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ORIGINAL ARTICLE

Increased serotonin transporter gene (*SERT*) DNA methylation is associated with bullying victimization and blunted cortisol response to stress in childhood: a longitudinal study of discordant monozygotic twins

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Background. Childhood adverse experiences are known to induce persistent changes in the hypothalamic–pituitary–adrenal (HPA) axis reactivity to stress. However, the mechanisms by which these experiences shape the neuroendocrine response to stress remain unclear.

Method. We tested whether bullying victimization influenced serotonin transporter gene (*SERT*) DNA methylation using a discordant monozygotic (MZ) twin design. A subsample of 28 MZ twin pairs discordant for bullying victimization, with data on cortisol and DNA methylation, were identified in the Environmental Risk (E-Risk) Longitudinal Twin Study, a nationally representative 1994–1995 cohort of families with twins.

Results. Bullied twins had higher *SERT* DNA methylation at the age of 10 years compared with their non-bullied MZ co-twins. This group difference cannot be attributed to the children's genetic makeup or their shared familial environments because of the study design. Bullied twins also showed increasing methylation levels between the age of 5 years, prior to bullying victimization, and the age of 10 years whereas no such increase was detected in non-bullied twins across time. Moreover, children with higher *SERT* methylation levels had blunted cortisol responses to stress.

Conclusions. Our study extends findings drawn from animal models, supports the hypothesis that early-life stress modifies DNA methylation at a specific cytosine–phosphate–guanine (CpG) site in the *SERT* promoter and HPA functioning and suggests that these two systems may be functionally associated.

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Introduction

Evidence from animal studies suggests that exposure to adverse environments early in life has long-term consequences on later behavioural and neurobiological functioning including hypothalamic–pituitary–adrenal (HPA) axis reactivity to stress (Suomi, 1997; Levine, 2005; Meaney & Szyf, 2005; Sanchez, 2006). In humans, childhood maltreatment has been associated with higher HPA axis activity (for a review, see Tarullo & Gunnar, 2006). There is also growing interest in understanding the origins of lower cortisol activity, another marker of disruption of the HPA axis (Heim *et al*. 2000; Gunnar & Vazquez, 2001; Fries *et al*. 2005). One prevailing hypothesis is that childhood maltreatment may induce stable changes in HPA axis activity and increase vulnerability to psychopathology (Susman, 2006; van Goozen *et al*. 2007; Yehuda *et al*. 2010). In addition to lower diurnal cortisol secretion (Cicchetti & Rogosch, 2001; Dozier *et al*. 2006; Bruce *et al*. 2009), accumulating research also indicates lower reactivity in relation to childhood adversity (Carpenter *et al*. 2007; Elzinga *et al*. 2008; Tyrka *et al*. 2008). Similarly, we reported blunted cortisol responses to stress in bullied twins in comparison

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with their non-bullied monozygotic (MZ) co-twins (Ouellet-Morin *et al*. 2011*a*). Examining discordant MZ twins reduces the possibility that differences between bullied and non-bullied children can be attributed to genetic variation or shared family environments, thus supporting the idea of an environmentally mediated effect of childhood victimization on cortisol reactivity. However, the mechanisms by which early adverse experiences shape the HPA axis remain unclear.

Research suggests that susceptibility to psychopathology partly arises from the interplay between childhood maltreatment and a polymorphism in the serotonin transporter gene (*SERT*) promoter (Canli & Lesch, 2007; Caspi *et al*. 2010; Uher & McGuffin, 2010). Notably, this polymorphism is also associated with cortisol responses to stress in newborns (Mueller *et al*. 2010) and female adolescents (Gotlib *et al*. 2008). This association was shown to be moderated by early adversity in adults (Alexander *et al*. 2009), although non-replication exists (Bouma *et al*. 2010). Because this polymorphism explains a relatively small proportion of *SERT* expression (<10%; Olsson *et al*. 2010), the investigation of other mechanisms affecting serotonergic neurotransmission, and potentially HPA axis reactivity, should be pursued. One possibility is that epigenetic alterations of *SERT* expression account for the environmentally mediated effect of childhood victimization on HPA axis reactivity. This hypothesis is consistent with accumulating evidence, mainly derived from animal studies, showing that epigenetic remodelling represents a mechanism by which adverse experiences disrupt reactivity to stress and health (Jirtle & Skinner, 2007; Mill & Petronis, 2007; Tsankova *et al*. 2007; Feinberg, 2008; Johnstone & Baylin, 2010; Meaney, 2010). Preliminary evidence of increased DNA methylation in the *SERT* promoter region of adults with a history of childhood abuse (Beach *et al*. 2010, 2011) supports the idea that *SERT* DNA methylation may link childhood victimization to HPA axis activity.

