *OXTR* non-promoter CpG sites, but not at promoter sites. A separate study by Unternaehrer et al. (2015) examining the effect of maternal care on *OXTR* DNA methylation also found an association between lower maternal care and higher methylation of specific CpG within *OXTR* exon 3 DNA segment. Moreover, a recent study by Smearman et al. (2016) found that early child abuse was associated with higher *OXTR* DNA methylation of two particular CpG sites located in exon 3 among African-Americans.

High ELA was associated with higher mean methylation in 4 CpG sites (Intron CpG 4, Intron CpG 5, Promoter CpG 3, and Promoter CpG 7), but lower mean methylation at Enhancer 1 CpG 2. An extensive literature indicate that greater methylation in specific regions (especially

the promoter) is associated with lower gene expression (Razin & Cedar, 1991; Weaver et al., 2004). Interestingly, we also observe a single lower methylated CpG site within the first enhancer element of *OXTR* in the high early adversity group, suggesting possible co-regulation of *OXTR* and the existence of a possible transcriptional repressor in this distal element. A lower methylation average of this enhancer CpG site in individuals with high early adversity may lead to the activation of such putative repressor, leading to the inhibition of *OXTR*. This may be possible when the enhancer in question contains an inhibitor binding sequence, where the binding of such inhibitor causes increased inhibition of *OXTR* (Blackwood & Kadonaga, 1998). More investigations are required to confirm this preliminary finding.

Furthermore, there were significant correlations between CpGs sensitive to ELA and teacher-rated childhood anxiousness trajectory in 4 CpGs (Intron CpG 5, Promoter CpG 3, Promoter CpG 7, and Enhancer 1 CpG 2). *Higher* methylation (Promoter CpG 3 and 7, Intron CpG 4 and 5) was associated with greater teacher-rated childhood anxiousness trajectory. In contrast, Smearman et al. (2016) found that different *OXTR* methylation interacted with early child abuse to predict depressive and anxiety symptoms, such that individuals with a history of severe ELA and *lower* CpG methylation at particular CpG sites in *OXTR* exon 1 reported *higher* anxious and/or depressive symptoms compared to individuals with *higher* CpG methylation at the same sites with a history of severe ELA. However, an interaction was also observed with other CpG sites along *OXTR* intron 3 (cg11589699) where individuals with *higher* CpG methylation and a history of severe ELA reported higher depressive and/or anxious symptoms compared to those with lower CpG methylation and a history of severe ELA at the same CpG sites. Methodological differences between Smearman et al. and our study may explain these differences. Whereas Smearman examines *older* low SES African-American adults self-reporting

depressive and anxiety symptoms *in adulthood,* our study examined teacher-rated anxiousness and disruptiveness childhood trajectories evaluated during *childhood* among younger adults of European-descent with a wide range of SES. Although the direction of observed methylation changes was different, both Smearman et al. and our study highlighted differences observed in promoter and intron 3 regions where no other previous studies have investigated. Other studies in patients with social anxiety disorder (SAD), and depression reported that *OXTR* DNA methylation was significantly associated with the severity of anxiety and depressive symptoms (Bell et al., 2015; Kimmel et al., 2016; Ziegler et al., 2015). Together, these data highlight an association between *OXTR* DNA methylation and anxiety and depression.

Interestingly, teacher-rated childhood anxiousness trajectory mediated the relationship between ELA and *OXTR* DNA methylation, suggesting a possible pathway whereby ELA led to increased anxiety. Indeed, Promoter CpG 7 significantly differed between the high and low ELA groups, and teacher-rated childhood anxiousness acted as a mediator of the methylation changes occurring on Promoter CpG 7 for women. This suggests a pathway of action whereby ELA affects teacher-rated childhood anxiousness trajectory, which in turn, leads to methylation changes observable in adulthood. Other studies suggest that such methylation changes, as a result of ELA, may lead to volumetric differences in specific areas of the brain implicated in social cognitive abilities (Rubin et al., 2016).

