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*BDNF*val66met affects neural activation pattern during fear conditioning and 24 h delayed fear recall

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Brain-derived neurotrophic factor (BDNF), the most abundant neutrophin in the mammalian central nervous system, is critically involved in synaptic plasticity. In both rodents and humans, BDNF has been implicated in hippocampus- and amygdala-dependent learning and memory and has more recently been linked to fear extinction processes. Fifty-nine healthy participants, genotyped for the functional *BDNF*val66met polymorphism, underwent a fear conditioning and 24h-delayed extinction protocol while skin conductance and blood oxygenation level dependent (BOLD) responses (functional magnetic resonance imaging) were acquired. We present the first report of neural activation pattern during fear acquisition 'and' extinction for the *BDNF*val66met polymorphism using a differential conditioned stimulus (CS)+ > CS− comparison. During conditioning, we observed heightened allele dose-dependent responses in the amygdala and reduced responses in the subgenual anterior cingulate cortex in *BDNF*val66met met-carriers. During early extinction, 24h later, we again observed heightened responses in several regions ascribed to the fear network in met-carriers as opposed to val-carriers (insula, amygdala, hippocampus), which likely reflects fear memory recall. No differences were observed during late extinction, which likely reflects learned extinction. Our data thus support previous associations of the *BDNF*val66met polymorphism with neural activation in the fear and extinction network, but speak against a specific association with fear extinction processes.

Keywords: amygdala; vmPFC; CBT; anxiety; fear recall; therapygenetics

INTRODUCTION

Brain-derived neurotrophic factor (BDNF) is the most abundant neutrophin in the mammalian central nervous system, and it is critically involved in synaptic plasticity (e.g. Bramham and Messaoudi, 2005). Initially, BDNF was implicated in hippocampus-dependent learning and memory in both rodents (for a review, see Tyler *et al.*, 2002) and humans (Egan *et al.*, 2003; Hariri *et al.*, 2003). Later, a similar role of BDNF for Pavlovian amygdala-dependent fear conditioning was shown in animals (Rattiner *et al.*, 2004, 2005; Ou and Gean, 2006). Also, human behavioural studies observed associations of a functional polymorphism in the *BDNF* gene (*BDNF*val66met) in a differential fear conditioning protocol (Lonsdorf *et al.*, 2010; but see Torrents-Rodas *et al.*, 2012), a fear generalization paradigm (Hajcak *et al.*, 2009; but see Torrents-Rodas *et al.*, 2012) as well as a context-fear generalization protocol (Mühlberger *et al.*, 2013).

During 'fear conditioning', an initial neutral stimulus (conditioned stimulus, CS) is repeatedly paired with an aversive event (unconditioned stimulus, US), and thereby acquires the ability to elicit a fear reaction (conditioned reaction, CR), which is similar to the unconditioned reaction elicited by the US itself. In differential protocols, one CS is predictive of the US (the CS+), whereas another one is not (CS−) and serves as a control stimulus. After repeated presentation of the CS+ without being followed by the US, the CR gradually weakens, a process referred to as 'extinction'.

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More recently, a role for BDNF has been demonstrated in extinction (learning) in animals (Andero and Ressler, 2012 (for a review); Peters *et al.*, 2010; Soliman *et al.*, 2010; Psotta *et al.*, 2013). In humans, the *BDNF*val66met polymorphism has been implicated in immediate fear extinction using functional magnetic resonance imaging (fMRI; Soliman *et al.*, 2010) but not in delayed extinction using psychophysiological measures (Lonsdorf *et al.*, 2010). Further, therapygenetic studies, which use genetic markers to predict the outcome of psychological treatment (for a review, see Lester and Eley, 2013), have reported associations of the met-allele with reduced responder rates in obsessive compulsive disorder patients (Fullana *et al.*, 2012), and reduced response to cognitive behavioral therapy (CBT) in patients suffering from post-traumatic stress disorder (Felmingham *et al.*, 2013).

In particular, the interdisciplinary study by Soliman and colleagues (Soliman *et al.*, 2010) has drawn a lot of attention. They studied fear extinction in knock-in mice as well as the functional *BDNF*val66met polymorphism in healthy humans. In the human research part of the study, a paradigm, consisting of a conditioning, reversal learning and an extinction phase that followed immediately upon each other was used in the fMRI. During reversal learning, the stimulus that had served as CS+ during conditioning, now served as the CS− and vice versa. In extinction, both stimuli were unpaired. During extinction, met-carriers showed decreased activation in the ventromedial prefrontal cortex (vmPFC) and enhanced activation of the amygdala to CS+s (that is, the CS− in the preceding reversal phase) relative to a fixation baseline. In addition, they report generally heightened skin conductance responses (SCRs) in met-carriers during fear conditioning and extinction. While this study currently represents the only evidence from the human neuroimaging field with respect to *BDNF*val66met and fear extinction, data on neural activation pattern during fear acquisition are still lacking. As extinction performance is dependent on acquisition learning, comparing activations between groups during both phases will allow for a more unequivocal