The objectives of the present study were to examine the impact of bullying victimization on *SERT* DNA methylation and to test whether DNA methylation was associated with lower cortisol responses to stress. Similar to maltreatment, bullying victimization has been associated with a wide range of mental health problems (Arseneault *et al*. 2010). Both types of victimization are characterized by intentional harm and involve repeated harmful actions between individuals where there is a power imbalance between the perpetrator and the victim whereby it is difficult for the victims to defend themselves (Arseneault *et al*. 2011). Specifically, we tested whether differences in *SERT* DNA methylation between bullied and non-bullied children were detectable in childhood using a longitudinal discordant MZ twin design to exert a strong control over genetic and environmental confounds (Rutter, 2009). Based on previous findings, we hypothesized higher *SERT* DNA methylation levels in bullied twins compared with their non-bullied MZ co-twins. Moreover, we examined *SERT* DNA methylation prior to victimization to rule out the possibility that bullied children already had higher DNA methylation levels. We then tested whether *SERT* DNA methylation was associated with lower cortisol responses to stress.

Method

Sample

Participants were recruited from the Environmental Risk (E-Risk) Longitudinal Twin Study, which tracks the development of a nationally representative birth cohort of 2232 British children (Moffitt & the E-Risk Study Team, 2002). The sample was drawn from a larger birth register of twins born in England and Wales in 1994–1995. The E-Risk sample was constructed in 1999–2000, when 1116 families with same-sex 5-year-old twins (93% of those eligible) participated in home-visit assessments. Follow-up home visits were conducted when the children were aged 7 (98% participation), 10 (96%) and 12 years (96%). Zygosity of the twins was determined with a standardized questionnaire which has been shown to have 95% accuracy (Price *et al*. 2000). Ambiguous cases were zygosity typed using DNA. Parents gave informed consent and children gave assent to participate in the study. Ethical approval was granted by the Joint South London and Maudsley and the Institute of Psychiatry National Health Service (NHS) Ethics Committee, UK.

From the E-Risk sample, we identified twin pairs eligible to participate in a substudy of cortisol if they met the following five criteria: (1) MZ twins; (2) one twin was bullied at least occasionally; (3) bullying was reported by both mothers and children at age 12 years; (4) bullying incidents involved harm, either psychological or physical; and (5) co-twins never experienced bullying victimization. From this substudy sample of 30 MZ twin pairs (Ouellet-Morin *et al*. 2011*a*), two pairs had missing data on *SERT* DNA methylation at the age of 10 years. The present study thus comprises 28 pairs of 12-year-old MZ twins discordant for bullying victimization with valid cortisol and DNA methylation data at the age of 10 years (42.9% males). Most twins were Caucasian (92.9%) and one in four families came from a low socio-economic background (25.0%). Children in this subsample had intelligence quotient (IQ) scores within the normal range when

they were 5 years old (from 69 to 134; mean = 100.1, S.D. = 15.4). We previously showed that one-third of the variance in bullying victimization is due to unique environments or random experiences (Ball *et al*. 2008). These factors possibly explain why genetically identical individuals could be differently exposed to bullying. For example, British twins are routinely separated into different classrooms in secondary schools, which may randomly place them at distinct risk for bullying victimization. Bullied and non-bullied MZ twins were similar on pre-existing risk factors (birth weight, IQ, behavioural and emotional problems), child-specific family environments (lifetime maltreatment, maternal warmth), concomitant factors (body mass index, pubertal maturity, bullying perpetration) and Psychosocial Stress Test (PST)-related measures (perceived stress and increased negative affect; Ouellet-Morin *et al*. 2011*a*). E-Risk discordant bullied MZ twins did not differ from concordant bullied MZ twins on socio-economic status, IQ or birth complications. A subset of 22 twin pairs had valid DNA data at both the ages of 5 and 10 years.

