DNA Methylation of *NR3C1* and *FKBP5* is associated with Posttraumatic Stress Disorder, Posttraumatic Growth and Resilience

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Abstract

Objective: Understandings of the biological mechanisms underpinning posttrauma responses is limited. This pilot study aimed to expand research in this area by examining the relationship between DNA methylation of stress genes Nuclear Receptor Subfamily 3 Group C Member 1 (*NR3C1*) and FK06 Binding Protein 5 (*FKBP5*) with an array of posttrauma responses of posttraumatic stress disorder (PTSD) symptom severity, posttraumatic growth (PTG) and resilience.

Method: First-year paramedicine students (*N*=47) completed self-report measures of PTSD symptom severity, PTG and resilience; and provided a saliva sample for methylation analysis. Surrogate variable analyses (SVAs) identified covariates after which generalized regression models were performed to identify genomic sites significantly associated with PTSD symptom severity, PTG or resilience.

Results: Methylation of different *FKBP5* and *NR3C1* sites was significantly associated with PTSD symptom severity, PTG and resilience. Methylation in *FKBP5* site cg07485685 was a predictor of both PTSD symptom severity and resilience in opposite directions.

Conclusions: This is the first study investigating methylation changes in PTG, and overall the results suggest that *NR3C1* and *FKBP5* methylation is associated with both positive and negative posttrauma responses.

Keywords: trauma, PTSD, PTG, Resilience, DNA methylation, *NR3C1*, *FKBP5*

Clinical Impact Statement: This study advances understanding of how experiences of trauma can influence the epigenome. With further research, *NR3C1* and *FKBP5* genes may act as specific and individualized targets for research, prevention, identification and treatment of various posttrauma responses as well as and health promotion.

People respond to trauma in different ways (Bonanno, 2004). Understanding these different trauma responses is important as potentially traumatic events (PTEs) are frequent (American Psychiatric Association [APA], 2013). It has been estimated that over 70% of people will experience a PTE (Benjet et al., 2016), however, it is uncommon for Posttraumatic Stress Disorder (PTSD) to develop as a result (>6% of cases; Koenen et al., 2017). Instead, positive responses such as resilience or posttraumatic growth (PTG) are far more likely (Bonanno, 2004; Tedeschi, Shakespeare-Finch, Taku & Calhoun, 2018). Resilience occurs when an individual is able to 'bounce back' after a traumatic (or some other challenging) event, and is typically characterised by adaptive coping abilities (Bonanno, 2004; Smith et al., 2008); and PTG is the emergence of positive transformative changes after trauma (Tedeschi, Shakespeare-Finch, Taku & Calhoun, 2018). Of these posttrauma responses, research has consistently demonstrated that resilience is the most common (e.g., Bonanno et al., 2012; Orcutt, Bonanno, Hannan & Miron, 2014).

Literature has extensively explored demographic, psychological, personality and social factors that are related to these posttrauma responses. Generally, PTG and resilience are associated with good pre-trauma mental wellbeing, greater emotion regulation skills, coping self-efficacy and greater levels of perceived social support among other factors (e.g., Armstrong, Shakespeare-Finch & Shochet, 2014; Bonanno & Diminich, 2013; Marshall, Frazier, Frankfurt, & Kuijer, 2015; Orcutt et al., 2014). On the other hand, PTSD is associated with greater levels of prior trauma exposure, neuroticism, rumination, behavioural disengagement, inflexible emotional regulation, and reduced social support (e.g., Armstrong et al., 2014; Bonanno & Diminich, 2013; Orcutt et al., 2014; Wild, Smith, Thompson, Bear, Lommen, & Ehlers, 2016).

