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Interactive effects of early life stress and *CACNA1C* genotype on cortisol awakening response

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Highlights

Investigated cortisol awakening response (CAR) in 109 healthy males

CACNA1C rs1006737 genotype mediated the effects of early adversity on CAR

Higher CAR in non-risk genotype carriers may indicate a more resilient HPA axis

More pronounced genotype-trauma interaction when stress experienced prior to adolescence

Abstract

The rs1006737 (A/G) single nucleotide polymorphism within the gene encoding the $Ca_v1.2$ subunit of the L-type voltage-dependent calcium channel (*CACNA1C*) has been strongly implicated in psychiatric disorders. In addition, calcium channels are sensitive to the effects of glucocorticoids and functional variation may contribute to altered stress responsivity. This study aimed to investigate the role of early life stress (ELS) and its interaction with *CACNA1C* rs1006737 in affecting the cortisol awakening response (CAR), an indicator of HPA-axis function. Salivary cortisol was measured in 103 healthy adult males (aged 21-63) on two consecutive days at awakening and 30 minutes later. The ELS measure investigated self-reported adverse life events prior to age 17. The results revealed a marginally significant main effect of *CACNA1C*, a significant main effect of ELS, and a significant genotype-by-ELS interaction on the CAR, whereby non-risk allele carriers (GG) who had experienced early adversity showed higher CAR compared to the other groups. Further exploratory analyses showed that this interaction may have arisen from individuals who had experienced ELS before adolescence (prior to age 13). This study is the first to provide evidence that the effect of ELS on CAR may be partially moderated via *CACNA1C* rs1006737 genotype, whereby the heightened CAR in the GG-ELS group may be an indicator of mental health resilience in response to ELS.

Keywords: *CACNA1C, calcium channel, early life stress, childhood trauma, cortisol awakening response*

Background

It has been proposed that early life stress (ELS) might contribute to dysregulation in the development of the hypothalamic-pituitary-adrenal (HPA) axis, the body's primary stress response system (McEwen, 1998), therefore rendering genetically at risk individuals vulnerable to trauma in later life and contributing to the development of psychiatric disorders (Aas et al., 2016; Holtzman et al., 2013; van Winkel, Stefanis, & Myin-Germeys, 2008). Indeed, recent meta-analyses indicate that rates of childhood traumatic events are markedly higher in schizophrenia and related disorders (Matheson, Shepherd, Pinchbeck, Laurens, & Carr, 2013; Varese et al., 2012), bipolar disorder (Palmier-Claus, Berry, Bucci, Mansell, & Varese, 2016) and depressive disorders (Mandelli, Petrelli, & Serretti, 2015) compared to healthy controls and numerous studies have reported HPA-axis dysfunction in these conditions (Belvederi Murri et al., 2016; Heim, Newport, Mletzko, Miller, & Hemeroff, 2008; Phillips et al., 2006).

In an attempt to discern biological markers of HPA-axis function, the cortisol awakening response (CAR) has gained considerable interest. The CAR refers to the typical rise in free cortisol levels during the first 30 minutes post-awakening (Clow, Thorn, Evans, & Hucklebridge, 2004). Although the exact function of CAR is uncertain, it might help to prepare the individual for the demands of the upcoming day, or indicate a response to the natural stressor of awakening, therefore reflecting HPA-axis responsivity (Fries, Dettenborn, & Kirschbaum, 2009). Increased CAR has been shown to predict the onset of depression (Adam et al., 2010) and anxiety disorders (Adam et al., 2014), and blunted CAR has been found in patients with severe depression symptomatology (Veen et al., 2011), first-episode psychosis and schizophrenia (Berger et al., 2016), whereas remission from depression after selective serotonin reuptake inhibitor (SSRI) treatment is associated with an increase in CAR, suggesting restoration of HPA-axis activity (Ruhe et al., 2015). However, the results for individuals at-

risk for psychosis have been inconsistent (Cullen et al., 2014; Day et al., 2014; Labad et al., 2015). Furthermore, studies investigating the CAR in the context of early adversity have also produced mixed findings reporting both blunted (Desantis, Kuzawa, & Adam, 2015; Kohrt et al., 2015; Mangold, Wand, Javors, & Mintz, 2010; Meinlschmidt & Heim, 2005) and increased CAR (Butler, Klaus, Edwards, & Pennington, 2017; Engert, Efanov, Dedovic, Dagher, & Pruessner, 2011; Lu et al., 2013; Mondelli et al., 2010). These diverse findings may be the result of factors such as the developmental timing of the ELS and the interaction between stress hormones and gene variants that impact on stress responsivity (Heim & Binder, 2012; Miller, Chen, & Zhou, 2007), with increasing evidence suggesting a role for calcium channel encoding genes (Landgraf, McCarthy, & Welsh, 2014).