Bullying victimization

We prospectively assessed bullying victimization for all E-Risk participants during the interviews conducted with mothers when the children were 7, 10 and 12 years old and with the children themselves at the age of 12 years. Before asking questions related to bullying victimization, we explained that 'Someone is being bullied when another child: says mean and hurtful things, makes fun or calls a person mean and hurtful names; completely ignores or excludes someone from their group of friends; hits, kicks or shoves a person, or locks them in a room; tells lies or spreads rumours about them; and does other hurtful things like these. We call it bullying when these things happen often, and when it is difficult to make it stop. We do not call it bullying when it is done in a friendly or playful way'. We asked mothers whether each twin had been bullied by another child, responding 'never', 'yes' or 'frequently'. We further asked mothers who reported bullying victimization whether the twin suffered physical harm (e.g. bruise, cut) or psychological distress (e.g. bad dreams or school avoidance) as a consequence of bullying, responding 'never', 'yes' or 'frequently'. During private interviews, we asked children 'Have you been bullied by another person?'. A senior investigator further reviewed all descriptions of the bullying events recorded by the interviewers to confirm instances of bullying by looking for evidence of (1) repeated harmful actions, (2) between children, and (3) where there was a power imbalance between the bully and the victim. A test–retest reliability of 0.87 was noted for 30 parents randomly selected from the total E-Risk sample and who were interviewed 3–6 weeks apart. Our findings indicate that both mothers and children are valid and reliable informants of bullying victimization and that they tended to agree with one another (Shakoor *et al*. 2011). In this subsample, mothers reported that 19.6, 28.6 and 51.8% of children were victims of bullying at the ages of 7, 10 and 12 years, respectively, while 32.1% of twins reported experiences of bullying since the beginning of formal schooling.

Psychosocial Stress Test

When they were 12 years old, twins from the substudy sample were invited to our research laboratory early in the afternoon. At 1 h after arrival, each twin took part in an adapted version of the Trier Social Stress Test for children, which included a social stressor (speaking in front of judges) and a cognitive stressor (mental arithmetic; Buske-Kirschbaum *et al*. 1997). The cognitive task was first administered using the Children's Paced Auditory Serial Addition Task (Dyche & Johnson, 1991), a serial-addition task used to assess sustained attention, rate of information processing and working memory. Children heard a random series of 61 numbers ranging from 1 to 9 and were instructed to add the numbers in pairs such that each number was added to the previous one. The time interval between each number was 2.4 s for the first series of numbers and 2.0 s for the second series. Before the task started, children were told to make as few mistakes as possible because they were in competition against their co-twin and the winner would get a prize. The research interviewer did not offer support and avoided eye contact to enhance the stressful aspect of the challenge. The public speaking task immediately followed. Children were told to stand and to recall their most unpleasant experience at school in front of an unknown and inexpressive judge and the interviewer. Children had 2 min to prepare in silence, standing in front of the camera, and were then asked to speak for 5 min. The PST lasted approximately 15 min. This stress paradigm was selected because a combination of public speaking and cognitive tasks has been shown to elicit reliable cortisol responses in laboratory settings (Buske-Kirschbaum *et al*. 1997; Dickerson & Kemeny, 2004). At the end, the interviewer told the twins that they did well and rewarded their efforts.

Cortisol

We collected five saliva samples to measure cortisol responses to the PST. Saliva was collected by asking

children to use a straw to pass through 1 ml of saliva into the cryovials. The first samples were collected 20 and 2 min prior to the PST. A third sample was collected immediately at the end. A fourth sample and a fifth sample were collected 25 and 35 min after the start of the tasks. Twins were asked to refrain from doing any vigorous exercise in the morning, to eat a light lunch before midday, avoiding dairy products and red meat. Saliva samples were stored at −20 °C in a freezer.