interpretation of extinction findings and prevent misinterpretation of findings. Thus, to date, it remains possible that the reported extinction-specific findings represent persistent group differences that arose during fear acquisition or preceding reversal learning and clarification is needed. In fact, fear acquisition processes have been linked to BDNF in animals (Rattiner *et al.*, 2004, 2005; Ou and Gean, 2006) and to the *BDNF*val66met polymorphism in humans (Lonsdorf *et al.*, 2010; but see Torrents-Rodas *et al.*, 2012), while studies reporting neural activation patterns during both fear acquisition and fear extinction in humans are missing. The importance of this becomes clear in light of our previous findings of significant CS+ startle potentiation against the intertrial interval (ITI) as well as CS+/CS− discrimination in val-homozygotes but not met-carriers in late acquisition. This was reflected in 24 h later in early extinction, which most likely reflects recall of the fear memory that was acquired the day before. No differences were found in late extinction, which reflects the outcome of online extinction learning.

To elucidate these questions, we explored the neural activation pattern of fear conditioning and extinction between *BDNF*val66met met-carriers and val-homozygotes using a differential imaging contrast (CS+ > CS−). Based on our previous fear-potentiated startle data (Lonsdorf *et al.*, 2010), we expected more pronounced neural discrimination in val-homozygotes in regions of the fear and extinction network (amygdala, insula, hippocampus, vmPFC) during acquisition (based on the results of Soliman *et al.*, 2010), that carry over to early but not late extinction.

METHODS

Participants

Sixty-seven participants were selected from a larger database of genotyped participants ($N \sim 500$, mainly healthy young university students), but analyses are based on 59 participants (32 females, mean age: 24.0 years, s.d. : 0.4, range: 19–31 years). Eight participants were excluded from the experiment because of technical problems ($N=5$), left-handedness ($N=2$) and pathological anatomy ($N=1$). In addition, four participants (one val-homozygote) were excluded for Day 1 because of artifacts, and three participants (two val-homozygotes) were excluded on Day 2 because of artifacts.

The majority of participants were selected for a different study arm (data to be published elsewhere and (same sample, but different experimental phase: Lonsdorf *et al.*, 2011) based on other genotypes (*5HTT*LPR [s-carriers *vs* l/l], *COMT*val158met [val-carrier *vs* met/met], while an additional two participants were specifically selected based on the *BDNF*val66met genotype ($N=3$ additional met/met genotype individuals) for the purpose of the study arm that is reported here. Participants of both study arms were scanned intermixed in a double-blind fashion. Therefore, genotypes of no interest for this article were included as covariates (*5-HTT*LPR and *COMT*val158met). In total, the sample consisted of 34 val-homozygotes and 25 met-carriers (whereof 5homozygotes). As previously done (e.g. Egan *et al.*, 2003; Hajcak *et al.*, 2009; Lonsdorf *et al.*, 2010; Soliman *et al.*, 2010; Torrents-Rodas *et al.*, 2012), carriers of one and two met-alleles were grouped together because of the low frequency of met-homozygotes despite of the pre-selection of two additional individuals with the met/met genotype. Chi-square tests confirmed that *BDNF*val66met genotype groups were equally distributed within the genotype groups of these two polymorphisms (that is, neither *5-HTTLPR* s-carriers nor *COMT* met-carriers were overrepresented within any of the *BDNF* genotype groups) both P's > 0.13. Further, genotype frequencies (excluding the three specifically for this study arm invited participants) did not differ from Hardy-Weinberg-Equilibrium (HWE) ($P=0.46$).

*BDNF*val66met genotype groups did not differ with respect to sex, age, State Trait Anxiety Inventory (STAI) state (Spielberger *et al.*, 2014) prior to or after fear conditioning or extinction, ratings of US valence or US arousal, salivary cortisol concentrations prior or post-conditioning or change of cortisol concentrations during conditioning (methods reported in Lonsdorf *et al.*, 2011), time of day the experiment was conducted, self-reported intake of oral contraceptives (for females) or menstrual cycle phase (for females not taking oral contraceptives; calculated by self-reported time since last menses and self-reported average cycle duration) all P's > 0.11.

Exclusion criteria were self-reported non-Caucasian, lifetime psychiatric or neurological disorders, pregnancy and non-removable metal parts in the body.

Participants were instructed to refrain from eating, drinking (except water), smoking, chewing gum and exercising for 2 h prior to the experiment. The study was approved by the Ethics Committee at the Karolinska Institutet and performed in compliance with the Declaration of Helsinki; all participants provided written informed consent. After completing the whole study (involving two additional experiments after extinction (Golkar *et al.*, 2012 and data to be reported elsewhere), participants were paid 400SEK. After complete description of the study, written informed consent was obtained.

Stimuli

Two angry male faces from the Karolinska Directed Emotional Faces (Lundqvist *et al.*, 1998) served as CSs (mean duration 7 s, jittered 6–8 s to reduce collinearity of the CS+ and the US in this 100% reinforcement paradigm), and a white fixation cross on a black background was presented during the ITI (mean duration 13 s, jittered 10–16 s).

