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Variation in DNA Methylation of the Oxytocin Receptor Gene Predicts Children's Resilience

to Prenatal Stress

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Abstract

Background: Emerging research in epigenetics has shown that there is variability in how environmental exposures "get under the skin" through mechanisms like DNA methylation to influence gene expression that may lead to differential adaptation to stress. This is the first study to examine prospectively the relationship between DNA methylation at birth and resilience to prenatal environmental stressors in several domains (conduct, hyperactivity, emotional problems and global symptomatology) in middle childhood. *Method:* We focused on DNA methylation in the vicinity of the oxytocin receptor (*OXTR*) gene as it has been previously associated with impairments in social-cognitive processes that may underlie a wide range of childhood psychopathology. Participants were 91 youth exposed to pre-and postnatal adversity with established conduct problem trajectories drawn from the Avon Longitudinal Study of Parents and Children (ALSPAC). *Results:* Consistent with our hypothesis, *OXTR* DNA methylation was predictive of resilience in the conduct problems domain in mid-childhood. DNA methylation profiles did not predict resilience in domains of emotional, hyperactivity, and global symptomatology suggesting a potential role for *OXTR* in the development of conduct problems in particular. However, individuals who were resilient to conduct problems were also broadly resilient across multiple domains. *Conclusions:* Future research should elucidate the biological pathways between *OXTR* DNA methylation and gene expression and its relation to impairments in social behavior.

Key Words: Oxytocin-receptor gene, DNA methylation, resilience, prenatal, conduct problems

Resilience is defined as successful emotional, behavioral, or social adaptation or adjustment despite experience of significant adversity, stress, or trauma (Luthar, Cicchetti, & Becker, 2000; Rutter, 2006). In children, researchers have operationally defined this adaptation in a myriad of ways such as mastering normative developmental tasks (Luthar, Crossman, & Small, 2015), absence of psychopathological outcomes (Martinez-Torteya, Bogat, Von, & Levendosky, 2009) or functioning that is "better than expected" given a level of exposure to risk (Kim-Cohen, Moffitt, Caspi, & Taylor, 2004). Some children show resilience across multiple domains of functioning. More commonly, however, at-risk children show resilience in one domain of functioning but not in others or they may be resilient at one time period but not another (Masten, 2012). Thus, resilience as a construct may be better defined as a dynamic process (not a trait or characteristic) that depends on the balance of risk and protective factors available to an individual at a given point in time (Jaffee & Gallop, 2007; Rutter, 2006; Rutter, 2012). Understanding the mechanisms that promote resilient functioning in addition to the processes that confer risk for psychopathology, and the dynamic balance between them, is essential to understanding how normative and maladaptive developmental trajectories form.

Researchers have long posited the importance of adverse life events during early critical periods in understanding risk and resilience. The prenatal period, specifically, is one in which the fetus is especially vulnerable to a wide range of environmental exposures that have the potential to confer risk for emotional, cognitive, and behavioral problems in childhood (Braithwaite, Murphy, & Ramchandani, 2014; Rice, Jones, & Thapar, 2006). A large number of studies have shown that exposure to prenatal maternal psychopathology such as depression and anxiety is associated with both internalizing and externalizing behavioral outcomes in children such as depression, anxiety, ADHD symptoms, and conduct problems (e.g. Barker, Jaffee, Uher, & Maughan, 2011; O'Connor, Heron, Golding,

Bereridge, & Glover, 2002; O'Donnell, Glover, Barker, & O'Connor, 2014; Robinson et al.,

2011; Van den Berg & Marcoen, 2004; Van den Berg et al., 2006). Prenatal exposure to

maternal stressful life events, such as death of a close relative or friend, divorce, marital

problems, and job loss, has also been linked to ADHD, behavioral problems, and

internalizing symptoms (Laucht et al., 2000; Pawlby, 2009; Ronald, Pennell, & Whitehouse,

2011).

It is clear that a range of stressors during the prenatal period increase risk for child

psychopathology. However, not all children exposed to environmental stressors in utero go

on to develop psychopathology and some children seem to be less vulnerable than others.

There are several hypotheses that can potentially account for this differential vulnerability.

One possibility is that a supportive postnatal environment can attenuate or reverse the effects

of prenatal stress. For example, researchers have found that sensitive caregiving moderates

the effect of prenatal maternal stress on infant fearfulness (Bergman, Sarkarm, Glover, &

O'Connor, 2008) and cognitive outcomes (Bergman, Sarkarm, Glover, & O'Connor, 2010).

A second possibility is that individual differences in genotype confer protection

against prenatal stressors. For example, researchers have found that variation in the

glucocorticoid receptor gene (*NR3C1*), a gene integral to the functioning of the

hypothalamic-pituitary-adrenal (HPA) axis which is involved in stress reactivity, moderated

the effect of prenatal maternal psychological symptoms on later emotional and behavior

problems, such that children whose mothers were depressed or anxious when they were

pregnant with the child had an increased risk of emotional and behavioral problems at age 3 if

they possessed the minor allele C (CC or CG), but not if they were homozygous for the major

allele (GG) (Velders, 2011). Using data from 1513 children in the Generation R cohort,

Pluess and colleagues (2011) found that infants whose mothers were more anxious during

pregnancy had higher scores on a measure of negative emotional temperament than infants

whose mothers were not anxious and this effect was significantly stronger for infants who carried the short 's' form of the serotonin transporter gene (5-HTTLPR) compared with infants who carried two copies of the long 'l' form of the gene. In addition, Oberlander and colleagues (2010) found that prenatal exposure to maternal anxiety predicted internalizing symptoms in children with 2 copies of the 5-HTTLPR 's' allele (but not in children who carried the 'l' allele). In contrast, a mother's anxiety during pregnancy predicted her child's externalizing problems only if her child had 2 copies of the 'l' allele and not if the child carried at least one 's' allele (Oberlander et al., 2010).

