

To our knowledge, this is the first analysis of *de novo* mutations in ASD, as indexed by birth order, as an explanation for the paternal age effect that simultaneously considered maternal age and family size. The SSC sample, which was limited to sporadic cases of ASD, presumed to be enriched for *de novo* mutations, is a particularly powerful sample to examine this question.

In conclusion, this work adds to the growing body of research that probes potential determinants of the association between *de novo* mutations and NDDs; however, the conflicting results between our work and that of Jaffe *et al.*¹⁴ highlight the complexity of factors that may influence the relationship between advanced paternal age, *de novo* mutations, and NDDs. We highlight the importance of considering both maternal and paternal age,¹⁸ and birth order,^{13,19} as well as the specificity of the findings for SZ and other NDDs.²⁰ Moreover, other factors such as birth interval,¹⁰ and sex¹¹ should also be considered in future studies, along with comparison to the mutation rate in control subjects. In fact, the lack of availability of the full range of potential explanatory variables within the same sample may be one source of the inconsistency of results in previous parental age research. For example, recent evidence suggests that epigenetic mechanisms rather than structural changes may be more strongly associated with paternal age.¹⁹ Direct examination of *de novo* mutations and other genetic variants, together with phenotype information, parental age information, and other relevant perinatal factors using translational epidemiological approaches may be a more fruitful line of investigation.²¹

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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BDNF Val66Met genotype determines hippocampus-dependent behavior via sensitivity to glucocorticoid signaling

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The BDNF gene Val66Met single-nucleotide polymorphism is carried by ~0.55% of Sub-Saharan Africans, 19.9% of Europeans and 43.6% of Asians, but may be carried by up to 72% of some Sub-Asian population groups.¹ The switch from a guanine to adenine nucleotide at position 196 within the pro-region of the BDNF gene causes a Valine (Val) to Methionine (Met) amino-acid residue substitution at codon 66 (Val66Met), resulting in diminished activity-dependent secretion of BDNF at the synapse.² The BDNF gene Val66Met polymorphism has been implicated as a modifier of hippocampal function and is a putative locus of risk for anxiety and affective disorders such as post-traumatic stress disorder (PTSD) and major depression.³ However, the literature suggesting that the loss of function 66Met variant is risk conferring for these disorders is inconsistent; and in some cases is even contradictory by suggesting that the wild-type 66Val allele provides risk as well.³ Likewise, a growing number of human studies have also failed to replicate the hippocampal deficits associated with the 66Met allele as reported by early studies, whereas meta-analyses have suggested that effect sizes of