An individually adjusted monopolar 100 ms direct current (DC)-pulse electric stimulation (STM200; Biopac Systems Inc, www.biopac.com) served as the US. Importantly, final intensity did not differ between *BDNF*val66met genotype groups, $F(1,57) < 1$.

Stimuli were presented via Presentation® (Neurobehavioral Systems Inc., Albany, CA) using fMRI-compatible goggles (NordicNeuroLab, Bergen, Norway).

Experimental Protocol

Day 1. Before fear conditioning, both pictures were presented six times to avoid that orienting responses to the stimuli affect neural activation during conditioning (habituation), and participants were explicitly informed that there would not be any US presentation yet. During fear conditioning, there were 15 presentation of each CS, and assignment of the two face picture to the CS+ and CS− was counterbalanced between participants. A reinforcement ratio of 100% was used. Participants were not instructed about the contingencies or the learning element of the experiment beforehand.

After conditioning, participants underwent a structured interview to assess CS–US awareness. All participants but one (val/val genotype) were able to correctly report the conditioning contingencies ("aware"). In our previous study (Lonsdorf *et al.* 2010), a higher percentage of unawares was observed despite of a nearly identical experimental protocol. It can only be speculated that differences in arousal (fMRI *vs* non-fMRI environment) might underlie this difference.

Day 2 (extinction). Participants returned for an extinction session ~24 h later during which they were presented with each CS 24 times (whereof 12 CS assigned to an early and late extinction phase, respectively). No other experimental task took place in between the conditioning and the extinction phase, and recall of the CS–US contingencies was not assessed again prior to the extinction session on Day 2. Participants were informed that they would continue the experiment from the previous day.

Genotyping

Genetic material was collected as 20 ml whole blood or as saliva samples using the Oragene®DNA SelfCollection Kit (DNA Genotek Inc., Kanata, Kanada). DNA extraction was performed as described earlier from either whole blood (Jensen *et al.*, 2009) or from saliva using the protocol and reagents supplied by Oragene®. Genotyping was performed as described earlier for *5HTT*LPR/rs25531 (Lonsdorf *et al.*, 2009a) and *COMT*val158met (Lonsdorf *et al.*, 2009b) and *BDNF*val66met (Lonsdorf *et al.*, 2010).

Data acquisition, response definition and data analysis

SCRs. SCRs were acquired with a BIOPAC MP150 digital converter (Biopac Systems, Goleta, CA) and fed into AcqKnowledge 4.0 software. SCRs were sampled at 250 Hz, and a 1 Hz lowpass filter was applied during acquisition. SCR responses were scored in AcqKnowledge 4.0 as the largest increase in SCR occurring 0.9–4 s post-stimulus onset with a minimal amplitude of 0.03 m Siemens.

A mixed model analysis of variance with stimulus (2) and time (2) as repeated factors and *BDNF*val66met genotype as between-subject factors was calculated. *5-HTT*LPR and *COMT*val158met genotypes were entered as covariates. The significance level for all analyses was set at $P < 0.05$.

fMRI. An anatomical scan and fMRI data were obtained using a GE Signa Echo Speed 1.5T scanner and an 8 channel headcoil. Functional wholebrain images were acquired using a gradient echo T2*-weighted echoplanar imaging scan, echo time = 40 ms, repetition time = 2.5 s, flip angle of 90°, 32 axial slices (thickness = 3 mm with 1 mm gap) and a field of view = 22 cm × 22 cm. The first scans were defined as dummy scans to allow for longitudinal T1-equilibrization, and these were not included in the analysis.

Pre-processing [SPM8 (www.fil.ion.ucl.ac.uk/spm) on Matlab R2009b (The MathWorks, Natick, MA)] involved realignment, unwarping and normalization to a sample-specific template, using DARTEL (Ashburner, 2007). A general linear model [for details, see (Friston *et al.*, 2006)] was set up for statistical first-level (single-subject) analysis. CS onsets were modeled as an event using a "stick" function. During acquisition, three regressors per CS type [one for the habituation, as well as a categorical and a parametric regressor (modulated with a linear decreasing function to capture responses over time)] were included as well as two nuisance regressors for the US and the ITI (eight regressors in total). Note that parametric regressors capture responses over time (Büchel *et al.*, 1998; Marschner *et al.*, 2008; Lonsdorf *et al.*, 2014) and higher parameter estimates are indicative of faster decaying responses. During extinction, a model including nine regressors ($CS+_{early}$, $CS+_{late}$, $CS-_{early}$, $CS-_{late}$ for both categorical and parametric, ITI) was set up. An additional extinction model captured the complete extinction phase through five regressors, two per CS type (categorical and parametric) and the ITI. All regressors were convolved with a canonical hemodynamic response function.