In addition to identifying structural variants in the genome that buffer against the effects of prenatal stress, new research in the field of behavioral epigenetics has started to elucidate the underlying biological mechanisms of the relationship between stress exposure and later developmental outcomes, including emotional and behavioral problems. Epigenetic research sits at the intersection of social and biological explanations for developmental psychopathology and has enormous potential for describing how stressful life events "get under the skin" and have lasting effects on mental and physical health. The epigenome describes the chemical switches that sit on top of genes and modulate gene expression. Stress-induced epigenetic modifications are typically measured by examining DNA methylation, where methyl groups are added to cytosine-guanine-phosphate (CpG) sites on the regulatory or promoter regions of genes to silence transcription factors or block access to recognition elements of a gene (Bick et al., 2012). DNA methylation is typically related to lower gene expression in promoter regions. However, DNA methylation in other genomic regions can have the opposite effects on expression (e.g. gene body) and there is little known about the functional role of DNA methylation in other locations such as the intergenic region (Jones, 2012). Studies have shown that DNA methylation patterns are under significant control – as evidence by the discovery of a large number of methylation quantitative trait loci

(mQTL; Gaunt et al., 2016; Jones, Fejes, & Kobor, 2013) – but are also sensitive to environmental influences (McGowan & Roth, 2015). Although the environment modifies the epigenome throughout the lifespan, there is some evidence that the in utero environment has the largest effect (Billack, 2012). These prenatal effects have been largely interpreted in terms of the fetal programming hypothesis in which the fetus adapts its phenotype – such as stress reactivity or metabolism – to what it anticipates its postnatal environment to be on the basis of the biological cues from the mother's environment (Gluckman, Hanson, & Thornburg, 2008).

The majority of studies investigating the association between prenatal exposure to maternal stress and methylation have focused on *NR3C1*. Prenatal stressors such as maternal depression (Condradt, Lester, Appleton, Armstrong, & Marsit, 2013; Hompes et al., 2013), exposure to intimate partner violence (Radtke et al., 2011), and exposure to war (Mulligan, Errico, Stees, & Hughes, 2014; Perroud et al., 2013) have been associated with increased methylation of *NR3C1* at birth. There is, however, variability in these methylation profiles, even among newborns whose mothers reported high levels of stress and this variability may be predictive of children's risk for emotional or behavioral health problems versus their resilience. To date, few biologically informed prospective studies have explored gene-specific methylation patterns in the context of resilience. We focus on methylation of the oxytocin receptor gene (*OXTR*).

Oxytocin is an essential neuropeptide and hormone in the regulation of social and affiliative behavior such as empathy, attachment, bonding, emotion recognition, and processing of social stimuli (Jack, Connelly, & Morris, 2012). Oxytocin has also been shown to have anxiolytic effects by dampening physiological, hormonal, and brain-level responses to stressful or aversive signals (Heinrichs, von Dawans & Domes, 2009). Thus, stress-related epigenetic changes in the oxytocin system may confer risk for the development of

psychopathology by shaping socio-emotional, socio-cognitive, and stress response systems that underlie temperament and children's relationships with peers and adults.

It is biologically plausible to predict that prenatal (or postnatal) stressors would be associated with increased DNA methylation and, in turn, increased emotional and behavioral problems. That is, if DNA methylation acts as a gene silencer, increased methylation in the promoter region of the gene would result in lower messenger RNA (mRNA) levels, blocking of transcription factors, and in turn, decreased gene expression and decreased circulating oxytocin in the blood. Indeed, a number of studies have found evidence for these relationships. For example, increased *OXTR* methylation leads to decreased *OXTR* mRNA expression in hepatoblastoma human cells (Kussui et al., 2001) and in murine cells (Mamrut et al., 2013) in the promoter region of the gene. Gregory and colleagues (2009) found increased promoter region *OXTR* methylation in peripheral blood as well as in temporal cortex tissue in individuals with autism compared with controls. Increased methylation resulted in a 20% reduction in mRNA expression.

Consistent with the possibility that *OXTR* methylation may be a mechanism by which prenatal exposures increase risk for psychopathology, Dadds and colleagues (2014) found that elevated methylation in *OXTR* in a sample of 4- to 16-year-olds was associated with lower levels of circulating oxytocin and higher levels of callous-unemotional traits. Similarly, in a sample of youth with early-emerging and persistent conduct problems, Cecil and colleagues (2014) found that higher methylation at birth at the *OXTR* locus was associated with higher levels of callous-unemotional traits at age 13, although the effect was only observed in youth with low levels of internalizing problems. Moreover, mothers' reports of behaviors that might have caused stress to themselves or the fetus (e.g., their own criminal behavior, their partner's criminal behavior, their own psychopathology and substance use) were associated with elevations in *OXTR* methylation at birth.

Despite the plausibility of a pathway by which prenatal stressors lead to increased *OXTR* methylation, a number of studies have found the inverse relationship between *OXTR* methylation and prenatal stress as well as psychological outcomes. For example, one study focusing on prenatal stress found that the more life-changing stressful events a mother experienced when she was pregnant, such as being a victim or witness of assault or experiencing the severe illness or death of a loved one, the *lower* the *OXTR* methylation levels in cord blood at birth (Unternaehrer et al., 2015). Reiner and colleagues (2015) found that depressed women had lower *OXTR* exon 1 DNA methylation levels compared to non-depressed women. Moreover, Ziegler and colleagues (2015) found in a sample of adults that decreased *OXTR* methylation was associated with a diagnosis as well as symptoms of social anxiety disorder, increased cortisol responses to a stress test, and increased amygdala responsiveness during social anxiety word processing. In addition, in a brain imaging study, researchers found that higher *OXTR* methylation was related to *increased* brain activity in areas associated with social perception such as the temporoparietal junction and the dorsal anterior cingulate cortex (Jack, Connolley, & Morris, 2012). Thus, the evidence is mixed as to whether prenatal stressors are associated with increased or decreased *OXTR* methylation and whether individual differences in *OXTR* methylation are associated with positive or negative child (or adult) outcomes. Direction of effects could be highly dependent on the location of the probes examined.

The present study is the first to make use of a longitudinal design to examine if *OXTR* methylation at birth can differentiate resilient and non-resilient youth as measured by hyperactivity, conduct problem, and emotional problem outcomes in mid-childhood. Additionally, a strength of the study is that only children with pre- and post-natal adversity were included which ensures that resilience is not driven by differences in the quality of the postnatal environment. Given mixed findings in the literature about the direction of the

relationship between stress in pregnancy and methylation levels at birth as well as the relationship between methylation levels and later behavior, we do not propose a directional hypothesis. Understanding plasticity at this critical period in development can help us examine how early stress can "get under the skin" and alter developmental trajectories. We hypothesize that this variability will be predictive of which newborns grow up to have low levels of psychopathology, despite their exposure to prenatal risk factors.