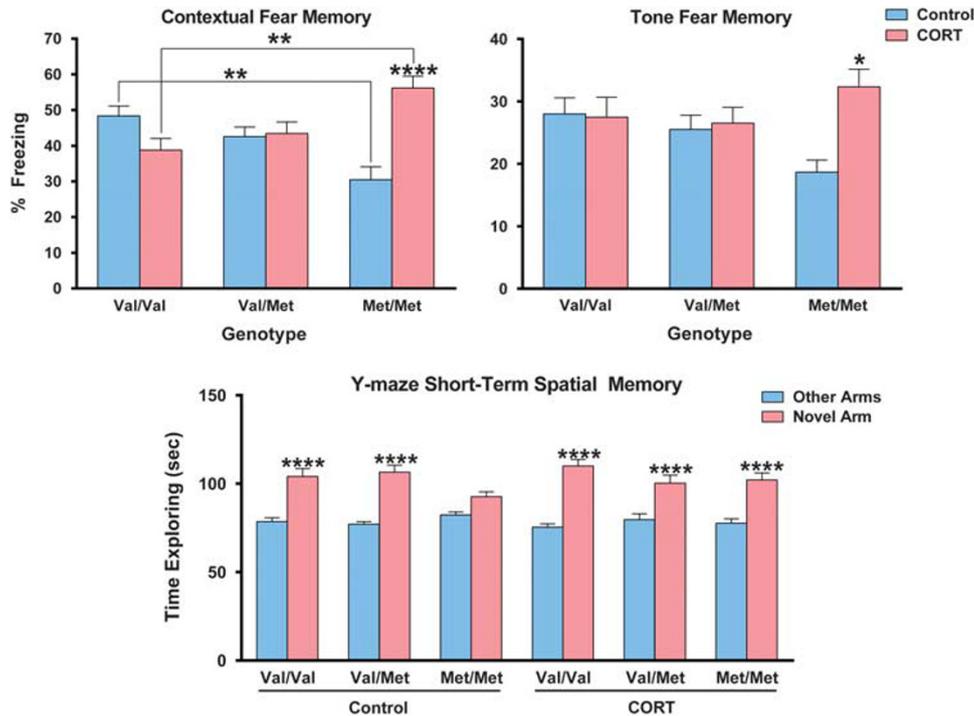


Figure 1. Fear conditioning was used as a measure of emotionally salient memory function. A significant genotype \times treatment interaction was observed for contextual and tone fear memory. At baseline, hBDNF^{Met/Met} mice had the worst task performance of all genotype groups, having significantly worse contextual fear memory than the wild-type hBDNF^{Val/Val} controls. Following CORT, this pattern of results was reversed. For both contextual and tone fear memory, the chronic CORT treatment selectively enhanced the memory function of hBDNF^{Met/Met} mice. The Y-maze, utilized here as a further test of hippocampus-dependent memory, revealed that a significant preference for the novel arm was absent amongst the hBDNF^{Met/Met} genotype group at baseline. Following the chronic CORT treatment, this deficit was reinstated to levels consistent with hBDNF^{Val/Val} controls. All data presented as mean \pm s.e.m.; * $P < 0.05$, ** $P < 0.01$, and **** $P < 0.0001$. Per group, $n = 22-41$.

the Val66Met polymorphism on both cognition⁴ and hippocampal structure⁵ may only be small – if they exist at all. Many explanations for these discrepant results have been suggested, such as a lack of power, regression towards the mean or biased sampling.³ Another account is that discrepant data may be the result of failing to control for complex gene–environment interactions, which may determine or unmask BDNF phenotypes.

We hypothesized that stress, specifically the action of glucocorticoid stress hormones acting on the glucocorticoid receptor (GR), may be one such factor that interacts with the BDNF Val66Met polymorphism to determine hippocampus-dependent behavior. To test this hypothesis, we modeled the long-term effects of chronic stress exposure in a novel mouse model that has been genetically modified to express a humanized BDNF (hBDNF) coding transcript via endogenous mouse promoters that has yet to be behaviorally phenotyped. Specifically, this mouse model has the Val66Met polymorphism knocked-in including an extended sequence of 11 nucleotides across a 274-bp region that humanizes the coding exon of the rodent *Bdnf* gene.⁶ To model stress, we used a chronic corticosterone (CORT) exposure paradigm to specifically induce GR signaling without the confounding effects of other physiological components of the stress response axis. Exposure to CORT was time locked to a developmental period coinciding with late adolescence (weeks 6–8), with the behavioral phenotyping of hBDNF^{Val66Met} mice occurring in adulthood (weeks 11–12) so to probe the long-term behavioral adaptation to mid-developmental GR recruitment.⁷ Tests of hippocampal function were the primary measures of the current study given that this is the primary phenotype of the Val66Met polymorphism and is a key component of the pathophysiology of both anxiety and affective disorders. Further details of our experimental design, the genetic

construct of our hBDNF^{Val66Met} mouse model and methodology can be found in the Supplementary Material.

To assess emotionally salient behavior, fear conditioning was used as a non-spatial, amygdala- and hippocampus-gated, memory paradigm. On day one, mice were conditioned using three tone-shock pairings, before being returned to their conditioning context 24 h later and to a novel context 48 h later to assess contextual and tone fear memory, respectively. A significant genotype \times treatment interaction was observed for hippocampus-dependent contextual fear memory when analyzing males ($F(2,76) = 7.0$, $P = 0.0016$) and females ($F(2,79) = 14.14$, $P < 0.0001$) separately as well as when combined ($F(2,161) = 16.19$, $P < 0.0001$). As this interaction occurred independent of sex, the combined dataset was used for between-group comparisons so to increase power of detecting diminutive effect sizes as predicted by meta-analyses. *Post hoc* analyses of this dataset revealed that hBDNF^{Met/Met} mice had significantly worse contextual fear memory relative to wild-type hBDNF^{Val/Val} mice at baseline ($P < 0.01$), however following the chronic CORT treatment this pattern was reversed with hBDNF^{Met/Met} mice having significantly better contextual fear memory than mice carrying the hBDNF^{Val/Val} genotype ($P < 0.01$). *Post hoc* comparisons also revealed that the CORT-treated hBDNF^{Met/Met} mice had significantly improved contextual fear memory than hBDNF^{Met/Met} mice allocated to vehicle treatment ($P < 0.0001$). For tone-elicited fear memory, none of the main effects reached significance, however, a significant genotype \times treatment interaction, following the same direction as that reported for contextual fear memory, was also observed amongst the sex-collapsed data set ($F(2, 161) = 4.779$, $P = 0.0096$). The only comparison to reach significance was the enhanced tone-elicited fear memory of CORT-treated hBDNF^{Met/Met} mice relative to

hBDNF^{Met/Met} controls ($P < 0.05$). These results highlight that the chronic activation of glucocorticoid receptors during late adolescence potentiates the fear circuitry in adulthood according to hBDNF^{Val66Met} genotype (see Figure 1).