In contrast, research into the biological predictors of posttrauma responses, particularly PTG and resilience, is limited and still developing (Mehta, Miller, Bruenig, David & Shakespeare-Finch, 2020; Schubert, Schmidt & Rosner, 2016). Current understandings of the biological response to trauma center around the hypothalamic-pituitary-adrenal (HPA)-axis. When an individual is exposed to stress, glucocorticoids are released by the HPA-axis resulting in physiological changes that assist in the 'fight or flight' response (e.g., elevated heart rate; Hing, Gardner & Potash., 2014). After the perceived threat has passed, the stress response is terminated via a negative feedback loop whereby heightened blood cortisol activates the glucocorticoid receptor (GR) which down-regulates HPA-axis activity (Hing et al., 2014; Yehuda, 2009). HPA-axis dysregulation (indicated by cortisol suppression) means that the body fails to return to physiologic homeostasis, extending the stress response (Hing et al., 2014; Yehuda, 2009). It has been proposed that HPA-axis dysregulation is related to PTSD after trauma (Hing et al., 2014; Yehuda, 2009); and by the same reasoning, it can be predicted that well-being after trauma (e.g., PTG and resilience) is related to healthy HPA-axis functioning.

The interaction between the environment (e.g., trauma exposure) and an individual's genome (epigenetics) offers one avenue to study the biological mechanisms underpinning trauma responses (Dudley, Li, Kobor, Kippin & Bredy, 2011). One of the most commonly studied epigenetic mechanisms is that of DNA methylation, a process whereby methyl groups attach to cytosine base/s within the CpG dinucleotides (Dudley et al., 2011). This can alter DNA without changing the sequencing of base pairs, altering DNA transcription and subsequently, gene expression (Dudley et al., 2011). Epigenetic studies have associated PTSD with differential DNA methylation and gene expression patterns in HPA-axis stress genes such as Nuclear Receptor Subfamily 3 Group C Member 1 (*NR3C1*) and FK06 Binding Protein 5 (*FKBP5*; e.g., Klengel, Pape, Binder & Mehta, 2014; Mehta et al., 2017).

The *NR3C1* gene encodes for the GR, and acts as a transcription factor to promote physiological responses to stress (Castro-Vale, van Rossum, Machado, Mota-Cardoso & Carvalho, 2016; Klengel et al., 2014). Similarly, *FKBP5* – the co-chaperone and regulator of *NR3C1* – regulates glucocorticoids (Castro-Vale et al., 2016; Klengel et al., 2014). Therefore, *NR3C1* and *FKBP5* play integral roles in activating and terminating the stress response after trauma exposure by regulating glucocorticoids and activating the negative feedback loop via the GR (Binder, 2009). As the HPA-axis is involved in terminating the stress response, it is conceivable that *NR3C1* and *FKBP5* may also be involved in positive posttrauma responses. For example, healthy HPA-axis function indicated by a diurnal cortisol slope has been significantly associated with heightened PTG in breast cancer survivors (Diaz, Aldridge-Gerry & Spiegel, 2014).

This pilot study is the first study to examine the epigenetic mechanisms underpinning PTG and resilience to provide a more holistic view of the biology of posttrauma responses using a salutogenic approach (Antonovsky, 1979). The salutogenic approach views health as a continuum ranging from ill-health to health and offers a way to acknowledge a broad range of posttrauma responses (Antovonosky, 1979). We hypothesised that *NR3C1* and *FKBP5* methylation would be associated with PTSD symptom severity, PTG and/or resilience.

Method

Participants

Participants were first year Bachelor of Paramedic Science Students (N=49) from two Australian universities. Data from two participants was excluded due to poor saliva quality meaning the final sample was comprised of data from 47 participants. Table 1 depicts the demographic characteristics of participants, who included both males (38.3%) and females (59.6%) ranging in age from 17 to 43 (M = 23.43, SE = 0.95). Participants were primarily female (59.6%), Caucasian (91.5%), single (51.1%), high-school educated (56.3%) and

financially stable (58.7%). The majority of participants were not taking medications (72.3%), were non-smokers (89.4%), did not use drugs (97.9%), and drank alcohol (70.2%; Table 1).

Measures

Participants reported demographic information including age, sex, ethnicity, relationship status, education, socio-economic status, height, weight, medication use, smoking status, drug use and alcohol consumption. Participants reported whether they had ever experienced a traumatic event, briefly described the event, their subsequent level of distress, and their perceived severity of the event on a rating scale ranging from 0 (not traumatic) to 9 (severely traumatic).