Methods

Participants

The *Avon Longitudinal Study of Parents and Children* (ALSPAC) is an ongoing epidemiological study of children born from 14,541 pregnant women residing in Avon, UK, with an expected delivery date between 1st April 1991 and 31st December 1992 – 85% of eligible population (Fraser et al., 2013). Ethical approval for the study was obtained from the ALSPAC Law and Ethics Committee and the Local Research Ethics Committees.The sample is representative of the general population (Boyd et al., 2013). The study website contains details of all the data that is available through a fully searchable data dictionary: http://www.bris.ac.uk/alspac/researchers/data-access/data-dictionary/.

The *Epigenetic Pathways to Conduct Problems Study* consists of a subsample of youth (n = 339, 50% female) nested within a larger study of DNA methylation in ALSPAC (www.ariesepigenomics.org) (Relton et al., 2015) who follow previously established trajectories of conduct problems (4-13 years) (Barker & Maughan, 2009) and have epigenetic data at birth and/or childhood. This subsample is comparable to the full trajectory sample (n = 7,218) in terms psychiatric comorbidity (Barker, Oliver & Maughan, 2010). DNA methylation measures were available for 326 youth at birth. Children with missing ethnicity information were removed, leaving a total sample of 321. Except for factor analyses, in which we used data from all youth, the present study only included youth who scored above

the sample average on our measure of prenatal and postnatal (birth to age 7) environmental

risk exposure. Although exposure to prenatal risk was the focus of our study, we wanted to

ensure that differences in the postnatal environment did not account for any observed

associations between methylation profiles at birth and resilience in middle childhood. These

measures of prenatal and postnatal risk are described below in the section "Environmental

Risk." The final analytic sample was $n = 91$, all of whom had complete data including DNA

methylation at birth, had been exposed to pre- and post-natal adversity, and for whom

information on emotional and behavioral outcomes was collected. See Figure 1 for a flow

chart representing which youth were included in the analysis sample.

Measures

DNA methylation data at birth. Five hundred nanograms of genomic DNA from cord blood

(birth) was bisulfite-converted using the EZ-DNA methylation kit (Zymo Research, Orange,

CA, USA). The protocol followed manufacturer instructions using the recommended

alternative incubation conditions for use with Illumina Infinium arrays. Illumina

HumanMethylation450 BeadChips (Illumina, USA) were run following the manufacturer's

protocol with no modifications and arrays were scanned using an Illumina iScan (software

version 3.3.28). Initial quality control of data generated was conducted using GenomeStudio

(version 2011.1) to determine the status of staining, extension, hybridization, target removal,

bisulfite conversion, specificity, non-polymorphic and negative controls. DNA methylation

data was only available on samples that passed this stage. Samples were quantile normalised

using the *dasen* function within the *wateRmelon* package (wateRmelon_1.0.3; 19) in R and

batch corrected using the ComBat package (Johnson, Li, & Rabinovic, 2007). Probes were

removed if they were cross-reactive, used for sample identification on the array or had a SNP

at the single base extension, leaving a total of 413,510 probes (Chen et al., 2013).

We extracted data for probes located within the *OXTR* CpG island (*n* = 12), as this area has been previously investigated and shown to play a key role in modulating the transcriptional activity of *OXTR* (Kusui et al., 2001). For each probe, methylation levels were indexed by beta values (corresponding to the ratio of methylated signal divided by the sum of the methylated and unmethylated signal). Factor analysis was used to reduce the 12 *OXTR* probes into a smaller set of factors, which accounted for shared variance between them. A 3-factor solution showed the best fit to the data as well as good temporal stability. Full details of the factor analysis procedure and results can be found in Cecil and colleagues (2014). We present findings relating specifically to Factor 1, three probes located in the 5'UTR region – Probe 1 (cg00078085), Probe 5 (cg03987506), and Probe 10 (cg12695586) – because Factor 2 and 3 scores were not significantly associated with any type of resilience.

Environmental risk. The prenatal risk score comprised items that were reported by mothers and summed to create 4 conceptually distinct but related domains: (i) *Life events* (e.g. death in family, accident, illness), (ii) *Contextual risks* (e.g. poor housing conditions, financial problems), (iii) *Parental risks* (e.g. maternal psychopathology, criminal involvement and substance use), (iv) *Interpersonal risks* (e.g. intimate partner violence, family conflict). Measures of post-natal environmental risk were created for the early childhood (birth-age 7) and middle childhood (age 8-9) periods. These included all domains represented in the prenatal risk composite as well as a measure of *Direct victimization* (e.g. child bullied by peers or physically hurt; available only postnatally).

Risk domains were positively and significantly correlated, both within and between developmental periods, with the majority of correlations ranging from $r =.20-.40$. For the prenatal and postnatal periods, we used confirmatory factor analyses (CFAs) to assess the internal reliability of the risk domains and to extract one global cumulative risk score for each developmental period, showing good model fit. Higher scores indicate greater environmental

risk exposure. See online supplement in Cecil and colleagues (2014) for full item descriptions, details of inter-correlations between risk domains and factor analysis fit indices. To ensure that youth who were defined as resilient or non-resilient had been exposed to at least some moderate level of environmental risk, the sample was restricted to youth who scored above the mean on the measures of prenatal and postnatal cumulative environmental risk, as described in the Participants section.

Internalizing and Externalizing Problems. Repeated assessments of conduct problems, hyperactivity, and emotional problems were made at ages 4, 7, 8, 10, 12, and 13 via maternal reports on the Strengths and Difficulties Questionnaire (SDQ) (Goodman, 1997). The SDQ is a widely used screening instrument with reliability and validity demonstrated in a large national sample (Goodman, 2001). The SDQ comprises five subscales, each consisting of five items rated by mothers as 'certainly true', 'somewhat true', or 'not at all true'. In the current study, we utilized the conduct problems subscale (e.g., 'often fights with other children or bullies them', 'often lies or cheats'), the hyperactivity/inattention subscale (e.g., 'restless, overactive, cannot stay still for long', 'constantly fidgeting or squirming') and the emotional problems subscale (e.g., 'often unhappy, down-hearted or tearful', 'many worries, often seems worried'). In order to obtain more robust and reliable estimates of symptomatology, we performed a confirmatory factor analysis for each of the three subscales that included data from age 4 to 13, so as to generate a single factor score for each subscale that accounted for shared variance across time points. We also created a 'global symptomatology' factor score combining all three SDQ subscales as a measure of more general overall functioning. See Figure 2 for summary statistics as well as full details of the confirmatory factor analysis.