Calcium channels are expressed widely across the nervous system (Berger & Bartsch, 2014; Bigos et al., 2010) with intracellular calcium implicated in an array of neuronal processes, including the formation and maintenance of neuronal connections during development, and hormone secretion, neurotransmitter release and gene transcription in adulthood (Heyes et al., 2015; Lidow, 2003). The gene encoding the pore-forming $Ca_v1.2$ subunit of the L-type voltage-dependent calcium channel (*CACNA1C)* has gained particular interest in the context of psychopathology (Bhat et al., 2012; Heyes et al., 2015; Ou et al., 2015), with a number of genome-wide association studies (GWAS) supporting its role in major depression, bipolar and schizophrenia spectrum disorders (Ferreira et al., 2008; Liu et al., 2011; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Sklar et al., 2008). A recent study demonstrated an interaction between two SNPs in *CACNA1C* (rs73248708 and rs116625684) and environmental stressors in increasing depression symptomatology in humans, whereas heterozygous deletion of *CACNA1C* from the forebrain glutamatergic neurons increased stress-induced anxiety-like and depression-like behaviours in mice (Dedic et al., 2018). Within *CACNA1C*, the rs1006737 (A/G) single nucleotide

polymorphism (SNP) positioned in the third intron has been most often investigated. The risk allele (A) has been associated with increased *CACNA1C* mRNA expression in the dorsolateral prefrontal cortex (PFC) in human post-mortem samples (Bigos et al., 2010), reduced hippocampal and striatal activity and diminished hippocampal connectivity during an episodic memory task (Erk et al., 2010; Krug et al., 2014), but increased hippocampal activation during emotional memory tasks at trend level (Bigos et al., 2010). Behaviourally, the risk allele has been associated with schizotypal traits in healthy individuals and the increased risk of schizotypal personality disorder (Roussos, Giakoumaki, Georgakopoulos, Robakis, & Bitsios, 2011; Roussos et al., 2013). Other studies have found increased anxiety, depression, interpersonal sensitivity and neuroticism scores, and consequently it has been proposed that the risk allele carriers may have heightened vulnerability to stress (Erk, Meyer-Lindenberg, Schmierer, et al., 2014; Erk et al., 2010).

Although the exact biological mechanisms leading to increased stress vulnerability in the rs1006737 risk allele carriers remain to be elucidated, animal studies have repeatedly demonstrated the high responsivity of calcium channels to glucocorticoid administration, which has been shown to result in increased expression of calcium channel mRNA and elevated levels of intracellular calcium (Bali, Gupta, Singh, & Jaggi, 2013; Karst et al., 2002; Kerr, Campbell, Thibault, & Landfield, 1992). The $Ca_v1.2$ subunit encoded by *CACNA1C* is one of the main targets of corticosteroid hormones. Studies on animal models have shown that chronic stress leads to elevated calcium current amplitude and increased $Ca_v1.2$ mRNA expression in the rat dentate gyrus, amygdala and CA3 areas (Maigaard, Hageman, Jorgensen, Jorgensen, & Wortwein, 2012; Qin, Karst, & Joels, 2004; van Gemert & Joels, 2006). Others have demonstrated increased $Ca_v1.2$ mRNA expression in the basolateral amygdala neurons after treatment with glucocorticoids *in vitro* (Karst et al., 2002). In addition, increased $Ca_v1.2$ mRNA expression in the rat hippocampal CA1 area has been reported in an acute stress model (Joels,

Velzing, Nair, Verkuyl, & Karst, 2003) and $Ca_v1.2$ protein upregulation in the CA1 area in response to corticosterone exposure has also been shown (Van Gemert et al., 2009). As the hippocampus plays a key role in HPA-axis activity and the CAR (Clow, Hucklebridge, Stalder, Evans, & Thorn, 2010; Fries et al., 2009), any deviation in calcium channel activity in this brain region is likely to lead to changes in stress response system functioning.