The Posttraumatic Stress Disorder Checklist for DSM-5 (PCL-5; Weathers et al., 2013) was used to measure PTSD symptom severity. The PCL-5 is a 20-item measure of PTSD symptom severity in the past month, where responses range from 0 (not at all) to 4 (extremely). Higher scores represent greater PTSD symptom severity. The PCL-5 has demonstrated internal consistency, test-retest reliability, convergent validity and discriminant validity in US trauma-exposed students (Blevins, Weathers, Davis, Witte & Domino, 2015). The current sample corroborated good overall internal consistency ($\alpha = .94$).

The Brief Resilience Scale (BRS; Smith et al., 2008) was used to measure resilience where resilience was defined as the ability to 'bounce back' after trauma. The BRS is a 6-item measure where responses range from 1 (strongly disagree) to 5 (strongly agree), and higher average scores represent greater resilience. The scale has previously shown high convergent and divergent validity, high discriminant predictive validity and strong reliability (Smith et al., 2008), and good reliability within an Australian paramedic population ($\alpha = .88$; Shakespeare-Finch & Daley, 2017). The current sample demonstrated good internal consistency ($\alpha = .86$).

The Posttraumatic Growth Inventory X (PTGI-X; Tedeschi, Cann, Taku, Senol-Durak & Calhoun, 2017) was used to measure PTG. Twenty-five items assess how much change occurred because of a traumatic or highly stressful event, ranging from 0 (change did not occur at all) to 5 (change happened to a very great degree). Higher summed scores represent greater levels of growth. The PTGI-X has shown high internal consistency in US (α = .97), Turkish (α = .96) and Japanese samples (α = .95; Tedeschi et al., 2017). The current sample verified strong reliability (α =.96).

Experimental procedures

DNA methylation data was obtained from participant saliva samples collected in Oragene kits (DNA Genotek). Saliva samples were sent to the Australian Genome Research Facility (AGRF) for DNA extraction and DNA methylation microarrays. At the AGRF, samples were stored at -20ºC. DNA was extracted from the 2mL saliva sample using MACHERY-NAGEL NucleoSpin L (MACHINERY-NAGEL GmbH & Co. KG, Düren, NRW, Germany). The quality of the extracted DNA was assessed through resolution on a 0.8% aragose gel at 130 V for 60 minutes. Samples were normalised to 200 ng of DNA in 4 µl.

To assess DNA methylation, samples were bisulphite converted using the Zymo EZ DNA Methylation kit. For the methylation analysis, GenomeStudio v2011.1 with Methylation module 1.9.0 software was used with default Illumina settings. DNA methylation was assessed using the Illumina EPIC DNA methylation arrays that analyzes over 850,000 CpG sites and encompasses 99% of annotated RefSeq genes. The EPIC array covers 96% of CpG islands and multiple sites within each island, as well as the shores (within 2 kb from CpG islands) and shelves (>2 kb from CpG islands). It also covers CpG sites outside of CpG islands, DNase hypersensitive sites and micro ribonucleic acid promoter regions.

Procedure and statistical analyses

Ethical approval was obtained from both recruiting university's Human Research Ethics committees (approval numbers 1700001104 and H18REA087). Participants were informed about the research through presentations at lectures and research team contact information was made available online. Participants were asked to stay hydrated and to not eat within one hour of participation in order to ensure the saliva sample was of high quality. After providing written informed consent, participants completed the surveys and provided a saliva sample.