After thawing, saliva samples were centrifuged at 3500 revolutions per min for 10 min, which resulted in a clear supernatant fraction of low viscosity. Saliva cortisol concentrations were determined using the 'Immulite 1000' model – Siemens' Immunoassay System (www.diagnostics.siemens.com; Mondelli *et al*. 2010). The assay had an analytical sensitivity of 0.2 nmol/l and inter-/intra-assay precision of less than 10%. All samples from each twin pair were analysed together. Cortisol measures were skewed and normalized using a $\log_{10}$ transformation.

DNA methylation analysis

All DNA samples were extracted from buccal cells using an established method that yields high-molecular-weight genomic DNA (Freeman *et al*. 2003). All DNA samples were tested for degradation and purity using spectrophotometry and gel electrophoresis. No samples were excluded because of poor sample quality. Genomic DNA (375 ng) was treated with sodium bisulfite using the EZ-96 DNA Methylation Kit (Zymo Research, USA) following the manufacturer's standard protocol. Bisulfite–PCR primers were designed using Sequenom EpiDesigner software (http://www.epidesigner.com). The *SERT* amplicon was amplified using standard Sequenom MassCLEAVE tagged primers (tags in lower case): 5′-aggaagagag TATTGTTAGGTTTTAGGAAGAAAGAGAGAG-3′ (forward) and 5′-cagtaatacgactcactatagggagaaggct AACCCTCACATAATCTAATCTCTAAATAACC-3′ (reverse) and encompassed 471 base pairs (NCBI build 36, chromosome 17: 25586879-25587349). Bisulfite–PCR amplification was conducted using Hot Star Taq DNA polymerase (Qiagen, UK) and cycling conditions of 45 cycles with an annealing temperature of 56 °C. All reactions were performed in duplicate and DNA methylation analysis was subsequently conducted using the Sequenom EpiTYPER system (Sequenom Inc., USA) as described previously (Coolen *et al*. 2007). The Sequenom EpiTYPER system is a highly reliable and quantitative technology for determining the density of methylated cytosines across specific genomic loci (Coolen *et al*. 2007). It uses base-specific cleavage followed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry in which the size ratio of the cleaved products provides quantitative methylation estimates for each cytosine–phosphate–guanine (CpG) unit, which contains either one or an aggregate of neighbouring CpG sites (see Supplementary Figs 1 and 2). Artificially methylated and unmethylated samples were included as positive and negative controls to ensure unambiguous PCR amplification of bisulfite-treated samples. All samples were processed blind to sample identification. Data generated from the EpiTYPER software were treated with stringent quality-control analysis where CpG units with low calling rates (<80%) were removed from analyses (none identified).

Statistical analyses

We conducted statistical analyses in four steps. First, we replicated previous findings indicating a blunted pattern of cortisol response to stress in bullied twins in comparison with their non-bullied co-twins using repeated-measures analysis of variance (ANOVA) in this subsample of 28 twin pairs. Second, we tested a promoter-wide difference in DNA methylation between bullied and non-bullied MZ twins using linear regressions. In the presence of a significant finding, we further explored site-specific differences. To control for non-independent observations and patterns of within-pair clustering due to shared genetic and environmental influences, linear regression analyses were adjusted with tests based on the sandwich or Huber/White variance estimator (Williams, 2000). Third, we investigated separately the bullied and non-bullied twins who had DNA at both the ages of 5 and 10 years (22 pairs) to test whether methylation levels changed over time using repeated-measures ANOVAs. Fourth, we explored the association between DNA methylation and cortisol responses to stress using Pearson correlation adjusted with the sandwich or Huber/White variance estimator. Cortisol responses to the PST were indexed using the standardized residuals (Z) of the area under the curve with respect to increase (AUC_i; Pruessner *et al*. 2003), calculated using the five cortisol measures and controlling for dairy product consumption and histaminic medication.