Increasing evidence, including a study from our research group have shown that genotype-by-ELS interactions impact on cognitive outcomes, potentially via effects on stress-sensitive brain structures (Klaus et al., 2017) and we have further shown that ELS is related to an increased CAR in the same sample of healthy males (Butler et al., 2017). However, no studies to date have investigated the interaction between *CACNA1C* and ELS on the HPA-axis activity. The aim of the current study was to investigate whether the rs1006737 polymorphism of the *CACNA1C* may partially moderate the effects of ELS on HPA-axis function as measured by CAR in a sample of healthy adult males. Recent studies have suggested that a more dynamic CAR in response to external challenges is linked to a more resilient stress response system, whereas severe stressors may lead to HPA-axis exhaustion as evidenced by blunted CAR (Chida & Steptoe, 2009; Jakobsen et al., 2016; Ruhe et al., 2015). We therefore hypothesised that whereas ELS leads to higher CAR overall, the magnitude of CAR would be lower in the rs1006737 risk allele A carriers from the ELS group. Furthermore, as trauma before adolescence is thought to have more profound effects on HPA-axis programming, we expected a larger effect of genotype in those who had experienced ELS before the age of 13 (Butler et al., 2017; Maercker, Michael, Fehm, Becker, & Margraf, 2004) in comparison to those with no ELS.

Methods

Participants

The sample overlapped with that described in Butler et al. (2017), but the current analyses were restricted to Caucasian participants only. Briefly, 103 healthy adult males (mean age=34.54 years, SD=10.8, range 21 to 63) were recruited from Lincoln, UK, and the surrounding areas as part of a larger ongoing programme of research. None of the participants had current diagnosis of a psychiatric disorder, drug or alcohol addiction problems, nor used steroid based medication at the time of recruitment as determined through self-report. Symptoms of psychopathology and current perceived stress levels were measured by the Hospital Anxiety and Depression Scale (HADS; Zigmond & Snaith, 1983) and the Perceived Stress Scale (PSS-14; Cohen, Kamarck, & Mermelstein, 1983), respectively. Participants additionally completed custom questionnaires on their psychiatric history, demographic information and sleep history. All participants gave written informed consent to take part and the study was approved by the School of Psychology Research Ethics Committee at the University of Lincoln.

Salivary cortisol collection and measurement

The methodology for cortisol collection, measurement and characteristics of the cortisol data can be found in Butler et al. (2017). In brief, saliva samples were collected at home using Salivette sampling devices (Sarstedt Ltd., Leicester, UK) over two consecutive days, immediately upon awakening and 30 minutes post-awakening. Inter and intra assay coefficients of variation were 17.14% and 9.67% respectively, which falls within a 20% cut-off used in earlier research (Gassling et al., 2012; Kershaw & Hall, 2016; Sink, Lochmann, & Fecteau, 2008). CAR for day 1 and day 2 was calculated by subtracting the waking cortisol concentration from the 30 minute post-awakening concentration. The CAR from day 1 and day 2 did not significantly differ, $t(102)=.218$, $p=.828$, and the measures were significantly

correlated, *r=.266, p=.007*, with the re-test reliability estimates similar to those reported previously (Almeida, Piazza, & Stawski, 2009; Hellhammer et al., 2007). Therefore, an overall mean CAR was calculated for each participant.

Early life stress

ELS was assessed using the Childhood Traumatic Events Scale (CTES; Pennebaker & Susman, 1988), which asks participants retrospectively about the occurrence of six categories of trauma: death of a close friend or relative, parental separation or divorce, traumatic sexual experience, physical violence, major illnesses or injuries, or other traumatic experiences prior to the age of 17. Participants recorded the age at which the event occurred, and rated the severity of the traumatic event and the extent that they remember confiding in others about it on a 7-point scale where 7 is extremely traumatic. According to the authors' guidelines, experiences rated as 6 or 7 on the trauma scale were classified as having experienced ELS.

***CACNA1C* genotyping**

DNA was extracted from saliva using Oragene prepIT L2P (DNA Genotek Inc., Ottawa, ON, Canada, http://www.dnagenotek.com) according to the manufacturer's protocol. Genotyping for *CACNA1C* rs1006737 was carried out using TaqMan® Pre-Designed SNP Genotyping Assay containing primers and fluorescent allele-specific probes (Applied Biosystems, Warrington, UK). The 25 µl reaction volume contained 30 ng of genomic DNA, 0.66 µl of 40X assay mix, 12.50 µl of TaqMan® Universal PCR Master Mix, and 11.25 µl of DNAse-free water (Cat #: 4502, Sigma-Aldrich, Dorset, UK). Amplification was carried out using ABI StepOne™Plus Real-Time PCR System with 96-well plates. The thermal profile was 60°C for 30 s, 95°C for 10 min, followed by 50 cycles at 92°C for 15 sec and 60°C for 90

sec. PCR software (StepOnePlus™ v2.0) measured SNP-specific fluorescence and genotyped each sample post-PCR.