Raw data from EPIC Illumina arrays was exported into R for statistical analysis. Methylation levels were represented by a calculated '*ß*-value' – the ratio of methylated versus unmethylated sites. Using the minfi Bioconductor R package version 1.10.2, intensity read outs, normalisation and methylation *ß*-values were calculated. SWAN normalisation removed technical differences between MINFI package Infinium I and Infinium II probes (Maksimovic, Gordon & Oshlack, 2012; Pidsley et al., 2013). Methylation status for each gene was recorded as a *ß*-value, ranging from 0 (low methylation) to 1 (high methylation). A detection *p*-value was calculated for all arrays, where *p*-value >0.05 indicates methylation that is not significantly different from background measurements. We used excluded probes with p-detection > 0.01 in 10% or more samples. Samples with probe detection call rates <95% as well as those with an average intensity value of either <50% of the experiment-wide sample mean or <2000 arbitrary units (AU) were excluded from further analysis.

Standardised methylation levels were uploaded into Statistical Package for the Social Sciences (SPSS) for analysis with self-reported data. Assumptions of the data were inspected, and no breaches were detected.

Surrogate variable analyses (SVAs) revealed 4 significant SVA vectors that were used as covariates in the model to correct for technical artefacts and hidden confounds (Leek &

Storey, 2007). Generalized regression models were performed to identify genomic sites significantly associated with PTSD, PTG or resilience after correcting for the SVAs. Sensitivity analysis was performed by excluding individuals with no reported trauma exposure and the results remained significant. Bonferroni multiple testing correction was performed to account for the number of CpGs tested across each gene.

Results

In terms of psychological variables (Table 2), the majority (80.85%) of participants had PTGI-X scores of 50 or over, indicating moderate to high growth after trauma. Resilience scores were typically high, with 89.36% of participants scoring 3 or above out of a potential 5. PTSD symptom severity scores were low, with 82.98% of participants scoring below the diagnostic cut-off score of 33 (Weathers et al., 2013).

DNA Methylation marks associated with posttrauma responses

To test if DNA methylation levels in *FKBP5* and *NR3C1*/GR were associated with posttrauma responses, the normalized beta values were regressed against the phenotype of interest (PTSD symptom severity, brief resilience scale or Posttraumatic growth scale) whilst co-varying for surrogate variance analysis vectors in the regression models. A total of 52 CpG sites within the *FKBP5* gene and 89 CpG sites within the *NR3C1* gene were tested for association with the posttrauma responses.

For PTSD (Table 3), a total of 3 CpG sites (2 in *FKBP5* and 1 in *NR3C1*) were significantly associated with PTSD symptom severity ($p<0.05$; Table 3). Of these, one CpG in the *FKBP5* gene, cg07485685, remained significant after Bonferroni correction for multiple testing.

For PTG (Table 3), a total of 3 CpG sites (1 in *FKBP5* and 2 in *NR3C1*) were significantly associated with PTG ($p<0.05$). For resilience (Table 3), a total of 7 CpG sites (2

in *FKBP5* and 5 in *NR3C1*) were significantly associated with resilience scores ($p<0.05$). Of these, none remained significant after Bonferroni correction.

The CpG site cg07485685 in gene *FKBP5* was significantly associated with both PTSD symptom severity and resilience, and the directions were opposite such that DNA methylation at the CpG site was associated with reduced PTSD symptom severity and increased resilience (Table 3).

Discussion

This research sought to expand biological research into posttrauma responses by examining the relationship between DNA methylation of stress genes *NR3C1* and *FKBP5* with the posttrauma responses of PTSD, PTG and resilience. Overall, methylation of different regions of both *NR3C1* and *FKBP5* were significantly associated with PTSD symptom severity, PTG and resilience.

Healthy HPA-axis functioning after trauma exposure terminates the stress response, while HPA-axis dysregulation results in an extended stress response after trauma (Hing et al., 2014; Yehuda, 2009). Both *NR3C1* and *FKBP5* play integral roles in activating and terminating this stress response, and so play a role in an individual's response to a traumatic event. Results demonstrated that methylation of promotor-associated region cg03906910 of *NR3C1* was significantly associated with heightened PTSD symptom severity. This replicates previous findings where methylation and expression of *NR3C1* promotor associated regions has been consistently implicated in the pathophysiology of PTSD and other related disorders such as depression (see Bakusic et al., 2017; Watkeys et al., 2018 reviews), and suggests that methylation of this region may be linked to poor HPA-axis function.

