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Long-lasting regulation of hippocampal *Bdnf* gene transcription after contextual fear conditioning

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Long-term memory formation requires *de novo* protein synthesis and gene transcription. During contextual long-term memory formation brain-derived neurotrophic factor (BDNF) gene expression changes in conjunction with alterations of DNA methylation in the *Bdnf* gene. However, little is known about the molecular mechanisms underlying the maintenance and persistence of contextual long-term memory. Here, we examined the transcription of specific *Bdnf* exons in the hippocampus for long periods after contextual fear conditioning. We found changes in transcription lasting for at least 24 h after contextual fear conditioning, with some sex-specific effects. In addition, hypomethylation at a CpG site in CpG island 2 located at the end of *Bdnf* exon III sequence was detected at 0.5 h and maintained for up to 24 h after contextual fear conditioning. The identification of these long-lasting changes in transcription and DNA methylation at the *Bdnf* gene suggests that BDNF might have a role for storage of contextual long-term memory in the hippocampus.

Keywords: Contextual fear memory, DNA methylation, gene expression, hippocampus, *Nr4a1*, sex differences

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Long-term memory formation requires *de novo* protein synthesis and gene transcription (Dudai 2004; Silva & Giese 1994). However, little is known about the molecular mechanisms underlying the maintenance and persistence of memory. Recent studies suggest that cellular development and memory processes have homologous molecular mechanisms (Day & Sweatt 2011). Thus, epigenetic coding, which is important for development, might be critical for memory. A key epigenetic mechanism mediating the dynamic regulation of gene transcription is DNA methylation occurring primarily at CpG dinucleotides in the genome and catalyzed by DNA methyltransferases (DNMTs) (Sweatt 2009; Wu & Zhang 2010).

Recent studies have indicated that DNA methylation regulates processes in the mature nervous system including synaptic plasticity and memory formation in adult rodents (Feng *et al*. 2010; Lubin *et al*. 2008; Martinowich *et al*. 2003; Miller & Sweatt 2007; Nelson *et al*. 2008). In contextual fear conditioning, where a neutral environment is associated with an aversive shock, DNMT inhibitors block memory formation (Lubin *et al*. 2008; Miller & Sweatt 2007; Monsey *et al*. 2011). Furthermore, contextual fear conditioning leads to hypermethylation and transcriptional silencing of the memory suppressor gene *PP1* and to rapid demethylation and transcriptional activation of the synaptic plasticity gene *reelin* (Miller & Sweatt 2007). Additionally, after contextual fear conditioning, DNA methylation regulates exon-specific transcription of the *Bdnf* gene (Lubin *et al*. 2008).

Brain-derived neurotrophic factor (BDNF) regulates not only the survival and differentiation of neurons during development, but also synaptic plasticity and memory in the adult brain (Cunha *et al*. 2010; Tyler *et al*. 2002; Yamada *et al*. 2002). BDNF plays an important role in hippocampus-dependent memory including contextual fear conditioning and spatial memory formation (Gorski *et al*. 2003; Lee *et al*. 2004; Liu *et al*. 2004). Furthermore, recent studies have shown that there is a novel protein synthesis- and BDNF-dependent phase in the hippocampus for the persistence of long-term memory storage (Bekinschtein *et al*. 2007, 2008b). This shows that both BDNF and protein synthesis are required not only for the formation of memories soon after training, but also for memory persistence days after training (Bekinschtein *et al*. 2008a). Additionally, reactivation of long-term memory can induce BDNF transcription in the hippocampus (Kirtley & Thomas 2010), although such transcription may not be essential for the maintenance of long-term memory (Lee *et al*. 2004).

The *Bdnf* gene is highly complex, consisting of nine 5′ non-coding exons each linked to individual promoter regions, and a 3′-coding exon (IX), which codes for the BDNF precursor-protein amino acid sequence (Aid *et al*. 2007). For example, *Bdnf* promoter IV regulates *Bdnf* gene transcription and is correlated with DNA methylation state at CpG sites within promoter IV during memory formation or stress in rats (Lubin *et al*. 2008; Roth *et al*. 2009, 2011).