Data analysis

A *post hoc* power analysis using G*Power version 3.1.9.2 (Faul, Erdfelder, Lang, & Buchner, 2007) suggested that the current sample of N=103 provides a power estimate of .71 to detect medium effect size (f=.25) in the primary analyses. For the secondary investigations into the developmental timing of ELS, *post hoc* power analysis based on the sample of N=103 suggested a power estimate of .60 to detect medium effect size. Given the limited sample size in the developmental timing analyses, this was treated as an exploratory data analysis. All data were analysed using SPSS version 21 (IBM Corp., Armonk, NY, USA). The main effects of *CACNA1C* genotype and ELS (categorised as ELS/no ELS) and their interaction on CAR were investigated using a two-way analysis of covariance (ANCOVA). Assumptions for a parametric test were checked by inspecting the normality of the residuals visually using histograms and Q-Q plots and by checking skew and kurtosis (Kim, 2013). Although CAR can have high inter-individual variability (Adam et al., 2010), all CAR values in the current fell within the mean ± 4 standard deviations (as in Carnegie et al., 2014), and no outliers were therefore removed. Awakening cortisol levels, waking time, sleep duration, age and current perceived stress score measured by PSS-14 were included as covariates due to their proposed effect on CAR (Chida & Steptoe, 2009; Stalder et al., 2016). Due to strong correlations between HADS and PSS-14 scores (HADS anxiety: r_s=0.718, p<0.001; HADS depression: r_s=0.543, p<0.001), HADS scores were not included as covariates. The same statistical methods were used to investigate the effect of developmental timing of stress and *CACNA1C* variation on CAR by using a cut-off of experiencing preadolescent stress <13 years in accord with previous studies (e.g., Butler et al., 2017; Maercker et al., 2004). The age at which ELS occurred was

not recorded for one participant and so this analysis was performed on 102 participants. *Post hoc* one-way ANCOVAs were used for pairwise comparisons of CAR magnitudes. *Alpha* was set at .05 and this level was used to determine significance in the main ANCOVA analyses. *Post hoc* tests were corrected for multiple comparisons using Bonferroni correction, whereby significance for the primary genotype-by-ELS group comparisons was set at $p<.008$ and for the secondary developmental timing analyses the significance was set at $p<.003$.

Results

All participants were successfully genotyped for *CACNA1C* rs1006737 and the genotype distribution conformed to the Hardy-Weinberg Equilibrium, $\chi^2(1, N=103)=.067$, $p=.795$. As the number of AA homozygotes was low ($n=10$), AA and AG carriers were combined for all analyses, therefore testing a dominant model similarly to a number of previous studies (Erk, Meyer-Lindenberg, Linden, et al., 2014; Erk, Meyer-Lindenberg, Schmierer, et al., 2014; Krug et al., 2010), including the original GWAS that implicated rs1006737 in psychiatric disorders (Ferreira et al., 2008). A two-way ANOVA revealed no difference in age, HADS anxiety and depression scores, PSS-14 scores, waking time, sleep duration or awakening cortisol levels between the two genotype groups, $p>.05$ in all cases. HADS anxiety and depression scores and PSS-14 scores did differ between ELS groups ($p<.05$ in all cases) as previously reported in Butler et al. (2017), but this difference did not vary between genotype groups. Chi-squared test showed no significant differences in the genotype distributions between ELS groups, $\chi^2(1, N=103)=.007$, $p=.934$ (see Table 1 for descriptive statistics). In the combined sample, the cortisol levels at 30 minutes post-awakening ($M=9.18$ nmol/L, $SD=5.38$) were significantly higher than awakening cortisol levels ($M= 7.40$ nmol/L, $SD=4.31$), $t(102) = -4.22$, $p < 0.001$. The overall mean CAR was 1.77 nmol/L ($SD=4.26$; 95% CI: 0.94-2.60).