Interestingly, low to moderate methylation of 2 *FKBP5* 5' untranslated regions (UTRs) was associated with reduced PTSD symptom severity. This finding tends to contradict previous findings where *FKBP5* methylation has been associated with PTSD (e.g.,

Yehuda et al., 2016; Methal et al., 2019). However, the functional outcome of UTR methylation remains uncertain (Aran et al., 2011; Martino & Saffrey, 2015). In this case, it could be tentatively suggested that 5'UTR methylation in these genes may be related to healthy HPA-axis function.

Low PTG after trauma was significantly associated with both *FKBP5* and *NR3C1* methylation. Hypomethylation of a promotor-associated *NR3C1* region and hypermethylation of a non-promotor *NR3C1* region were significantly associated with reduced PTG. These two different methylation patterns tend to increase gene expression (Bakusic et al., 2017; Dudley et al., 2011), and have previously been associated with heightened distress and PTSD symptoms (see Watkeys et al., 2018 review). Therefore, methylation of *NR3C1* may result in heightened gene expression, reduced PTG after trauma and dysfunctional HPA-axis function.

It is important to note that methylation of *NR3C1* and *FKBP5* were only associated with low levels of PTG and not heightened PTG. This may be due to low power of a small sample size. Alternatively, this result may suggest that stress genes and the HPA-axis are not related to the experience of growth after trauma; that these genes are related only to low PTG; or that some other system is related to the experience of higher levels of growth after trauma. Clearly, further research into the biological mechanisms underpinning PTG is required.

Resilience was significantly associated with methylation of different regions of both *NR3C1* (5 sites) and *FKBP5* (2 sites). Methylation of 3 *NR3C1* sites was significantly associated with reduced resilience, while methylation of 2 other *NR3C1* sites was significantly associated with heightened resilience. There were no observable patterns in associations between reduced or heightened resilience and methylation levels/ regions. Individuals who are resilient after trauma may still experience distress, however this distress does not disrupt their ability to function in a healthy way (Bonanno & Diminich, 2013).

Therefore, *NR3C1* methylation being both positively and negatively associated with resilience may reflect this process. This seemingly contradictory result highlights the need for further research into positive posttrauma responses.

Interestingly, methylation of the same *FKBP5* site (cg07485685) was significantly associated with PTSD symptom severity and resilience in opposite directions. That is, methylation of this site was significantly associated with reduced PTSD symptom severity and heightened resilience. This finding therefore provides biological evidence for the inverse relationship between PTSD and resilience (Streb, Häller, Michael, 2014); and also lends further credibility to the HPA-dysregulation hypothesis of PTSD. That is, appropriate HPA-axis activity after trauma is associated with reduced PTSD symptom severity and heightened resilience after trauma.

This pilot study has made an important contribution to the literature by examining positive posttrauma responses rather than focusing only on potential disorder as is common in trauma and epigenetic research. For example, Mehta and colleagues (2020) systematic review of DNA methylation and gene expression patterns associated with positive and negative posttrauma responses identified 51 studies, of which none examined PTG, and only two measured resilience (Azadmarzabadi et al., 2018; Kohrt et al., 2016). Therefore, this pilot study addressed this pathogenic research bias by including positive posttrauma responses of PTG and resilience.

Some limitations are acknowledged that are all related to the fact that this was a pilot study. The sample was small, and therefore had limited power to detect significant results. However, significant initial results despite low power are encouraging, and support the need for further research. The sample showed low diversity, influencing the generalisability of findings. The study is also limited by its cross-sectional candidate gene design. That is, a longitudinal genome-wide approach is an avenue for future research in identifying novel

genes given that methylation of PTG and resilience had not been previously examined (Mehta et al., 2019). Based on these limitations, it is suggested that future research replicate and extend these findings using a larger, more diverse sample, and a longitudinal, genome-wide approach. Further investigations into the broad spectrum of trauma responses in interaction with biology can help uncover the complex process that is involved in resolving trauma experiences, and may provide more specific and individualized targets for research, prevention, identification and treatment of various posttrauma responses as well as and health promotion. Taken together, the findings of this pilot study are encouraging, and warrant further examination in larger cohorts.