Absolute sex differences in *OXTR* DNA methylation were also present. Promoter CpG 7, Intron CpG 2, Intron CpG 3, and Promoter CpG 9 methylation were significantly different between males and females. Similarly, Unternaehrer's findings also suggest absolute sex differences in *OXTR* DNA methylation located in the exon 3 region, where men have lower DNA methylation compared to women. The small mean gender differences we observe among

female and male subjects irrespective of adversity groups is not surprising as an abundance of animal and human literature suggest such sex-related genome-wide and gene-specific epigenetic differences (McGowan et al., 2011).

The association between *OXTR* DNA methylation and early abuse was only significant among females. These data parallel findings on the sexually dimorphic role of oxytocin in social behavior in different rodent species (Uhl-Bronner et al., 2005). There exists differential *OXTR* methylation in the exonic *OXTR* region with respect to childhood maternal care in both men and women (Unternaehrer et al., 2015). Further, Rubin et al., (2016) observed sex-specific associations between *OXTR* methylation and behavior. Moreover, some studies report gender differences in the effect of administration (Ditzen et al., 2013; Fischer-Shofty, Levkovitz, & Shamay-Tsoory, 2013; Rubin et al., 2016; Tseng et al., 2014). Findings in humans, however, are more mixed. In other behavioural tasks such as self-perception, there were no gender differences in the effects of administration of oxytocin (Cardoso, Ellenbogen, & Linnen, 2012). Nevertheless, women's greater sensitivity to the impact of ELA on *OXTR* methylation than men may partially explain the epidemiological evidence highlighting the doubled risk for women to develop anxiety and/or depression related disordered compared to men (Nolen-Hoeksema & Girgus, 1994).

OXTR DNA methylation can interact with other genetic variations such as single nucleotide polymorphisms (SNP). Recent research on *OXTR* genotype and methylation in older women showed that *OXTR* methylation is increased for women with anxiety and depression only when a specific genotype was present (Chagnon, Potvin, Hudon, & Preville, 2015). Another study in clinically depressed female patients showed similar results where *OXTR* methylation within exon 1 was lower under the presence of specific rs53576 genotype (GG) when compared

to both AA/AG depressed patients and non-depressed female controls (Reiner et al., 2015). In addition, interactions between rs53576 and increasing *OXTR* DNA methylation was shown to increase risk to develop post-partum depression in women who did not experience prenatal depression, while no such GxE interaction existed for women who experienced prenatal depression (Bell et al., 2015). Similar results were also observed in a primarily female African-American population where both altered DNA methylation at *OXTR* CpG site within Intron 3 and specific *OXTR* SNPs rs237897 and rs7629329 were associated with increased anxiety and depression symptoms in samples who experienced varying levels of early life trauma (Smearman et al., 2016). This suggests *OXTR* polymorphisms as possible moderators of the effect of *OXTR* methylation on anxiety and depression.

In this study, ELA-related differences in *OXTR* methylation were observed more than 20 years after the initial study assessment. The observed methylation differences between groups were at most 8%, with the average difference between all significant sites at 5%. The Cohen's d effect sizes of the methylation difference between ELA groups are medium to large for selected CpGs among females. The observed between groups methylation difference is consistent with other studies involving early life adversity, DNA methylation, and psychiatric disorders (depression, BPD, PTSD) whereby mean methylation differences related to phenotypic or environmental factors ranges from 1% to 5% using salivary or blood samples (Martín-Blanco et al., 2014; Melas et al., 2013; Perroud et al., 2011; Yehuda et al., 2015). Nevertheless, as the participants' blood samples were collected in adulthood while early life adversity was assessed many years prior to sample collection, it indicates that the methylation signature of early life adversity persists after prolonged period of time.

Animal models examining the effect of early maternal deprivation in monkeys reveal system-wide epigenetic changes associated with the different rearing conditions that persist into adulthood, from14-30 days, until age 7 (Provencal et al., 2012). Similarly in humans, McGowan et al. (2009) demonstrated that methylations levels of specific CpG dinucleotides in the glucocorticoid receptor gene (*GR*) were higher for adult individuals with a history of childhood abuse than those without a history of early abuse. In addition, other research has found persistent DNA methylation differences in another important gene, brain-derived neurotropic factor (BDNF), in subjects who experienced childhood maltreatment compared to those without a history of such experiences (Essex et al., 2013). Similarly, epigenome-wide studies among adults also identified multiple differentially methylated gene promoters that associated to early life trauma and persisted into adulthood (Labonte et al., 2012; Suderman et al., 2014). These converging lines of evidence suggest that exposure to early life adversity leads to persistent changes in DNA methylation in adulthood.