Effects of CACNA1C genotypic variation and ELS on CAR

Two-way ANCOVA with *CACNA1C* genotype (AA/AG vs GG) and ELS group (ELS vs No ELS) as fixed factors and age, sleep duration, waking time, awakening cortisol levels and PSS-14 score as covariates revealed a marginally significant main effect of genotype, $F(1, 94)=3.678$, $p=.058$, partial $\eta^2=.038$, a significant main effect of ELS, $F(1, 94)=8.977$, $p=.003$, partial $\eta^2=.087$, and a significant genotype-ELS interaction, $F(1, 94)=5.060$, $p=.027$, partial $\eta^2=.051$ (see Figure 1). *Post hoc* one-way ANCOVAs showed that GG-ELS group exhibited significantly higher CAR than GG-No ELS group, $F(1, 40)=7.634$, $p=.009$, partial $\eta^2=.160$, and AA/AG-No ELS group, $F(1, 46)=8.658$, $p=.005$, partial $\eta^2=.158$, and also higher CAR than AA/AG-ELS group at the margin of significance, $F(1, 32)=3.601$, $p=.067$, partial $\eta^2=.101$.

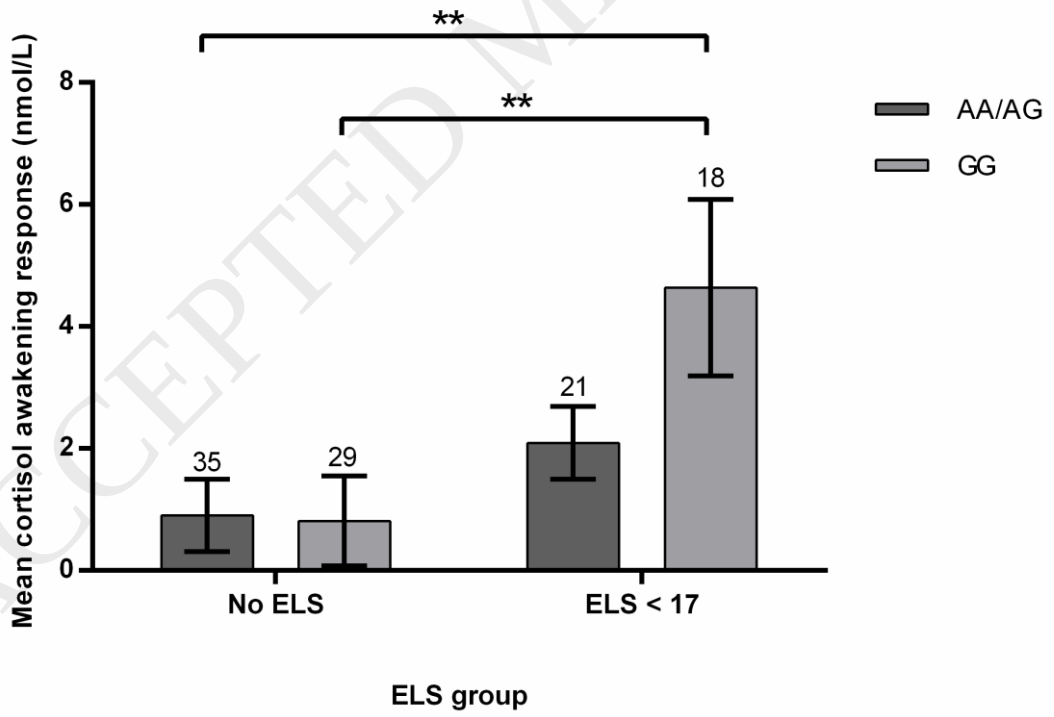

Figure 1. Effect of *CACNA1C* genotype (AA/AG vs GG) and early life stress (ELS vs No ELS) before the age of 17 on mean cortisol awakening response (nmol/L). Trauma categories are based on Childhood Traumatic Events Scale (Pennebaker & Susman, 1988), where ELS= a

rating of 6-7 on trauma scale and No ELS= a rating of 0-5. Error bars show +/- 1 standard errors. **p<.01

As the developmental timing of ELS has been found to affect HPA-axis function, in particular prior to adolescence (Lupien, McEwen, Gunnar, & Heim, 2009), we further investigated whether the observed genotype-ELS association stemmed from the individuals who had experienced ELS before age 13. Two-way ANCOVA with *CACNA1C* genotype and three ELS groups (No ELS, ELS <13, ELS 13-17) as fixed factors showed a significance of the main effect of genotype at trend level, $F(1, 91)=2.599$, $p=.110$, partial $\eta^2=.028$, a significant main effect of ELS, $F(2, 91)=4.716$, $p=.011$, partial $\eta^2=.094$, and a marginally significant genotype-ELS interaction, $F(2, 91)=2.775$, $p=.068$, partial $\eta^2=.057$ (see Figure 2).