On the second level, random effects full factorial models included regressors for the CS+ and the CS− during conditioning and extinction, respectively, and a group variable (*BDNF*val66met genotype). *5-HTT*LPR and *COMT*val158met genotypes were entered as covariates. Our contrast of interest was the differential contrast CS+ > CS−. To make sure that both genotype groups did not differ in neural activation pattern elicited by angry male faces prior to conditioning, an additional second-level model including categorical regressors for picture presentations during the habituation phase was set up. Again, *5-HTT*LPR and *COMT*val158met genotypes were entered as covariates, and BDNFval66met genotype was entered as group variable.

All analyses were restricted to pre-defined regions of interest (ROIs) using small volume correction (SVC) based on Gaussian random field theory [family-wise error rate method (FWE; Friston *et al.*, 2006)] at an α-level of $P < 0.05$. A priori ROI selection was based on expected genotype differences in regions ascribed to the fear network and previously linked to the *BDNF*val66met polymorphism such as the amygdala (Montag *et al.*, 2008; Soliman *et al.*, 2010), the anterior cingulate/vmPFC (Soliman *et al.*, 2010) as well as the (anterior) insula (Mukherjee *et al.*, 2011) and the hippocampus (e.g. Hariri *et al.*, 2003; Hashimoto *et al.*, 2008; Dennis *et al.*, 2011). Probabilistic anatomical masks (0.7 probability) derived from the Harvard–Oxford cortical and subcortical structural atlases were used (http://www.cma.mgh.harvard.edu; threshold 0.7; Desikan *et al.*, 2006). The provided insula masks were cut at y = 0 to form anterior insula ROIs.

Parameter estimates plots from peak voxels were created using RFXplot (Gläscher, 2009). In addition, beta values were extracted from peak voxels and correlations were performed in Matlab.

RESULTS

SCRs

Prior to conditioning, SCRs to the pictures of angry males did not differ between both genotype groups ($F < 1.6$, $P > 0.2$).

During conditioning and extinction, no main or interaction effect involving the factor *BDNF*val66met genotype was observed, all Fs < 2.0, all Ps > 0.16 (see Figure 1A and B) either when considering the whole phase or when separating extinction into early and late phases (all Fs < 1.4, all Ps > 0.25, data not reported in detail).

During acquisition, generally successful acquisition was indicated by a significant main effect of stimulus, $F(1,51) = 15.75$, $P < 0.001$, $Eta^2 = 0.24$. In addition, a main effect of time indicates habituating SCRs over time, $F(1,51) = 18.61$, $P < 0.001$, $Eta^2 = 0.27$, in the absence of a significant stimulus x time interaction, $F(1,51) = 1.41$, $P = 0.24$, reflecting rapid acquisition (see Figure 1).

During extinction, a main effect of stimulus indicated generally successful recall of conditioning contingencies learned 24 h before, $F(1,52) = 14.55$, $P < 0.001$, $Eta^2 = 0.22$, and a main effect of time indicated habituating SCRs over time, $F(1,52) = 68.59$, $P < 0.001$, $Eta^2 = 0.57$. In addition, a stimulus x time interaction indicated faster decreasing responses for the CS− than for the CS+, $F(1,52) = 3.48$, $P = 0.024$ $Eta^2 = 0.06$. CS+/CS− discrimination weakened over the course of time and was only significant in the first block, $F(1,52) = 14.05$, $P < 0.001$ $Eta^2 = 0.22$, trend level in Blocks 2 and 3, $F(1,52) = 3.93$, $P = 0.053$ $Eta^2 = 0.07$ and $F(1,52) = 3.30$,

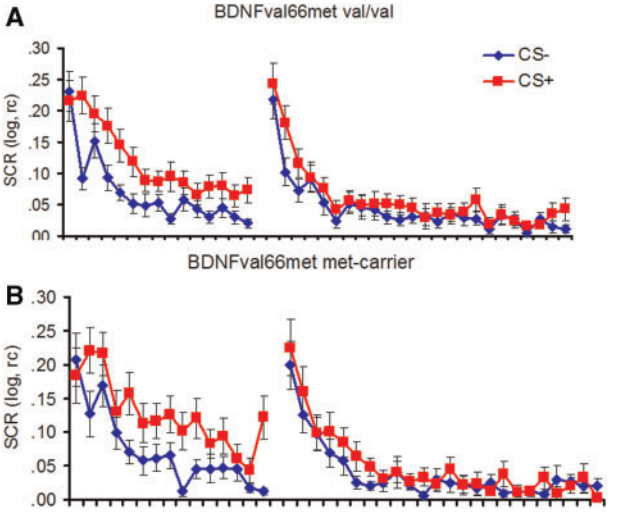

Fig. 1 SCRs to the CS+ and the CS− during fear acquisition and extinction in BDNF val-homozygotes **(A)** and met-carriers **(B)**. Error bars represent s.e.m.