Psychosocial functioning. We used factor scores from the peer problems (e.g. 'rather solitary and tends to play alone', 'generally liked by other children') and prosocial behavior

(e.g. "considerate of other people's feelings', 'kind to younger children') subscales from the SDQ. We also utilized a six item callous-unemotional traits questionnaire completed by mothers when the child was 13 (e.g., 'makes a good impression at first but people tend to see through him/her after they get to know him/her', and 'shallow or fast changing emotions') (Moran, Ford, Butler, & Goodman, 2008). Items were rated on a three-point scale ranging from 'not true' to 'certainly' true. Social cognition was assessed using the 12-item Social Communication Disorder Checklist (Skuse, Mandy, & Scourfield, 2005) completed by mothers when the child was 7 years old. Items included for example: 'not aware of other people's feelings', 'does not notice the effect of his/her behavior on other members of the family'. Higher scores indicate lower social cognition.

Classification of Resilience. Resilience to prenatal risk was first defined in terms of conduct problems, then co-morbid problems of hyperactivity and emotional problems as well as a measure of global symptomatology across all domains. In order to classify the sample into resilient (1) and non-resilient (0) groups, we conducted four ordinary least squares regressions to predict (i) global, (ii) conduct problems, (iii) hyperactivity, and (iv) emotional problems, respectively, from the prenatal cumulative risk factor score. We utilized residuals from these regressions to classify youth into resilient and non-resilient groups in each domain. Specifically, youth with negative residual scores were classified as resilient (indicating that they had lower-than-predicted levels of psychopathology, given their exposure to prenatal risk) and youth with non-negative residual scores were classified as non-resilient (indicating that they had predicted or higher-than-predicted levels of psychopathology, given their exposure to prenatal risk). One subject with conduct, hyperactivity, and global symptomatology residual scores > 3 s.d. from the mean was removed from all the analyses. Retention of the outlier results in a non-normal distribution of resilience residuals although findings remain unchanged with the subject's inclusion. For all

domains, the distributions of the residuals were normal. See Figure 4 for resilience classification.

For resilience as defined by global problems, $n = 44$ (48%) youths were classified as resilient and $n = 47$ (52%) were not resilient. For resilience as defined by conduct problem scores, $n = 44$ (48%) youth were classified as resilient and $n = 47$ (52%) were not resilient. For resilience as defined by hyperactivity scores, $n = 50$ (55%) youth were classified as resilient and $n = 41$ (45%) were not resilient. Finally for resilience as defined by emotional problem scores, $n = 50$ (55%) youth were classified as resilient and $n = 41$ (45%) were not resilient.

Data analysis

Factor analyses were conducted in Mplus version 6.1.128 and all other analyses in SPSS 21. Regression analyses were conducted to test whether resilience (defined globally and in terms of specific domains) was associated with the Factor 1 methylation score. Post-hoc analyses were conducted to test whether resilience was associated with the individual probes (Probes 1, 5, 10) that make up Factor 1. Covariates in all models included sex and cell-type composition, estimated using the approach described in Houseman and colleagues (2012). Analyses were bootstrapped 10,000 times. Bootstrapping is advantageous with small samples as it derives an approximation of the sampling distribution via repeated resampling of the available data to yield bias corrected 95% confidence intervals (CI). Significant associations were only presented if they survived bootstrapped confidence intervals. Then, further analysis on any resilient domains that had significant methylation results was conducted. Resilient and non-resilient groups in that domain were compared on additional psychosocial functioning factors.

Results

As shown in Table 1, children who had lower conduct problem scores than predicted given their exposure (to pre-natal environmental risk; i.e. resilient group) had a higher *OXTR* methylation Factor 1 score than non-resilient children. In contrast, when resilience was defined globally or in terms of hyperactivity or emotional problems, resilience scores were not associated with *OXTR* methylation.

When examining the individual probes that make up the *OXTR* methylation factor (Probes 1, 5, and 10), we found that youth who were resilient in terms of conduct problems had significantly higher methylation levels across all three probes compared to the non-resilient group. Interestingly, resilience as defined in terms of global problems and hyperactivity problems predicted increased methylation only within one probe (Probe 5). Figure 1 highlights percent methylation differences across groups who were resilient versus non-resilient in terms of conduct problems.

Table 2 provides descriptive information regarding the groups who were resilient and non-resilient in terms of conduct problems. The groups did not differ in terms of gender or in environmental risk at any developmental period (prenatal – age 9). Furthermore, in an ANCOVA controlling for sex, we found that youth who were resilient in terms of conduct problems also had lower hyperactivity, emotional problems and callous-unemotional traits, higher prosocial behavior and better social cognition as compared to youth who were non-resilient in terms of conduct problems. Thus, youth who were resilient to prenatal risk in terms of having relatively low levels of conduct problems were functioning well across multiple domains that are typically compromised when youth have conduct problems.

Post-hoc analysis: Exploring potential genetic influences

We explored potential genetic factors that may influence the DNA methylation sites associated with resilience to conduct problems. Because our sample was underpowered to directly examine genetic polymorphisms (SNPs) affecting DNA methylation, we used the

mQTLdb resource (http://www.mqtldb.org/) to search for known methylation quantitative trait loci (mQTLs) associated with our methylation sites of interest. The mQTLdb database contains the results of a large-scale study based on the ARIES sample in ALSPAC (from which our subsample is derived), characterizing genome-wide significant *cis* effects (i.e. SNP within ±1000 base pairs of the DNA methylation site) and *trans* effects (i.e. ±1 million base pairs) on DNA methylation levels across Illumina 450k probes at five different life stages, including cord blood DNA methylation at birth (Gaunt et al., 2016). Here, we searched for mQTLs based on results from the conditional Genome-wide Complex Trait Analysis (GCTA), which was used to identify mQTLs with the most representative, independent effect on each DNA methylation site in order to account for linkage disequilibrium (Gaunt et al., 2016). Based on mQTLdb search, we found that 2 out of 3 of Factor 1 probes (Probe 1 and Probe 10) were associated with known cis SNPs, suggesting that DNA methylation levels across these sites are likely to be under considerable genetic control. Interestingly, Probe 1 and Probe 10 are specific to conduct problems, while Probe 5 was significant in both hyperactivity and global problems. This suggests that these probes are likely to be influenced by genetic factors as well as environmental adversity and may suggest a specific GxE effect for conduct problems. See Table 3 for more details on SNP influences on Probe 1 and 10.