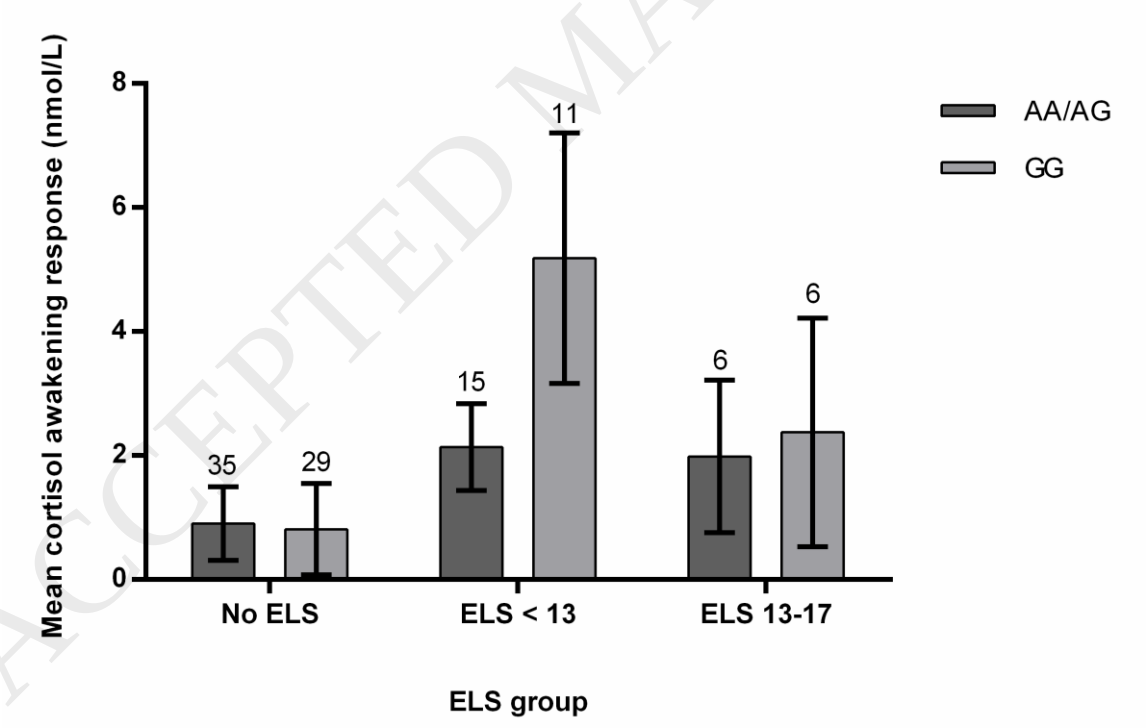

Figure 2. Effect of *CACNA1C* genotype (AA/AG vs GG) and early life stress (No ELS, ELS before the age of 13, ELS age 13-17) on mean cortisol awakening response (nmol/L). The number of participants in each group is given above the bars. Trauma categories are based on

Childhood Traumatic Events Scale (Pennebaker & Susman, 1988), where ELS= a rating of 6-7 on trauma scale and No ELS= a rating 0-5. Error bars show +/- 1 standard errors.

Conclusions

This study aimed to investigate whether *CACNA1C* rs1006737 genotype partially moderates the effect of ELS on the CAR in a sample of healthy males, and further to investigate whether this association is driven by ELS experienced prior to adolescence. In agreement with our hypothesis, we found a genotype-by-ELS interaction whereby CAR profiles in the ELS group differed according to the *CACNA1C* genotype, with the schizophrenia non-risk genotype (GG) carriers showing significantly elevated CAR. The CAR did not differ by *CACNA1C* genotype in the no-ELS group. This builds on our previous findings in this dataset (Butler et al., 2017) and provides further evidence for a role of calcium channel signalling in HPA-axis functioning. Furthermore, secondary exploratory analyses suggested that the significant genotype-ELS interaction may have arisen from the ELS encountered prior to the onset of adolescence. As the analyses on the developmental timing included small groups and the results were only marginally significant, these findings should be treated with caution. However, they are in agreement with previous evidence of the role of calcium signalling in the establishment of neural circuitry and stress sensitivity of neuronal connectivity during key stages of brain development (Heyes et al., 2015; Molet et al., 2016).