Results

Similarly to our previous findings (Ouellet-Morin *et al*. 2011*a*), we observed distinct patterns of cortisol response to stress between bullied and non-bullied twins (time × bullying: $F_{2.23,115.68} = 2.94$, $p = 0.05$). While non-bullied twins showed the expected cortisol increase after the PST ($F_{1.92,47.92} = 4.91$, $p = 0.01$), bullied

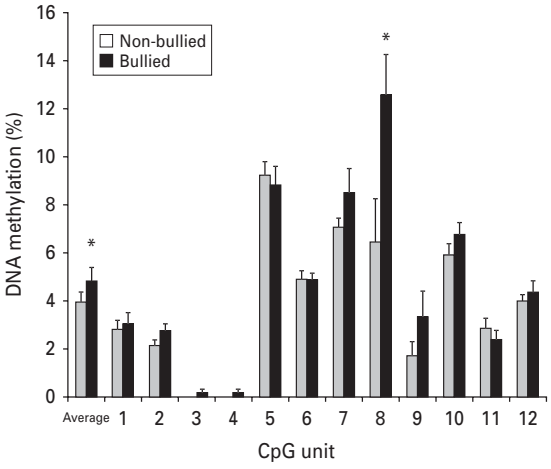

Fig. 1. Average and cytosine–phosphate–guanine (CpG) unit serotonin transporter gene (*SERT*) DNA methylation in bullied and non-bullied monozygotic twins at 10 years of age (28 twin pairs). Values are means, with standard errors represented by vertical bars. * Mean value was significantly different from that for non-bullied twins ($p < 0.05$).

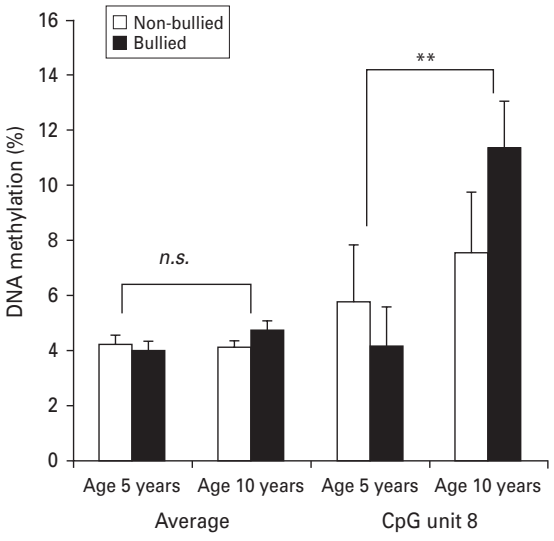

Fig. 2. Average and cytosine–phosphate–guanine (CpG) unit serotonin transporter gene (*SERT*) DNA methylation in bullied and non-bullied monozygotic twins from the age of 5 to 10 years (22 twin pairs). Values are means, with standard errors represented by vertical bars. ** Mean value was significantly different from that at age 5 years ($p < 0.01$).

twins did not exhibit this increase ($F_{2.55,63.75} = 0.56$, $p = 0.61$). Fig. 1 shows that bullied twins had higher *SERT* DNA methylation at the age of 10 years, averaged across the 12 CpG units compared with their non-bullied MZ co-twins ($t_{27} = 2.49$, $p = 0.02$). Additional tests showed that this difference emerged primarily from a site-specific difference (CpG8 in our assay) ($t_{27} = 2.39$, $p = 0.02$). Notably, both groups had similar methylation levels prior to bullying victimization at the age of 5 years (across the promoter region: $t_{21} = 0.56$, $p = 0.58$; CpG8: $t_{21} = 0.57$, $p = 0.58$). Fig. 2 shows that while bullied twins had increased levels of *SERT* methylation from the ages of 5 to 10 years at CpG8 ($F_{1,21} = 9.48$, $p = 0.006$), their non-bullied co-twins did not show that increase ($F_{1,21} = 0.32$, $p = 0.58$). DNA methylation between the ages of 5 and 10 years remained unchanged across the promoter region for both bullied and non-bullied twins ($F_{1,21} = 1.88$, $p = 0.18$ and $F_{1,21} = 0.10$, $p = 0.76$, respectively; see Fig. 2). We previously showed that bullied and non-bullied twins did not differ on child-specific family environments (lifetime maltreatment, maternal warmth and lifetime stressful life events), individual risk factors prior (birth weight, IQ, externalizing and internalizing problems) and concomitant to bullying experiences (body mass index, pubertal maturity and bullying perpetration) or related to the PST (perceived stress and increase in negative affects; Ouellet-Morin *et al*. 2011*a*). Therefore, these factors cannot account for differences in DNA methylation between bullied and non-bullied twins. Furthermore, Fig. 3 shows that