Discussion

Our goal in this study was to examine whether variability in *OXTR* DNA methylation profiles at birth predicted resilience as defined by psychopathological outcomes that were better than expected based on prenatal risks. Consistent with our hypothesis, Factor 1 methylation – as well as methylation of the individual probes (1, 5, 10) that make up the factor – was predictive of resilience to conduct problems in mid-childhood. In contrast, *OXTR* DNA methylation profiles did not predict resilience in domains of emotional, hyperactivity, and global symptomatology suggesting a potential role for *OXTR* in the

development of conduct problems in particular. This is consistent with the fact that many social-cognitive processes such as empathy, attachment, bonding, and emotion recognition are disturbed in children with conduct problems. In addition, problems in social cognition associated with conduct-disordered behavior are typically marked by deficits in oxytocin levels.

Children who were resilient in the conduct problems domain in mid-childhood also had significantly fewer hyperactivity, emotional, and peer problems, higher levels of prosocial behavior, better social cognition, and lower scores on a measure of callous-unemotional traits compared with non-resilient youth. Thus, the group that was resilient to conduct problems was broadly resilient across multiple domains. However, this was probably not due to *OXTR* methylation profiles, which were not predictive of resilience as defined by emotional or hyperactivity problems. One possibility is that children who have fewer-than-expected conduct problems get along better with their peers, are both innately more prosocial and observe higher levels of prosocial behavior in their interactions with peers, and are thus buffered against the emergence of other forms of psychopathology relative to children with higher levels of conduct problems (Oland & Shaw, 2005; Patterson, DeBaryshe, & Ramsey, 1989). The role of *OXTR* DNA methylation in resilience beyond the conduct problems domain remains unclear.

It is important to note that there were no significant differences between resilient and non-resilient youths in levels of environmental risk in any of the developmental periods from prenatal to age 9. This rules out the possibility that resilient youth exhibited fewer conduct problems than non-resilient youth because they were exposed to less environmental risk after they were born. If epigenetic modifications in *OXTR* are consequences of exposure to stress, why would youth with similar levels of exposure to prenatal adversity vary in terms of *OXTR* methylation profiles? Recently, researchers have recognized that DNA methylation patterns

may be allele-specific and the relationship between exposure to stress and DNA methylation

may be moderated by gene variants. For example, one study found that adolescents that were

homozygous for the l-allele of *5HTTLPR* and experienced more stressful life events had

higher levels of *5HTTLPR* methylation. Stressful life events were not associated with

methylation for s-allele carriers (Van der Knapp et al., 2015). Another study found that

decreased DNA methylation in the FK506 binding protein 5 *(FKBP5)* gene depended on

early childhood abuse and the rs1360780 risk allele (Klengel et al., 2014). Although we could

not examine direct SNP effects because of small sample size, our post-hoc analyses using the

mQTLdb demonstrated that methylation of Probes 1 and 10 is significantly influenced by

SNPs rs62243375 and rs237900 respectively. Interestingly, our results showed that Probes 1

and 10 were only related to conduct problems, while Probe 5 was related to global problems

and hyperactivity. This provides indirect evidence for *OXTR* genotype moderating the

relationship between adversity and DNA methylation in conduct problems. However, studies

examining allele specific DNA methylation effects earlier in child development, especially in

the prenatal/neonatal period are lacking. More research is needed to examine the integrative

effects of *OXTR* genotype and DNA methylation on the oxytocin pathway, especially during

the critical prenatal period.

Increased methylation of *OXTR* is associated with decreased gene transcription and

protein expression, which theoretically represents the molecular building blocks for

behavioral phenotypes (Kuisi et al., 2001; Kumsta, Hummel, Chen, & Heinrichs, 2013;

Mamrut et al., 2013). Interestingly, our results showed that *higher* levels of DNA methylation

of *OXTR* at birth predicted resilience to conduct problems in mid-childhood. This pattern was

unexpected in light of results showing that elevations in *OXTR* methylation are also

associated with relatively high levels of callous-unemotional traits (Dadds et al., 2014; Cecil

et al., 2014). However, this traditional view has been recently challenged with more and

more studies finding an inverse relationship, highlighting the complexities in predicting behavioral phenotypes from DNA methylation (Jack, Conolley, & Morris, 2012; Reiner et al., 2015; Ziegler et al., 2015). In a human cohort, researchers found that only a minority of individual CpG sites had significant negative correlations with mRNA signaling across individuals and in a number of genes, higher DNA methylation was associated with higher gene expression (Lam et al., 2012). This can also be because the relationship between methylation, transcription, and expression can vary depending on the location of the CpG site. Of note, the three probes in our study mapped onto the 5' UTR region of gene, where an inverse correlation between DNA methylation and mRNA expression has previously been reported (Eckhardt et al., 2006). Thus, although we might theoretically predict that higher methylation would be associated with a lack of resilience to conduct problems, the mechanics of methylation are likely to be more complex than this.

Interestingly, our findings conflict with Cecil and colleagues (2014) work also using data from the ALSPAC sample in which they found that higher *OXTR* methylation at birth was associated with higher callous-unemotional traits at age 13. Of note, Cecil et al (2014) found this relationship in *OXTR* probes that make up Factor 2, while Factor 1 probes were not associated with callous-unemotional traits in their study. Furthermore, the sample (N=39) was highly selected to include only youth who had early-onset and persistent conduct problems and the relationship between higher *OXTR* methylation at birth and callous-unemotional traits was only observed in the subgroup with low levels of internalizing profiles. Thus, although our analysis sample and Cecil et al's ostensibly come from the same cohort, they reflect very different groups of children.

The present findings should be interpreted in light of a number of limitations. In most behavioral epigenetic studies of human *OXTR* DNA methylation including this one, researchers have used peripheral blood as a tissue source. It is unknown to what extent

methylation profiles from peripheral tissue are correlated with DNA methylation in the brain, where most behaviorally relevant epigenetic changes are thought to take place. Although there is growing evidence that peripheral methylation patterns can correlate with patterns in the brain, tissue- and gene-specific variations do exist (Bakulski, Halladay, Hu, Mill, & Fallin, 201; Byun et al., 2009; Davies et al., 2012; Masliah, Dumaop, Galako, & Desplats, 2013; Walton et al., 2016).