This research demonstrates for the first time that increased CAR in response to ELS is not uniform in all individuals, but may be partially moderated by the *CACNA1C* genotype. The analyses also showed a significant main effect of ELS and a marginally significant main effect of *CACNA1C* genotype, but these main effects were largely driven by the non-risk GG homozygotes from the ELS group. It could be argued that the elevated CAR in the GG-ELS group were due to current stress levels (Pruessner, Hellhammer, & Kirschbaum, 1999) or

indicate vulnerability to developing depressive disorders (Adam et al., 2010). Indeed, a recent study demonstrated an association between two *CACNA1C* SNPs and stressful life events on depressive symptomatology in healthy adults (Dedic et al., 2018). However, the results from our ANCOVA model on the association between ELS and *CACNA1C* were significant even when controlling for current perceived stress, and remained unchanged after further controlling for current anxiety and depression levels (data not presented), suggesting that the underlying mechanisms of glucocorticoid response may be determined during development (Lupien et al., 2009). Indeed, the interaction between *CACNA1C* genotype and ELS, whereby the CAR was markedly higher in the GG-ELS group compared to other groups, was predominantly seen when the stressor was encountered prior to adolescence. Although these results are treated as exploratory, these findings may be expected, as voltage-gated calcium channels are essential for early brain development (Heyes et al., 2015), and whereas structures involved in HPA-axis function, such as the hippocampus, amygdala and PFC continue maturing postnatally (Gogtay et al., 2006; Lupien et al., 2009), both the hippocampus (Lupien et al., 2009) and the amygdala may be most sensitive to stress before adolescence (Pechtel, Lyons-Ruth, Anderson, & Teicher, 2014).

Although the exact function of the CAR is still debated, the results presented here may suggest that increased CAR in those GG carriers exposed to early trauma may be thought of as a formerly adaptive response that serves to prepare the individual for the challenges of the upcoming day during childhood and is carried on to adulthood (Lupien et al., 2009). A more dynamic CAR may therefore indicate a flexible HPA axis that responds adaptively to external stressors (Jakobsen et al., 2016). The adaptive nature of a dynamic CAR is further supported by the findings that patients remitting from depression in response to SSRI treatment experience an increase in CAR, suggesting restoration of HPA-axis activity (Ruhe et al., 2015). On the other hand, the risk allele A carriers from the ELS group showed CAR levels

comparable to the No ELS group. As high levels of stress, or chronic stress can lead to CAR levels similar to that of the non-stressed individuals (Wardenaar et al., 2011), this potentially suggests hypocortisolism due to increased vulnerability to stressors in the A carriers (Erk et al., 2010) and lends evidence to the nonlinear nature of cortisol which is further moderated by genetic variation.

The mechanisms by which calcium channels mediate the effects of ELS on HPA-axis dynamics are still to be elucidated and are likely to be complex. Several studies have shown that glucocorticoid exposure increases $Ca_v1.2$ mRNA and protein expression in the hippocampal areas (Joels et al., 2003; Maigaard et al., 2012; Van Gemert et al., 2009) and elevated L-type calcium channel expression potentiates additional glucocorticoid release, creating a positive feedback loop. Consequently, increased intracellular calcium exposure over an extended period of time may lead to deleterious effects associated with stress, including cell death and associated hippocampal damage (Bali et al., 2013; Erk et al., 2010; van Gemert & Joels, 2006). A more recent study showed that heterozygous *CACNA1C* knockout in the mouse forebrain leads to increased susceptibility to anxiety when subjected to environmental stressors during development, but this study failed to find an effect of calcium channel expression on basal and stress-induced corticosterone levels (Dedic et al., 2018). Studies on humans have shown that *CACNA1C* rs1006737 risk allele is associated with increased *CACNA1C* mRNA expression in the PFC (Bigos et al., 2010), but decreased expression in the cerebellum (Gershon et al., 2014), suggesting different regulatory mechanisms of this SNP depending on the brain region. Further work needs to be conducted on the effects of glucocorticoids and genetic variation in *CACNA1C* on $Ca_v1.2$ expression in brain regions involved in HPA-axis function.