We next examined short-term spatial memory using the Y-maze as an alternative test of hippocampus-dependent memory function that is independent of the fear circuitry. Briefly, mice were allowed to explore two open arms of a three-arm maze for 10 min. One hour later, mice were returned to the maze but allowed to explore all three arms, with intact spatial memory being quantified by exploration time of the previously blocked 'novel' arm. There was no significant main effect or interaction comprising sex on time spent in the novel or other arms, so males and females were once more analyzed together to increase power. A significant interaction between the factors of 'Group' and 'Arm' ($F(2,160) = 5.2$, $P = 0.0065$) was detected. Within-group analysis revealed that hBDNF^{Val/Val} ($P < 0.0001$) and hBDNF^{Val/Met} ($P < 0.0001$) mice showed a highly significant preference for exploring the novel arm, relative to the other arms, indicating intact short-term spatial memory performance. On the other hand, hBDNF^{Met/Met} mice showed a lack of preference for exploring the novel arm relative to the other arms, suggesting that the short-term spatial memory of these mice is subtly disrupted at baseline. Although, the chronic CORT treatment had no effect on Y-maze performance of hBDNF^{Val/Val} and hBDNF^{Val/Met} mice, the disrupted short-term spatial memory of hBDNF^{Met/Met} mice was rescued by CORT treatment ($P < 0.0001$) to levels consistent with hBDNF^{Val/Val} controls. Further experimentation determined that this effect was not the result of altered anxiety-related exploratory drive (see Supplementary Material). This subtle Y-Maze result confirms the specificity of late adolescent glucocorticoid signaling as a long-term modifier of hippocampus-dependent behavior in hBDNF^{Val66Met} mice.

The implications for the novel data reported here is that it is the first to provide experimental evidence that a history of glucocorticoid signaling during adolescence, a bottom-up model of chronic stress, determines adult hippocampus-dependent memory function according to BDNF Val66Met genotype. Outside of already identified sampling factors (e.g., underpowered clinical studies),³ these results suggest that discrepant clinical data on the topic of hippocampus-dependent memory function may be explained by a failure to stratify samples for stressful life events, in that the 66Met allele may be associated with poor memory function at baseline but may recover to the levels similar to, if not better than, controls following a history of stress. In particular, it appears although this effect occurs via the innate susceptibility of Met/Met homozygotes to CORT due to an increase in the expression of glucocorticoid receptors in the dorsal hippocampus during adolescence (see Supplementary Figure 2), which has long-lasting effects on hippocampus-dependent behavior into adulthood.

The data reported here could be interpreted as a protective mechanism of stress in 66Met carriers; whereby, in the absence of glucocorticoid exposure during late adolescence the brain may be more vulnerable to stress in adulthood. However, the clinical literature would suggest that the data reported here is more likely to represent an 'undesirable gain of function' than a 'positive adaptation'. Specifically, risk of depression has been selectively linked to the 66Met allele in females with a history of childhood stress,⁸ while a history of adverse events interacts with 66Met genotype to increase rumination.⁹ In non-humanized 66Met mice exposed to acute stress, there is also an increase in depression-related behavioral markers such as learned helplessness on the forced-swim test.¹⁰ Ultimately, the enhanced memory of fear – while possibly related to depressive disorders – is likely to hold more relevance to anxiety disorders such as PTSD where stress is a requisite factor in the pathogenesis of the disorder.

Although the Val66Met polymorphism has been only briefly investigated in PTSD patients, there is evidence that the 66Met allele is carried with a two- to threefold higher frequency in 'probable' PTSD probands¹¹ and confers resistance to exposure therapy.¹² Further, the 66Met allele has been associated with the persistence of fear in an extinction-learning paradigm in both man and mouse.¹³ Adding to this literature, our data suggest that there is a long-term effect of glucocorticoids in 66Met carriers that potentiates the fear circuitry into adulthood, which may increase susceptibility to trauma, events with negative emotional valence and related psychopathology.

Although clinical studies are required to confirm that the phenotype described here replicates in humans, the current data provides the first evidence for a long-term glucocorticoid-mediated 'switch' of hippocampus-dependent behavior in the hBDNF^{Val66Met} mouse, as well as a theoretical framework from which to resolve a putative role of BDNF in anxiety and affective disorders.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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