This study also focused specifically on DNA methylation of annotated probes located within the CpG island of *OXTR* and it is likely that differences across groups may be found in other genes (i. e. glucocorticoid or serotonergic pathways). Future studies may employ an epigenome-wide approach that would enable researchers to examine group differences in DNA methylation across the genome. In addition, we did not examine RNA expression and cannot explore the functional relevance of the probes in regards to gene expression and downstream biological mechanisms. However, we did select a region of *OXTR* that has previously demonstrated to be functional in utero. Although we provided indirect evidence for a potential GxE effect on DNA methylation via the mQTLbase data, we could not directly test it due to sample size. In general, the findings are based on a relatively small sample of youth, which limits statistical power to detect effects.

In summary, this is the first longitudinal study to examine the role of *OXTR* methylation in resilience across multiple domains. Our findings show that *OXTR* methylation at birth is exclusively related to resilience in the conduct problems domain in middle childhood. This may be potentially reflective of a GxE effect where genotype moderates the relationship between environmental stressors and DNA methylation. These findings highlight the importance of the prenatal period for the development of childhood psychopathology and suggest a potential mechanism by which early experiences may be biologically embedded. Because of the important role of oxytocin in social impairment, understanding individual

variations in *OXTR* methylation patterns might increase insight into risk and resilience factors that can bridge translational efforts in treatment and intervention approaches.

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Tables

Table 1
Multiple Linear Regression Predicting Factor 1 Methylation and Individual Probes at Birth by Types of Resilience

	Global		**Conduct**		Hyperactivity		Emotional	
Parameter	β	95% CI	**β**	**95% CI**	β	95% CI	β	95% CI
Factor 1 Methylation	.220	.000-.025	**.323**[**]	**.006-.031**	.154	-.006-.024	.015	-.013-.013
Probe 1	.153	-.009-.040	**.245**[*]	**.003-.051**	.051	-.022-.033	.022	-.022-.025
Probe 5	.274	.004-.041	**.283**[*]	**.006-.042**	.280[*]	.005-.042	.027	-.018-.020
Probe 10	.073	-.012-.021	**.244**[*]	**.002-.035**	.055	-.015-.024	-.042	-.021-.014

Note: β = Beta Weights; CI = Bootstrapped Confidence Intervals; Analyses controlled for sex and cell type
[*]$p < .05$, [**]$p < .01$, [***]$p < .001$.

Table 2
Descriptive Statistics of Resilient and Non-Resilient groups in Conduct Problems

	Resilient (*N* = 44)	Non-Resilient (*N* = 47)	
	N (%)	*N* (%)	
Gender			
Male	20 (46.5)	23 (53.5)	
Female	24 (50.0)	24 (50.0)	
	M (SD)	*M (SD)*	*T-test*
Environmental Risk			
Prenatal	0.54 (0.46)	.047 (.429)	-0.750
Ages 0-7	5.96 (4.46)	6.58 (5.80)	0.564
Ages 8-9	0.85 (1.81)	0.99 (1.76)	0.387
	M (SD)	*M (SD)*	*F-test*
Psychopathology			
Hyperactivity	-0.55 (1.10)	0.45 (1.22)	16.56***
Emotional Problems	0.00 (0.67)	0.31 (0.70)	4.90*
Peer Problems	-0.10 (0.67)	0.17 (0.63)	3.72^t
Prosocial Behavior	0.61 (0.64)	-0.40 (1.03)	31.18***
Social Cognition (Age 7)	2.24 (2.33)	5.16 (3.65)	18.14***
Callous-Unemotional Traits (Age 13)	1.79 (0.54)	2.33 (0.61)	16.87***

Note: All psychopathology outcomes controlled for sex
**p < .01, ****p* < .001.

Table 3
OXTR Single Nucleotide Polymorphism (SNP) effects on Probe 1 and Probe 10

Timepoint	SNP	SNP chr	SNP pos	A1	A2	CpG site	CpG chr	CpG pos	Beta	T-stat	Effect Size
Birth (Probe 1)	rs62243375	3	8810462	T	C	cg00078085	3	8810592	0.61340	0.00000	0.00794
Birth (Probe 10)	rs237900	3	8808696	A	G	cg12695586	3	8810077	-0.32780	0.00000	0.00439