There were some limitations in the current study. Firstly, the overall sample and the subgroups were limited in size, particularly in the secondary analyses on the developmental timing. Additionally, the power calculation was based on a medium effect size and as such we

were not powered to detect small effect sizes. We therefore acknowledge that a larger sample is needed to replicate these findings with greater power and to detect small effect sizes. Furthermore, owing to the small number of AA homozygotes (*n*=10), we pooled AA homozygotes with AG heterozygotes for the current analyses, which might have obscured any effects attributable to AA homozygosity. In addition, rs1006737 is in complete linkage disequilibrium with several other SNPs positioned in the third intron of *CACNA1C* (Nyegaard et al., 2010) and we therefore cannot disentangle the effects specific to the SNP investigated in this study. We also acknowledge that other genes previously implicated in cortisol function, such as the fk506 binding protein encoding *FKBP5* (Kohrt et al., 2015), brain derived neurotrophic factor encoding *BDNF* (Shalev et al., 2009), and serotonin transporter encoding *5-HTT* (Wust et al., 2009) may, possibly additively, also affect HPA-axis activity. Moreover, both glucocorticoids and calcium channel activity can affect gene transcription (Lupien et al., 2009; West, Griffith, & Greenberg, 2002), meaning that the effects seen here could be due to the regulation of other genes not investigated in this study.

It should also be noted that the mean CAR reported in this paper is rather low. As previously acknowledged, there are many factors which can influence the CAR (Chida & Steptoe, 2009; Stalder et al., 2016). Of these, we did adjust for awakening cortisol levels, waking time, sleep duration, age and current perceived stress levels in our analyses. Yet other factors might have affected CAR, such as socioeconomic status and body mass index (Stalder et al., 2016) which we did not have access to in our dataset. Further discussion on the factors influencing CAR magnitude, such as gender (Pruessner et al., 1997; Vreeburg et al., 2009; Wust et al., 2000), undictated waking times (Kudielka & Kirschbaum, 2003) and the sampling schedule have been discussed in Butler et al. (2017). However, it has also been proposed that negative CAR is a legitimate phenomenon (Stalder et al., 2016). Our previous study demonstrated that CAR magnitude may also be associated with the type of traumatic event

experienced and cumulative trauma exposure (Butler et al., 2017), but due to the small sample size investigation into these aspects of trauma was not feasible and could be addressed in future studies. Additionally, CAR is known to have high intra-individual variability due to both trait and state factors (Hellhammer et al., 2007). Although the CAR re-test reliability in the current study was comparable to previous reports (Almeida et al., 2009; Hellhammer et al., 2007), future studies could collect cortisol data over more days to increase the reliability of the CAR. Finally, as gender differences in HPA-axis function after ELS have been reported (DeSantis et al., 2011; Kudielka & Kirschbaum, 2003; Kudielka & Kirschbaum, 2005), future studies are needed to investigate the ELS-*CACNA1C* interactions on CAR in female samples.

In sum, this is the first study to demonstrate that the effect of ELS on CAR may be partially moderated by *CACNA1C* genotype, whereby the non-risk GG homozygotes who have experienced ELS exhibit higher CAR, potentially supporting the idea of the adaptive nature of heightened CAR (Fries et al., 2009), but the risk allele A carriers subjected to ELS show a pattern suggestive of hypoactive CAR. We further provided some preliminary evidence that this effect may be more profound if the adversity has been experienced before adolescence. In light of the previously controversial findings in this area, the results from the current study emphasise the importance of considering the individual's genetic background when investigating the effect of environmental stressors on HPA-axis function and provide a potential mechanism by which ELS may affect CAR.

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Table 1. Age, HADS and PSS-14 scores, waking time, sleep duration, awakening and +30 cortisol levels divided by ELS group (before age 17) and *CACNA1C* genotype.

Characteristic	**No ELS**		**ELS**	
	AA/AG (*n*=35)	**GG (*n*=29)**	**AA/AG (*n*=21)**	**GG (*n*=18)**
Age (Years)	35.23(10.84)	32.90(10.88)	35.24(10.44)	35.06(11.62)
HADS Anxiety	5.71(3.30)	5.90(3.31)	8.00(4.01)	7.28(4.30)
HADS Depression	2.71(2.14)	2.59(2.61)	3.67(2.82)	4.5(2.68)
PSS-14	18.83(8.35)	20.06(7.38)	23.10(9.16)	22.78(7.57)
Waking Time	7:29(1:11)	7:14(0:54)	7:16(0:53)	7:46(1:34)
Sleep Duration (Hours)	6.77(1.17)	6.66(1.09)	6.63(1.67)	6.63(1.43)
Awakening Cortisol (nmol/L)	7.30(4.70)	7.86(4.16)	6.15(2.10)	8.34(5.46)
+30 Cortisol (nmol/L)	8.20(5.34)	8.67(3.45)	8.24(3.52)	12.97(8.00)

Notes. Data shown are means and standard deviations for early life stress experienced before the age of 17. ELS = Early life stress, HADS = Hospital Anxiety and Depression Scale, PSS-14 = Perceived Stress Scale.