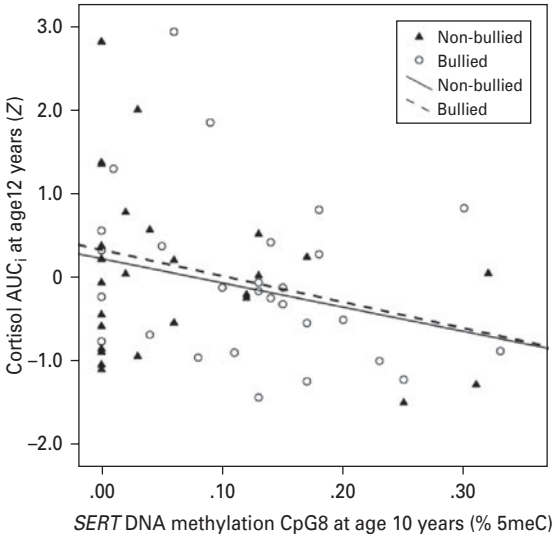

Fig. 3. Association between cytosine–phosphate–guanine (CpG) unit 8 serotonin transporter gene (*SERT*) DNA methylation and cortisol response to the Psychosocial Stress Test (56 twins). Standardized residuals (*Z*) were used to take into account the cortisol covariates. AUC$_i$, Area under the curve with respect to increase; % 5meC, % 5-methylcytosine.

twins with higher methylation at CpG8 (age 10 years) exhibited lower cortisol responses to the PST at the age of 12 years ($r = -0.28$, $p = 0.02$).

Discussion

We showed for the first time in humans that childhood victimization is associated with increased *SERT* DNA methylation in the absence of pre-existent differences between bullied and non-bullied children, providing longitudinal support that epigenetic processes are dynamic and responsive to early social environments. Our findings are consistent with higher levels of *SERT* methylation reported in adults exposed to harsh parenting, and physical and sexual abuses in childhood (Beach *et al*. 2010). Previous studies have also shown that *SERT* DNA methylation is associated with a lifetime history of major depression (Philibert *et al*. 2008; Olsson *et al*. 2010), antisocial behaviour (Beach *et al*. 2011) and unresolved trauma, a known risk factor for post-traumatic stress disorder (PTSD) (Bakermans-Kranenburg & van IJzendoorn, 2009), suggesting the potential role of *SERT* methylation in psychopathology.

The present study extends current knowledge in three ways. First, the discordant MZ twin design makes it unlikely that group differences in DNA methylation at this locus are attributable to genetic or shared environmental factors such as parents' psychopathology (Rakyan *et al*. 2011). This key feature of our study design further supports, in the absence of random assignment, that childhood victimization exerts an environmentally mediated effect on *SERT* DNA methylation. Second, our longitudinal design provides indirect evidence for directionality between victimization and methylation; only bullied twins showed increased DNA methylation from the age of 5 to 10 years, suggesting that it is not just a mere reflection of pre-existing differences. This result corroborates earlier findings showing that *SERT* DNA methylation is largely attributable to uniquely experienced environments (Wong *et al*. 2010) and substantiates the presumed impact of childhood victimization on higher *SERT* DNA methylation noted in retrospective studies of adults (Beach *et al*. 2010, 2011). Third, our findings suggest that the effect of early victimization on *SERT* DNA methylation can already be detected in childhood. This is consistent with the differential clustering patterns of DNA methylation reported across the genome between children raised in institutional care and controls (Naumova *et al*. 2011) and as a function of early maternal stress (Essex *et al*. 2011). Future studies examining DNA methylation prior to, concurrently and following naturally occurring adverse experiences could determine whether these changes are long lasting and are uniquely explained by early victimization.