The exact mechanism of how early life experiences are translated into DNA methylation changes is not yet clear. It has been postulated that external stimuli may trigger specific signaling pathways, which then recruit methylation-specific proteins to exert its actions on specific locations (Szyf et al., 2008). For example, it is believed that maternal behaviour triggers specific serotonin signaling pathways in the brain, followed by release of secondary messenger signals (cAMP), which recruits methylation-specific enzymes that are targeted to specific genome locations and methylates/demethylates the corresponding genomic regions (Szyf & Bick, 2013). This is thought to lead to active transcription or repression of gene expression. According to Szyf & Bick (2013), DNA methylation of *OXTR* may then affect other downstream effectors within

the oxytocinergic and/or vasopressinergic system and other genes involved in same functional circuitry responsible for the regulation of social behavior.

Early life adverse events may affect DNA methylation signatures not just within candidate genes, such as *OXTR*, but rather the effect may be more genome-wide and system-wide (Suderman et al., 2014). Given that genes do not act individually but in clusters of functional circuitries, it is likely that changes in one candidate gene will have ramifications in other genes within the same functional pathway (van Weerd, Koshiba-Takeuchi, Kwon, & Takeuchi, 2011). There is evidence showing system-wide methylation changes in response to maternal care (Provencal et al., 2012). In the present study, CpG dinucleotide spanning thousands of base pairs apart were influence by ELA. Other studies found more than 900 differentially methylated gene promoters in over 1000 genes spanning the whole genome in association with maternal care and childhood abuse in both animal and human models (Provencal et al., 2012; Suderman et al., 2014). Furthermore, these differences were enriched in regulatory and developmental genes and encompass multiple functional categories such as chromatin modification, histone modification, transcription binding as well as signaling pathways, all of which are crucial during early developmental periods (Suderman et al., 2014). The exact cascade of genes modulated by ELA, however, still needs to be elucidated.

STRENGTHS & LIMITATIONS

This study possesses several unique strengths. The study participants were selected from a longitudinal cohort that was recruited at the same age and from the same racial background (French-Canadian with European descent), where age-related and race-related genetic differences are minimized. The second strength is the prospective assessment for early life adversity during childhood and adolescence. Finally, teachers, minimizing the risk for gene-

environment correlation associated with mother- or participants-reports, rated the measures for childhood behaviour trajectory.

While this study has several unique strengths and provides some unique insights, it is not without limitations. Because this study is a pilot study, the number of subjects tested is relatively small (n=46). In terms of statistical analysis, we did not perform multiple testing adjustments as the current study is exploratory in nature and is aimed at selecting novel CpG dinucleotides of interest in the *OXTR* for further investigation. Given the small sample, statistical adjustment would enhance the risk of type II error. Replications of the present results are thus paramount.

Moreover, because *OXTR* exerts most of its modulatory effects in the brain, using blood as our primary tissue provides limited insights into the exact changes of DNA methylation within the brain itself. However, there is evidence showing that DNA methylation is correlated among different tissue samples including the brain and blood (PBMC), which highlights the use of blood samples as a reliable and potentially non-invasive method of estimating the methylation levels in brain samples (Ma et al., 2014; Provencal et al., 2012; Smith et al., 2015; Wang et al., 2012). One study has found concordance between peripheral blood and temporal cortex among individuals with autism, while another study presented correlations between the serotonin transporter (SLC6A4) methylation and *in vivo* differences in brain imaging activity (Gregory et al., 2009; Wang et al., 2012). Recent studies also found evidence that changes in DNA methylation within the *OXTR* promoter in response to maternal care are concordant between peripheral tissues and the brain (Beery, McEwen, MacIsaac, Francis, & Kobor, 2016). While significant DNA methylation variations may be observed across tissues, there is a growing body of evidence indicating that DNA methylation from whole blood may reflect ELA-related changes in *OXTR* DNA methylation inside brain cells.