Table 1 Imaging results

Phase	Contrast	cat/pm	Comparison	Region	x,y,z	Z(SVC)	k(SVC)	p(SVC)	p(uc)
Conditioning	CSP>CSM	cat.	met-carrier > val/val	L amygdala	−20,0,−18	3.94	17	0.002	<0.001
			val/val > met-carrier	R ACC/vmPFC	2,40,−2	3.35	15	0.045	<0.001
		pm.	val/val > met-carrier	R amygdala	26,−4,−12	3.37	27	0.023	<0.001
				R ACC/vmPFC	8,42,6	3.67	34	0.023	<0.001
				L ant. hippocamp	−22,−10,−24	3.32	30	0.06	0.001
Extinction (1st half)	CSP>CSM	cat.	met-carrier > val/val	R amygdala	22 −4 −14	3.59	24	0.013	<0.001
				L ant Insula	−36, 6, 0	3.43	83	0.023	<0.001
				R ant Insula	36, 4, 8	3.68	33	0.012	<0.001
					36,2,−12	3.41	34	0.028	<0.001
				L hippocampus	−26 −10 −20	3.57	22	0.023	<0.001
				R ACC	2 12 40	3.27	25	0.089	0.001
			val/val > met-carrier	none					
		pm.	val/val > met-carrier	L ant. Insula	−34 14 −10	3.66	41	0.012	<0.001
					−38 4 −12	3.08	4	0.070	0.001
Extinction (2nd half)	CSP>CSM	cat.	met-carrier > val/val	none					
			val/val > met-carrier	none					

Note. cat. = categorial regressor; pm. = parametric (linear decreasing) regressor; L = left; R = right cluster detection threshold of 0.01.

$P = 0.075$, $Eta^2 = 0.06$, respectively, had disappeared in the last block, $F(1,52) = 1.93$, $P = 0.17$ $Eta^2 = 0.04$.

fMRI - Fear conditioning

Prior to conditioning (i.e. during the preceding habituation phase), no genotype-dependent differences in any of the ROIs were observed in response to pictures of angry males.

During fear conditioning, CS discrimination (CS+ > CS−) differed between genotype groups as reflected in significantly higher left amygdala reactivity (categorical) in met-carriers as opposed to val-homozygotes ($P = 0.002$ $FWE_{(SVC)}$; see Table 1, Figure 2A), mainly driven by more pronounced amygdala reactivity to the CS+ in met-carriers (see Figure 2A). An exploratory analysis taking all three genotype groups into account (val/val, val/met and met/met) finds the same result ($P = 0.011$ $FWE_{(wholebrain)}$, see Supplementary Material) and suggests an allele-dose effect (see Supplementary Material and Supplementary Figure S1). No differences in the right amygdala were observed even at more lenient thresholds.

No time x stimulus effect (linear decreasing parametric regressor) was observed for met-carriers> val-homozygotes.

In val-homozygotes CS discrimination (CS+ > CS−) was reflected in more reactivity (categorical) in the right (perigenual) ACC/vmPFC ($P = 0.045$ $FWE_{(SVC)}$; see Table 1, Figure 2B) as compared with met-carriers, mainly driven by reduced deactivation to the CS+ than to the CS− in this area in met-carriers while the opposite pattern (reduced deactivation the CS− as compared to the CS+) was found in val-homozygotes (Figure 2A). An exploratory analysis taking all three genotype groups into account demonstrates this pattern for both carriers of one and two met-alleles, suggesting a dominant effect of the met-allele on vmPFC activation (Supplementary Material and Supplementary Figure S1).

A Stimulus × Time interaction (parametric regressors) indicated faster decaying responses (higher parameter estimates) for the CS+ as compared with the CS− in val-homozygotes as compared with met-carriers in the right amygdala, right perigenual ACC/vmPFC and trend-wise in the left (anterior) hippocampus ($P = 0.023$, $P = 0.023$ and $P = 0.06$, respectively, all P's $FWE_{(SVC)}$; see Table 1).

Amygdala activation was observed in both genotype groups but with different temporal profiles and laterality, categorical in met-carriers and decreasing over time in val-homozygotes.

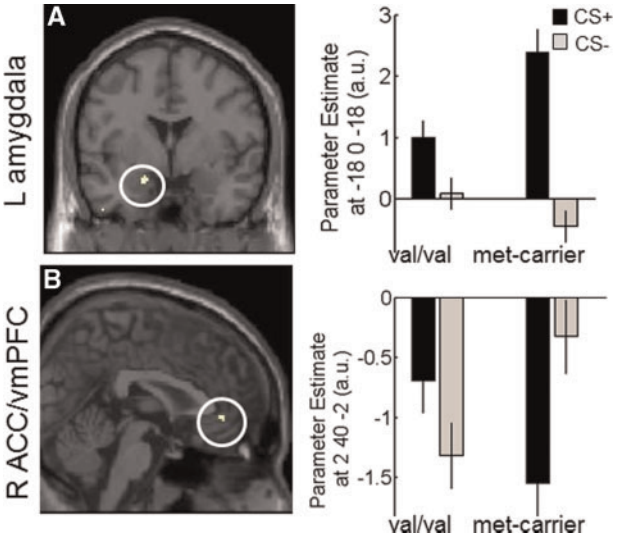

Fig. 2 Activation in the contrast CS+> CS− (categorical) for met-carriers > val/val in the left amygdala **(A)** and for val/val > met-carriers in the vmPFC **(B)**. Images are thresholded at $P < 0.001$(UC) for illustrative purposes. Error bars represent s.e.m. and beta estimates are derived from peak coordinates.