Our findings suggest that *SERT* DNA methylation may be involved in the association between childhood victimization and cortisol responses to stress. Specifically, the increased *SERT* DNA methylation shown in bullied twins and lower cortisol responses reported in children with higher *SERT* methylation are in line with previous suggestions of an environmentally mediated effect of bullying victimization on HPA axis reactivity (Ouellet-Morin *et al*. 2011*a*). Two other investigations examined the association between early adversity, DNA methylation and the HPA axis in humans (Oberlander *et al*. 2008; Tyrka *et al*. 2012). The first study showed increased DNA methylation of the glucocorticoid receptor gene (*GR*) in infants prenatally exposed to maternal depressive/anxious moods which was, in turn, associated with higher cortisol reactivity. The second study also reported increased *GR* DNA methylation in adults with a history of childhood adversity and lower HPA reactivity (although not in the same CpG sites). Contrasting effects of adverse experiences on HPA axis functioning are thought to arise according to the nature, duration and timing of exposure (Miller *et al*. 2007; Lupien *et al*. 2009). Also consistent with a time-variant impact of adversity, maternal depressed/anxious moods during the 2nd trimester (but not in the 3rd) was associated with lower *SERT* DNA methylation (Devlin *et al*. 2010). Similar patterns of findings have been detected elsewhere in the epigenome as a function of famine exposure around the time of conception but not in late gestation (Heijmans *et al*. 2008; Tobi *et al*. 2009) and maternal stress in the year following birth but not during the preschool years (Essex *et al*. 2011). The timing of exposure to bullying victimization (middle childhood) may explain why we detected higher rather than lower *SERT* DNA methylation in bullied children. Altogether, these findings are consistent with the possibility that epigenetic processes affect HPA axis reactivity. Longitudinal research should investigate the possibility that heterogeneous epigenetic and HPA axis reactivity profiles emerge in children exposed to adversity taking place at distinct periods of development.

More generally, our findings are in line with a series of experiments conducted with rodents indicating that naturally occurring variation in maternal care mediated, independently from DNA sequence, epigenetic modifications in the hippocampus and resulted in long-lasting changes in HPA axis reactivity (Meaney & Szyf, 2005; McGowan & Szyf, 2010; Bagot & Meaney, 2010; Champagne, 2010). Specifically, rodents exposed to low maternal care (e.g. licking and grooming) were shown to have increased *GR* DNA methylation of the exon 1_7 promoter, lower hippocampal *GR* expression and higher HPA axis reactivity (Liu *et al*. 1997; Francis *et al*. 1999; Weaver *et al*. 2004). A similar finding in

victims of suicide with a history of maltreatment supports the idea that analogous biological pathways may be present in humans (McGowan *et al*. 2009) and could jeopardize physical health (Filiberto *et al*. 2011). Additional findings suggest that early-life stress also alters DNA methylation of the arginine vasopressin gene (Murgatroyd *et al*. 2009) and the brain-derived neurotrophic factor gene (Roth *et al*. 2009). Past research thus suggests that early-life stress induces epigenetic remodelling of several genes directly or indirectly regulating the HPA axis. Genome-wide mapping of epigenetic alterations induced by early-life stress may help to uncover the complexity of the biological pathways underlying vulnerability to stress and psychopathology.

Our study raises the possibility that *SERT* methylation is involved in the recalibration of neuroendocrine stress reactivity following early adverse experiences and thus represents a molecular basis of vulnerability to stress and psychopathology. Lower cortisol responses to stress have recurrently been documented in individuals with externalizing problems (van Goozen *et al*. 2007; McCrory *et al*. 2010) or PTSD (Yehuda *et al*. 2010), especially in the context of childhood victimization (Meewisse *et al*. 2007; Ouellet-Morin *et al*. 2011*b*). The 'attenuation hypothesis' suggests that early adversity induces persistent cortisol elevation followed by the down-regulation of HPA axis reactivity (Susman, 2006). Lowering the set-points for initiating a stress response could be adaptive when exposed to uncontrollable and unpredictable harsh living circumstances although it may exert long-term constraints on neural circuits and brain structures regulating stress, emotion reactivity and social behaviour (Fairchild *et al*. 2008; Feder *et al*. 2009). It is not clear whether our findings are specific to bullying victimization or could be generalized to other forms of harmful experiences such as maltreatment by an adult. On the one hand, these experiences are both characterized by intentional harm and power imbalance (Arseneault *et al*. 2011). On the other hand, it is possible that these experiences have distinct effects on DNA methylation, especially if they occur at different times during development. More research is needed to identify which features of childhood victimization affect epigenetic regulation and later vulnerability to psychopathology.