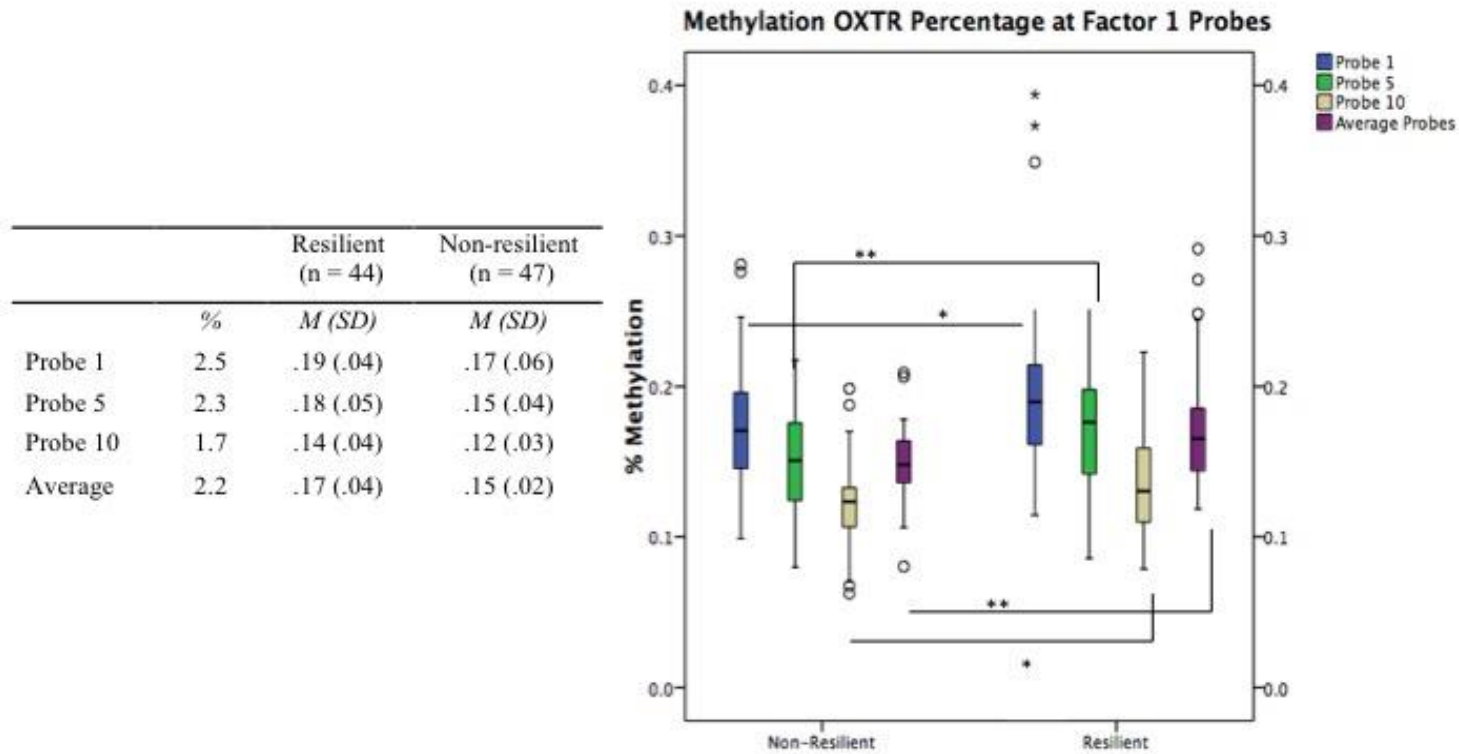Note: chr = chromosome, pos = position

Figures

Figure 1. *This figure shows mean methylation percentages at each individual probe that makes up Factor 1 controlling for sex and estimated cell–type composition.* $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$.

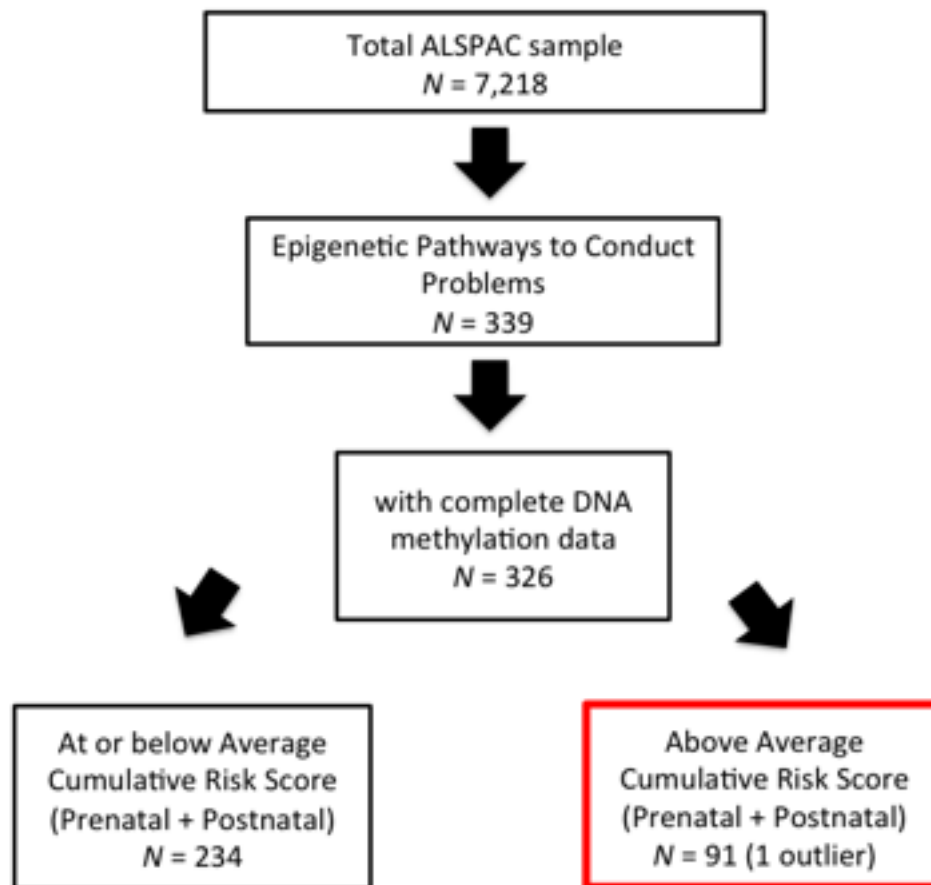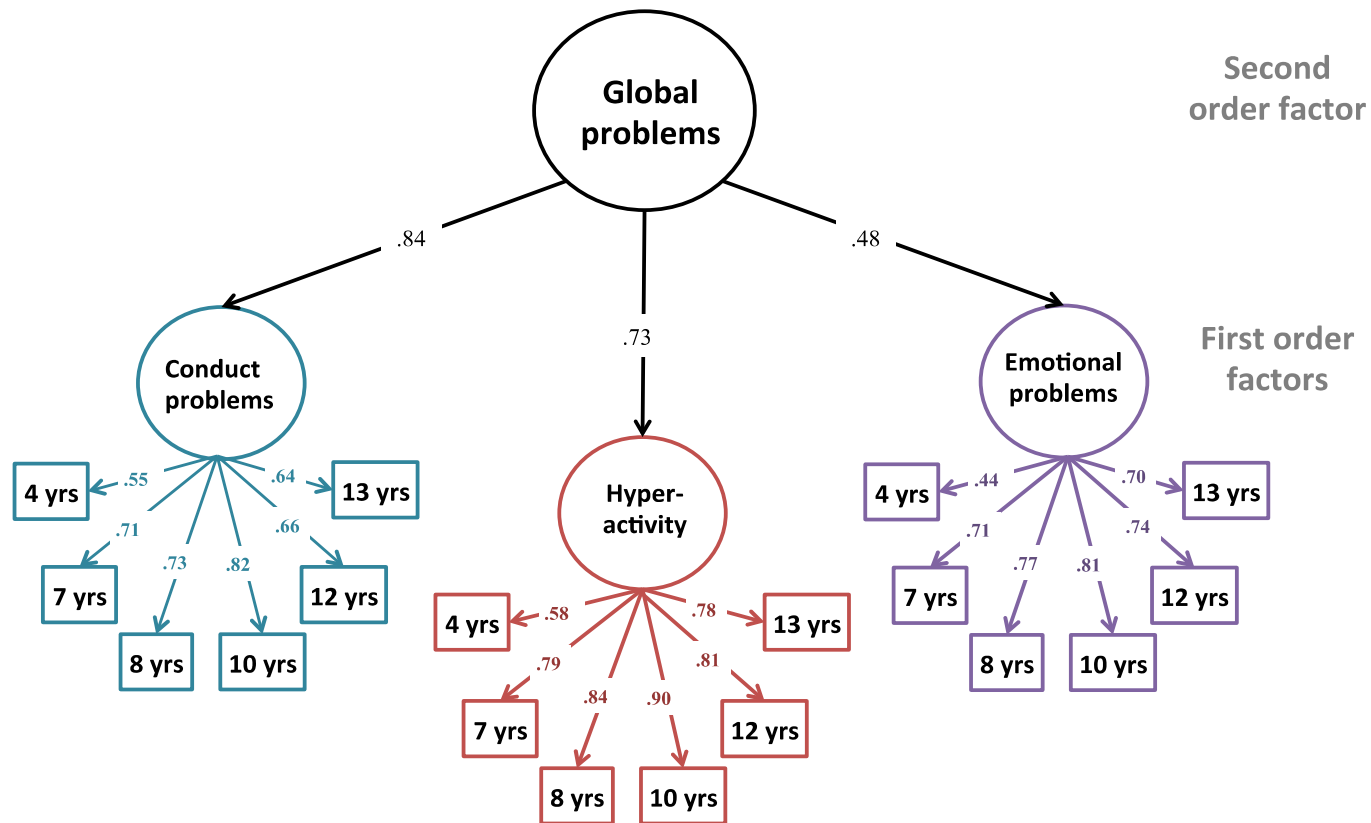|	%	Resilient (n = 44) M (SD)	Non-resilient (n = 47) M (SD)
Probe 1	2.5	.19 (.04)	.17 (.06)
Probe 5	2.3	.18 (.05)	.15 (.04)
Probe 10	1.7	.14 (.04)	.12 (.03)
Average	2.2	.17 (.04)	.15 (.02)