In any of the two genotype groups the discrimination index ([CS+] − [CS−]), derived from beta estimates for the CS+ and the CS− extracted from the peak voxels for the amygdala (Table 1) were correlated with beta estimates extracted from the subgenual anterior cingulate cortex/vmPFC peak voxel after exclusion of one outlier, both P's > 0.38.

See Supplementary Table S1 for exploratory whole brain analyses.

fMRI—fear extinction

During early extinction (first half), which can be taken to reflect fear memory recall 24 h after conditioning CS discrimination, met-carriers showed enhanced (categorical) responses in areas previously implicated in the fear network such as the bilateral insula (both P's < 0.028 $FWE_{(SVC)}$; see Table 1, Figure 3A), right amygdala ($P = 0.013$ $FWE_{(SVC)}$; see Table 1, Figure 3B), left hippocampus ($P = 0.023$ $FWE_{(SVC)}$; see Table 1, Figure 3C) and trendwise in the

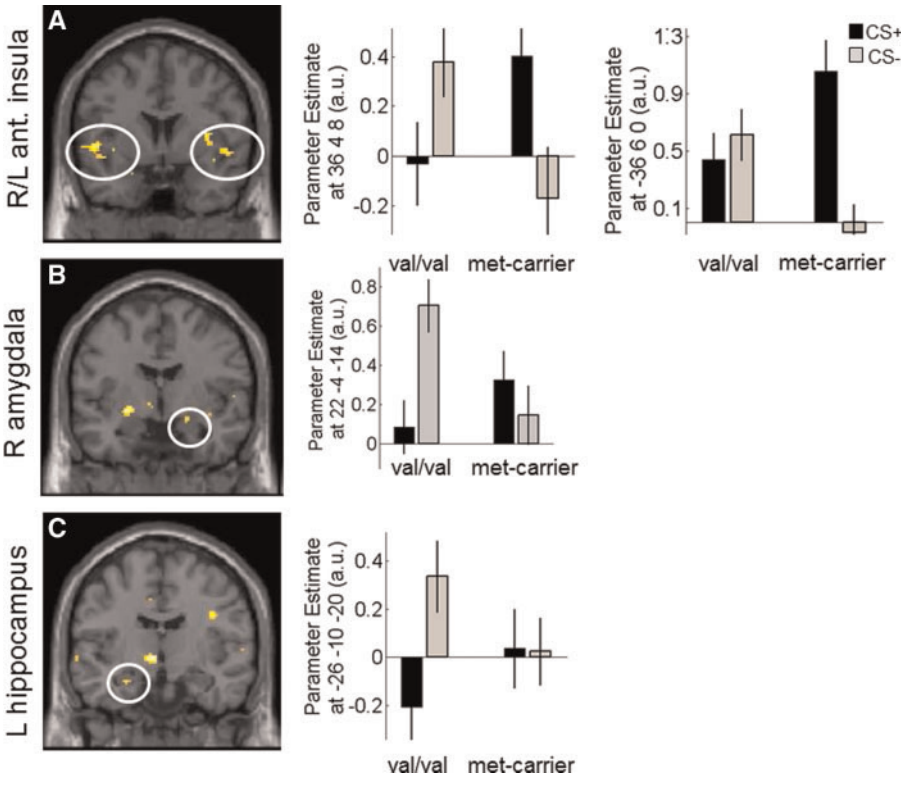

Fig. 3 Activation in the contrast CS+> CS− for met-carriers > val/val in the bilaterial anterior insula **(A)** right amygdala **(B)** and left hippocampus **(C)**. Images are thresholded at $P < 0.001$(UC) for illustrative purposes. Error bars represent s.e.m. and beta estimates are derived from peak coordinates.

(right) dorsal ACC (Table 1), while no areas were more activated in val-homozygotes as compared with met-carriers, suggesting enhanced activation in brain areas associated with fear memory recall in met-carriers than val-homozygotes. While only group differences in the insula reflected CS+ > CS− discrimination in met-carriers, all other significant activation clusters were driven by larger responses to the CS− than the CS+ in val-homozygotes (see parameter estimates Figure 3). A Time × Stimulus interaction as observed in the (left) anterior insula that showed faster declining responses in val-homozygotes as compared with met-carriers. In late extinction in turn, there were no more differences (categorical) between the genotype groups.

Collapsing extinction data across early and late extinction phases, revealed enhanced bilateral activation of the anterior insula in met-carriers as compared with val-homozygotes (both $Ps < 0.039$ $FWE_{(SVC)}$) during CS discrimination.

See Supplementary Table S1 for exploratory whole brain analyses.

DISCUSSION

Our results add an important new piece of the puzzle in understanding the involvement of the human *BDNF*val66met polymorphism in fear and extinction-related processes and thereby complements and extends prior human work. First, we provide the first data on neural activation pattern during fear conditioning depending on the *BDNF*val66met polymorphism and second, report on the stability of this effect by investigating fear recall and extinction 24 h later.