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Table 1

Demographic Information of the Sample

Demographic Characteristic	*n* (%)
Biological Sex	
Female	28 (59.6)
Male	18 (38.3)
Intersex	1 (2.1)
Ethnicity	
Caucasian	43 (91.5)
Asian	2 (4.3)
African American	1 (2.1)
Aboriginal/ Torres Strait Islander	1 (2.1)
Relationship Status	
Single	24 (51.1)
In a relationship but unmarried	17 (36.2)
Married/DeFacto	4 (8.5)
Divorced/Separated	1 (2.1)
Educational Background	
Year 10 or Below	1 (2.1)
Year 12/HSC	25 (53.2)
Certificate or Diploma	11 (23.4)
Bachelors Degree	9 (19.1)
Other	1 (2.1)
Financial Situation	
Very uncomfortable	9 (19.1)
A Little uncomfortable	10 (21.3)
Satisfactory	22 (46.8)
A Little Comfortable	5 (10.6)
Trauma exposure	39 (83)
Medication use	13 (27.7)
Drug use	1 (2.1)
Alcohol Consumption	33 (70.2)
Smoker	5 (10.6)

Note. HSC = High School Certificate.

Table 2

Descriptive Statistics for Psychological Variables

			Range	
Scale	*M*	*SE*	Potential	Observed
Trauma Severity	6.34	0.33	0-10	2-9
PTGI-X	72.40	4.28	0-125	6-120
Relating to Others	2.82	0.19	0-5	0-4.86
New Possibilities	2.91	0.22	0-5	0-5
Personal Strength	3.40	0.18	0-5	0-5
SEC	2.38	0.19	0-5	0 – 4.83
AL	3.43	0.18	0-5	0-5
BRS	3.61	0.09	1-5	2-5
PCL-5	16.81	2.14	0-80	0-58
Cluster B	3.47	0.61	0-20	0-18
Cluster C	1.89	0.33	0-8	0-8
Cluster D	6.23	0.85	0-28	0-22
Cluster E	5.21	0.60	0-24	0-14

Note. $N = 47$. *M* = Mean. CI = Confidence interval. *SE* = Standard Error. PTGI-X = Posttraumatic Growth Inventory X. SEC = Spiritual and Existential Change. AL = Appreciation of Life. BRS = Brief Resilience Scale. PCL-5 = Posttraumatic Stress Disorder Checklist for DSM-5.

Table 3

Associations between methylation of gene sites with PTSD symptom severity, PTG and Resilience

Trauma Response	*NR3C1* (chr.5)	*FKBP5* (chr. 6)	*p*	ß	Basepair	Location
PTSD SS	cg19645279		.041	>.001	142702733	Body
		cg07485685	>.001*	-	35696061	5'UTR;Body**
		cg03546163	.031	>.001	35654363	5'UTR
				-		
				>.001		
PTG	cg17860381		.003	-	142783569	5'UTR;TSS1500**
	cg16586394		.043	>.001	142757011	Body
		cg06087101	.004	-	35551932	Body; 3'UTR
				>.001		
				-		
				>.001		
Resilience	cg25579735		.019	-.018	142807171	5'UTR
	cg19645279		.024	-.014	142702733	Body
	cg08423118		.028	.050	142808610	5'UTR
	cg20728768		.040	.012	142696594	Body
	cg21702128		.042	-.003	142784721	TSS1500;5'UTR**
		cg07485685	.007	.013	35696061	5'UTR; Body**
		cg16052510	.040	-.024	35603143	Body

Note. Only significant CpG sites reported. Chr = chromosome number. SS = Symptom Severity. TSS =Transcription Start Site. Numbers after TSS represent the number of base

pairs the CpG is from the TSS. UTR = untranslated region. * Survived Bonferroni Adjustment. ** Promoter associated.