CONCLUSIONS

The results of the current study support the association between early life adversity and *OXTR* DNA methylation. The oxytocin receptor gene is a promising target for future investigations as a potential biomarker for anxiety and depression. It is the only receptor for oxytocin currently, and preliminary studies highlight its sensitivity to both genetic and epigenetic regulations. Given the association between *OXTR* methylation and anxiety, DNA methylation may act a mechanism responsible for translating negative early experiences into biological signals that have long lasting impacts on psychosocial development. DNA methylation may be an important marker to consider when examining risk factors for anxiety and/or depressive disorders. Future studies focusing on epigenetic regulation and single nucleotide polymorphisms of the *OXTR* gene and the closely related vasopressin receptor families and system may provide a complete biological mechanism mediating the impact of the social environment on risk for anxiety and depression.

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APPENDICES

Appendix A. Supplementary Tables

PCR Primers		
Promoter	OXTR_Promo_F1	GTTGGTTTTAGAGTTTTAATAAATGGG
	OXTR_Promo_R1	/5Biosg/ATTTTTAAATCCTAACCTTTTTTTCTAACT
	OXTR_Promo_8CG_F2	ATGGGTTTATTTTGTAGTGGTTTAA
	OXTR_Promo_8CG_R2	/5Biosg/AATAACCCCTCTCCCAACACTACTTAAAA
	OXTR_Promo_7CG_F3	ATTAGAAATGGTTTTTATTTTAAGTAGTGT
	OXTR_Promo_7CG_R3	Same as OXT_Promo_R1
Intron	OXTR_Intron_F1	GAGAGATTTTAGTTTAGTATTATATTAG
	OXTR_Intron_R1	CCCTAAACTTCCACAACACCTAC
	OXTR_Intron_F2	GTAGTTGGGTGTTAAGTAGGTAG
	OXTR_Intron_R2	/5Biosg/CAAACCCTAACATAAACACCTCC
Enhancer 1	OXTR_Enh1_F1	GTTTTGAGTTTTTGGTGATTTTTTTT
	OXTR_Enh1_R1	TTTCCCTACCCCTTAAACTTCAACA
	OXTR_Enh1_F2	GGTTTTTTGTTTTTTAGTTTTTTTATTTGT
	OXTR_Enh1_R2	/5Biosg/CACTCTAACCAAAATCTATATATATC
Pyrosequencing Primers		
Promoter	Promo_SQ1	GTAGTGGTTTAAAATT
	Promo_SQ1_2	GAGAGGGAGGGAATT
	Promo_SQ2	GGTTTAGAAGTTTTTG
	Promo_SQ2_2	AGAAGTTTTTGATTT
	Promo_SQ3	ATTTTTAGTTTGATGTAG
	Promo_SQ4	GGAATATTTTTGTTTTTATT
	Promo_SQ5	GTAGTGTTGGGAGAG
	Promo_SQ6	AGTGATTTTGTTTGTTT
	Promo_SQ6_2	GTTTAAGAGT
Intron	Intron_SQ1	TTAAGTAGGGGTGGA
	Intron_SQ2	GGTGGTGGGGTGTTT
	Intron_SQ3	TTGTAAAGTGATTTT
	Intron_SQ4	TATTTGGGTTTAAAG
Enhancer 1	Enh1_SQ1	GAGTGTGTGGTTAAT
	Enh1_SQ2	TTATATTTTAAAAGTA

Supplementary Table 1. *OXTR* Targeted Regions Primers List

Plasmid Primers		
	Promo_S_F1 Promo_S_R1	TCACGGATCCAAATGGGTTTATTT GTGAAAGCTTCTCAAGTCTCTCCAC
	Promo_AS_F1 Promo_AS_R1	TCACGGATCCTCAAGTCTCTC GTGAAAGCTTTTTGCAGTGGTTTAA
Plasmid Sequencing Primers		
	Promo_SQ_F	GGGTTTATTTTGCAGTGGTTTAAAAC

Supplementary Table 2. Plasmid Primers List