Mechanistically, activity-dependent elevations of BDNF levels during/after fear conditioning may mediate emotion-induced synaptic plasticity and thereby restructuring of synapses at critical sites of the fear network. The *BDNF*val66met polymorphism has been shown to affect activity-dependent BDNF secretion (e.g. Egan *et al.*, 2003) and dendritic arborization and thus has the capacity to affect learning and memory-dependent processes.

The present fMRI results, as our previous results from fear potentiated startle (FPS), suggest an association of *BDNF*val66met with fear acquisition (Lonsdorf *et al.*, 2010; but see Torrents-Rodas *et al.*, 2012) and 24 h-delayed fear memory recall (as indicated by early extinction) but not (delayed) extinction processes (as indicated by late extinction phases) (Lonsdorf *et al.*, 2010).

The observed differences in neural activation pattern, namely enhanced activation of the left amygdala in met-carriers and enhanced perigenual ACC/vmPFC activation in val-homozygotes, nicely mirrors the genotype group differences observed by Soliman *et al.* (2010). While this replicates an association of *BDNF*val66met with reactivity in these brain areas, the present study observed these genotype group differences during 'fear acquisition', while results are derived from the extinction phase in the Soliman *et al.* study (2010), where fMRI data from either the preceding fear acquisition or the reversal learning phases were not reported. Thus, it cannot be excluded that Soliman's results reflect the continuation of genotype group differences that emerged during acquisition or reversal learning, as might be suggested by our results. In addition, the reversal manipulation immediately preceding the extinction phase may have significantly impacted the results. Further complicated by this design, the critical contrast for differential fear conditioning studies CS+ > CS− could not be reported and results are based on the CS+ > fixation baseline contrast, which cannot control for orienting responses and sensitization effects, which affect both CS+ and CS− likewise and thus generally heightened CS-responsivity in met-carriers might be an additional alternative explanation.

In addition to the regions already implicated in genotype-dependent differences by Soliman *et al*, we also observed higher activation in the

bilateral anterior insula and the left hippocampus to the CS+ (>CS−), which are regions implicated in the fear network during early extinction, which likely reflects fear recall. These genotype group differences were largely (with the exception of the anterior insula) driven by an enhanced response to the CS− in val-homozygotes. This is in line with previous work, as genotype differences in insula activation during threat processing have been reported previously (Mukherjee *et al.*, 2011). As no genotype group differences were observed during late extinction, our data speak in favour of a more general association of the *BDNF*val66met polymorphism and emotional learning beyond extinction processes. As extinction occurs rapidly in this paradigm, future studies should investigate genotype-dependent differences in paradigms designed to slow down extinction (e.g. through the use of a lower reinforcement ratio during acquisition).

Previously, we had observed attenuated FPS CS+/CS− discrimination and CS+ potentiation during fear acquisition in contingency aware met-carriers. Based on these previous results and the assumption that FPS is a fear-specific measure and represents an immediate output of the amygdala (Davis and Whalen, 2001), we had expected heightened amygdala responses in val-homozygotes during fear acquisition and fear recall 24 h later. However, contrary to our expectations, we observed heightened left amygdala responses to the CS+ as compared with the CS− in met-carriers. Amygdala activation in val-homozygotes displayed a different temporal profile as evident from faster decaying responses during acquisition. During fear recall 24 h later, we again observed enhanced, however, right amygdala activation in met-carriers as opposed to val-homozygotes. Enhanced activation to the CS+ in brain areas associated with emotional responding in met-carriers is in line with animal work showing that BDNF met knock-in mice show intact and possibly enhanced cue-dependent fear conditioning (Chen *et al.*, 2006) and human work showing stronger amygdala activation in the right hemisphere in response to emotional stimuli in met-carriers (Montag *et al.*, 2008) and in anxious and depressed adolescents to emotional faces (Lau *et al.*, 2010).

At first glance, these results for fear acquisition are, however, at odds with attenuated CS+ potentiated startle reactions (Hajcak *et al.*, 2009; Lonsdorf *et al.*, 2010) and this requires clarification. It is generally accepted that the startle reflex is potentiated during aversive motivational states, while it is attenuated during appetitive motivational states (Lang *et al.*, 1990). Recently however, this clear distinction has been challenged by accumulating reports about paradoxically diminished and not potentiated startle reactions in situations of enhanced autonomic arousal during imminent or prolonged threat (Löw *et al.*, 2008; Richter *et al.*, 2012; Dunning *et al.*, 2013). For example, patients suffering from anxiety disorders characterized by 'discrete' fear (e.g. specific phobias) display 'enhanced' FPS, while disorders characterized by longer lasting 'diffuse anxiety' display 'diminished' FPS (McTeague and Lang, 2012). Such paradoxically reduced startle reactivity has been interpreted as deficient threat mobilization. As the organisms' defensive behaviour is known to depend on threat proximity (predator-imminence model; Fanselow, 1994; Lang *et al.*, 1997), it is tempting to speculate that the observation of enhanced amygdala activation to the CS+ in met-carriers (this study), co-occurring with attenuated CS+ specific FPS in a nearly identical paradigm (Lonsdorf *et al.*, 2010) might be explained though such paradoxical deficiencies in threat reflex mobilization in the face of imminent threats (e.g. it can be speculated that a 100% reinforcement ratio may represent an imminent threat). Future studies should use experimental paradigms explicitly designed to test this model (e.g. Mobbs *et al.*, 2007). If this speculation would be correct, the interpretation of our previous results would need to be revised. In this case, the lack of fear-potentiated startle reactions in met-carriers may not indicate deficient amygdala-dependent fear learning (as discussed in Lonsdorf *et al.*, 2010) but might rather reflect deficient threat reflex mobilization. This would be indicative of 'enhanced' diffuse anxiety-like behaviour, which in turn would be in line with enhanced amygdala reactivity during fear conditioning.