We speculate that increased *SERT* DNA methylation following childhood victimization affects HPA axis reactivity over time through the disruption of serotonin (or 5-hydroxytryptamine; 5-HT) neurotransmission. This hypothesized 'cascade' effect of altered serotonin neurotransmission on HPA axis activity following early-life stress is consistent with the known facilitating and inhibiting effects of serotonin neurotransmission, tryptophan depletion and the serotonin transporter on HPA axis reactivity (Li *et al*. 1999; Lowry, 2002; Vielhaber *et al*. 2005). The proposed role of *SERT* expression on poor stress coping strategies is also suggested in studies conducted with infant rhesus macaques exposed to early-life stress (Miller *et al*. 2009; Kinnally *et al*. 2010*a*, *b*). Evidence in humans also suggests that factors influencing serotonergic activity could modulate reactivity to stress through cortical–limbic regulation of emotions (Herman & Cullinan, 1997; Ochsner & Gross, 2005). For example, genetically based differences in *SERT* expression have been associated with increased amygdala activity to fearful stimuli (Hariri *et al*. 2002) and perturbed functional connectivity in the anterior cingulate cortex and the amygdala (Pezawas *et al*. 2005). It is also possible that persistent initial cortisol elevations triggered by childhood victimization affect *SERT* DNA methylation since glucocorticoids also regulate *SERT* expression (Lesch *et al*. 1996). Experiments conducted in rodents modelling the pharmacological use of glucocorticoids in premature babies support this possibility. Dexamethasone- and hydrocortisone-treated animals had lower *SERT* expression compared with controls, which could represent an adaptive mechanism that compensates for lower 5-HT levels (Vazquez *et al*. 2012). Childhood experiences triggering repeated and prolonged HPA axis activations, such as bullying victimization, may thus shape the 5-HT system in ways that it disrupts reactivity to stress and health. More research is needed to explore the biological pathways and temporal sequence of the complex bidirectional influences taking place between serotonergic pathways and the HPA axis during development.

The present findings provide support for the impact of childhood victimization on *SERT* DNA methylation and suggest an association between this epigenetic signal and cortisol reactivity; however, further tests are needed. First, the functional role of CpG8 methylation on *SERT* expression was not investigated. However, increased DNA methylation in the *SERT* promoter has been shown to decrease mRNA transcription (Philibert *et al*. 2008; Olsson *et al*. 2010). Second, we examined genomic DNA from buccal cells. Although buccal cells are a uniform cell population and of common embryonic origin with neuronal cells, it is not known whether the findings generalize to other tissues (Illingworth *et al*. 2008; Rakyan *et al*. 2008). Interestingly, a post-mortem investigation of 11 tissues (not buccal cells or blood) suggests, overall, a homogeneous pattern of DNA methylation across tissues (Byun *et al*. 2009). There are also preliminary indications of correlated epigenetic signals between blood and buccal cells for X-chromosome inactivation

(Monteiro *et al*. 1998; Rosa *et al*. 2008) and in candidate genes such as the corticotropin-releasing hormone gene (*CRH*) (Talens *et al*. 2010). Third, we measured *SERT* DNA methylation from samples collected at the ages of 5 and 10 years while cortisol was assessed when twins were aged 12 years. Concurrent measures of *SERT* DNA methylation and HPA axis reactivity repeatedly collected over time would help to establish the directionality of this association. Finally, the study was conducted in a small sample and should be replicated in larger studies. The discordant MZ twin design, however, allowed for a strong control of genetic and shared environmental potential confounds hardly ever taken into account in human studies (Rutter, 2009).

Our findings show prospective evidence that bullying victimization is associated with increased *SERT* DNA methylation. Moreover, children with higher *SERT* methylation exhibited lower cortisol responses to stress. Our study extends findings drawn from animal models and raises the possibility that early experiences of victimization modify the neuroendocrine response to stress through the alteration of *SERT* DNA methylation. This epigenetic mechanism may serve as an interface between childhood victimization, later vulnerability to stress and psychopathology.

Supplementary material

For supplementary material accompanying this paper visit http://dx.doi.org/10.1017/S0033291712002784.

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Declaration of Interest

None.

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