Figure 2. Flowchart of sample selection.

Model Fit: X^2 (132) = 462.16; p <.001; CFI = .88; TLI = .86; RMSEA = .08, 90% CIs = .08, .09

Figure 3. Confirmatory factor analysis of Strengths and Weaknesses Questionnaire (SDQ) subscales of conduct problems, hyperactivity, and emotional problems and global problems.
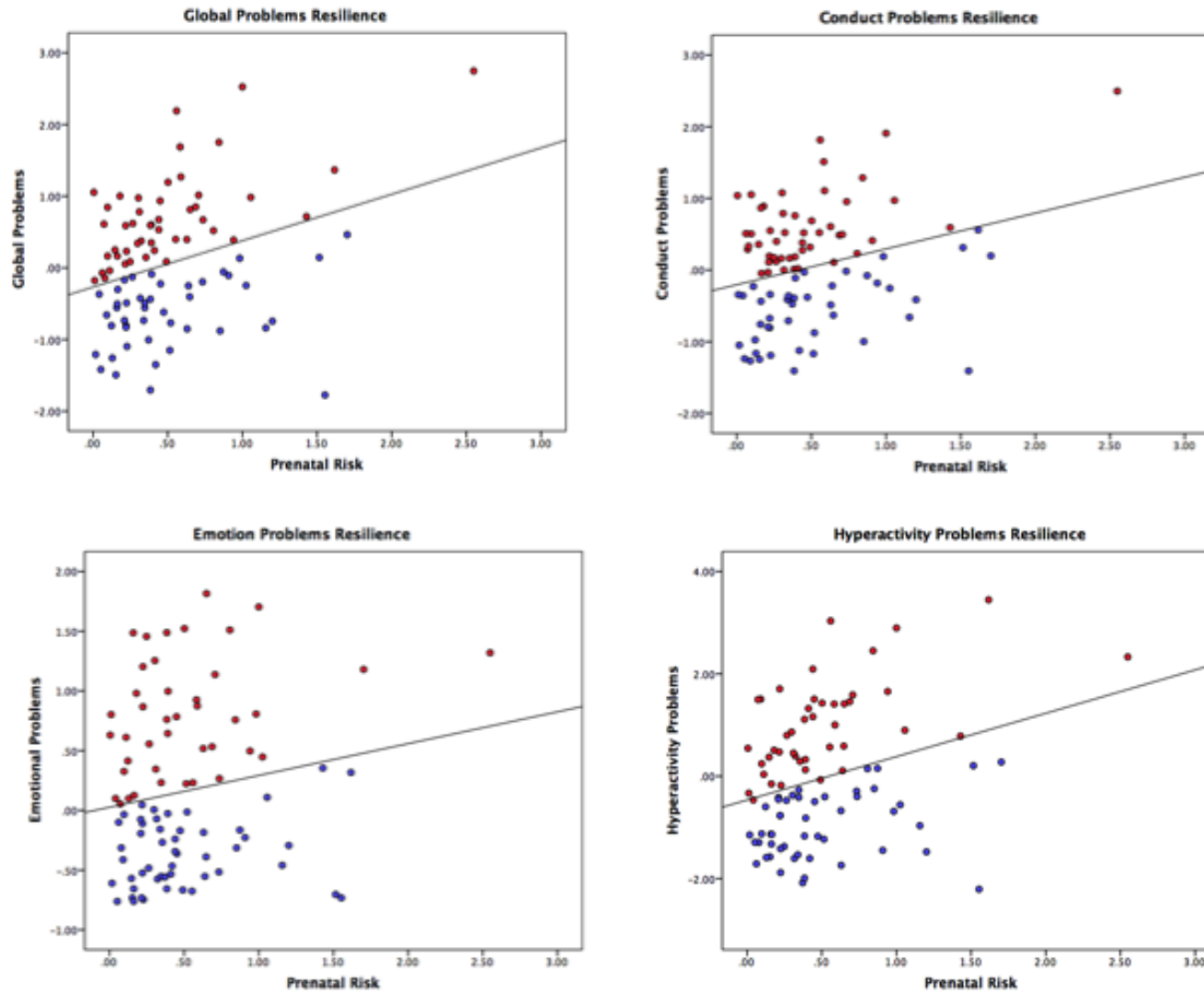
Figure 4. Linear Regression models used to classify resilient and non-resilient groups to global, conduct, hyperactivity, and emotional problems. Red (top half) dots represent the non-resilient group while the blue (bottom half) represent the resilient group.