As in previous work (Lonsdorf *et al.*, 2010; Torrents-Rodas *et al.*, 2012), we did not observe any genotype group differences in SCRs, which might not be sensitive and fear-specific enough to detect small group differences in fear conditioning and extinction, while FPS (Lonsdorf *et al.* 2010 but see Torrents-Rodas *et al.* 2012) taps a basic affective level of fear conditioning, largely independent of higher cognition (Hamm and Weike, 2005; Sevenster *et al.*, 2014). This interpretation is supported by other studies that find dissociations between results for SCRs and fMRI in Imaging Genetics studies (e.g. Lonsdorf *et al.*, 2011a; Klucken *et al.*, 2012; Klumpers *et al.*, 2012) and findings that SCRs can be dissociated from amygdala activation during human fear conditioning (Tabbert *et al.*, 2006) and largely reflects contingency awareness (Hamm and Weike, 2005).

Having discussed our present findings in the context of the literature and previous work, it becomes evident that the work on *BDNF*val66met and fear conditioning and extinction processes is paved by divergent findings (for a review see Lonsdorf and Kalisch, 2011b). While Torrents-Rodas *et al.* (2012) did not observe any associations, two fMRI studies report associations with fear conditioning (this study) and fear extinction (Soliman *et al.*, 2010). It may be possible that the aversive fMRI environment may facilitate that genotype-dependent differences become evident. On the other hand, two small behavioural studies also observed genotype-dependent differences in FPS (Hajcak *et al.*, 2009; Lonsdorf *et al.*, 2010), which renders this interpretation unlikely. It is possible that subtle methodological variations (e.g. reinforcement ratio, neutral or emotional CSs) may underlie these differences.

In closing, some limitations of the current study should be mentioned: First, life-time mental disorders and corresponding exclusion of participants was based on the participants self-report, which has to be considered somewhat imprecise. Second, angry male faces served as CSs. It remains therefore unexplored if results would be identical with neutral CSs [e.g. geometrical symbols as in (Hajcak *et al.*, 2009; Soliman *et al.*, 2010; Torrents-Rodas *et al.*, 2012)]. Related to this, it cannot be completely excluded that social events related to angry male individuals occurring in the 24 h between the conditioning and the extinction session may impact extinction results in a genotype-dependent fashion. However, this is rather unlikely. Future studies using delayed extinction tasks should account for this by acquiring information about experiences in between both experimental sessions. Third, most of the participants included in the current study were selected a priori based on other genotypes (*5-HTT*LR/rs25531 and *COMT*val158met) for another study arm, while only a minority was selected specifically for this study arm. Even though, these genotypes were included as covariates, it cannot be excluded that this might affect the results slightly. Fourth, while the genotype differences in the amygdala in acquisition and early extinction/fear recall as well as the left insula activation during early extinction are strong and would even survive a Bonferroni correction for the number of ROIs used [for conditioning and for extinction 4 ROIs each yielding significance threshold of $P = 0.012$ (0.05/4)], all other genotype-dependent differences as well as lateralization of the findings in neural activation pattern should be considered preliminary. Last, but not least, owing to restrictions on acquiring EMG signals inside the scanner, it was not possible to measure FPS in the current study, which would be necessary to directly compare the results with those of our prior work. However, recent technical developments recently resulted in first attempts of simultaneously assessing FPS and fMRI during a fear conditioning experiment, suggesting startle discrimination to be reflected

in amygdala and dorsal PFC activation (van Well *et al.*, 2012). As FPS was not measured, that the behavioural relevance of the present findings remains unclear until future studies indexing both neural and behavioral differences between BDNFval66met genotype groups have validated the interpretations put forth here.

In sum, the present data support a general association of *BDNF*val66met with a broader range of emotional memory processes than merely or selectively for fear extinction. Our preliminary data on all three genotype groups suggest that both an allele-load and a dominant effect of the met-allele may be observed depending on the brain region and function studied. The exact role for the *BDNF*val66met polymorphism in fear conditioning and extinction processes, however, as well as in general emotional memory formation still requires further investigation.

SUPPLEMENTARY DATA

Supplementary data are available at *SCAN* online.

Conflict of Interest

None declared.

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