In this study, we examined whether exon-specific *Bdnf* gene transcription is induced for long periods in the hippocampus after contextual fear conditioning and, if so, whether transcription is recapitulated with reactivation of the long-term memory. We also tested whether long-lasting changes in *Bdnf* messenger RNA (mRNA) expression are

linked to altered DNA methylation. All experiments were performed in both male and female mice, because some molecular mechanisms in memory formation are known to be sex-specific (Mizuno & Giese 2010). We identified significant upregulation in transcription of the *Bdnf* gene that persisted for at least 24 h after contextual fear conditioning. These changes correlated with altered DNA methylation at a number of specific CpG sites in CpG islands associated with the *Bdnf* gene.

Materials and methods

Animals

C57BL/6J mice (10 weeks old) were obtained from Charles River Laboratories. Mice were housed of 4–5 mice group per cage under a 12:12 light/dark cycle with foods and water *ad libitum*. All animal procedures were conducted under the United Kingdom Animals (Scientific Procedures) Act, 1986.

Contextual fear conditioning

All animals used for experiments were handled 5 min/day for 3 days before conditioning. All experiments were performed during light cycle. Each mouse was placed into the conditioning chamber (Med Associates Inc., St. Albans, VT, USA) in a soundproof box. After a 148-seconds introductory period, the mouse received a 2-second foot shock (0.75 mA), followed by two foot shocks with a 30-second interval between each. After an additional 30 seconds, the mouse was returned to the home cage. For contextual re-exposure, the mice were brought back to the conditioning chamber for 10 min.

The mice were divided into four groups (males $n = 5$, females $n = 5$ for each group): (1) naïve; (2) C0.5, consolidation group, killed 0.5 h after training; (3) C24.5, consolidation group, killed 24.5 h after training and (4) R0.5, memory reactivation group (reconsolidation), re-exposure to the training context for 10 min 24 h after training and killed 0.5 h after re-exposure. Re-exposure of 10 min to the context has been reported to cause the reactivation of memory, but not extinction after three shocks conditioning (Suzuki *et al*. 2004). A reconsolidation group was included as previous work has highlighted transcriptional differences between consolidation and reconsolidation (von Hertzen & Giese, 2005).

The behavior of the mice (R0.5 group) during re-exposure was videotaped and freezing was scored every 5 seconds for 2 seconds during the initial 5 min if no movements other than respiratory movements were detected.

Quantitative real-time polymerase chain reaction

Hippocampi were fresh-frozen on dry ice and stored at −80°C. Total RNA and genomic DNA were simultaneously purified using the AllPrep DNA/RNA/Protein mini kit (Qiagen, Crawley, UK). Total RNA (2 μg) was reverse transcribed using superscript II reverse transcriptase (Life Technologies, Grand Island, NY, USA). The obtained complementary DNA (cDNA) was diluted 1:10 and stored at −20°C. The cDNA for each sample was checked for genomic DNA contamination using a polymerase chain reaction (PCR) that distinguishes between genomic DNA and cDNA for the hypoxanthine phosphoribosyltransferase (*HPRT*) gene. The primer sequences used were *HPRT* forward 5′-GCTGGTGAAAAGGACCTCT-3′ and *HPRT* reverse 5′-CACAGGACTAGAACACCTGC-3′, and PCR amplification conditions were 93°C for 2 min, 35 amplification cycles (93°C for 30 seconds, 58°C for 45 seconds and 72°C for 1 min) and 72°C for 10 min. The specific *Bdnf* primers used for quantitative real-time PCR (qPCR) are listed in Table S1.

qPCR was performed in triplicate on the DNA Engine (Bio-Rad, Hemel Hempstead, UK) using SYBR Green (PrimerDesign Ltd., Hants, UK) and analyzed using Opticon Monitor analysis software 3.1 (Bio-Rad). Reactions were performed in 96-well ABgene PCR Plates (Thermo Scientific, Hampshire, UK) capped with cap strips. The cycle conditions were 95°C for 10 min, followed by 50 amplification cycles (95°C for 15 seconds and 60°C for 1 min). Validation experiments were performed to show that the efficiency of target and reference amplicons was approximately equal throughout a range of cDNA dilutions after the optimization of primer concentrations. For each sample, the mean threshold cycle (Ct) was determined for target and reference genes. If the absolute value of the slope of log input amount against differences between target and reference gene (dCt) was less than 0.1, then these primer combinations were used for qPCR. The comparative Ct method was used to normalize target mRNA amount to reference (*HPRT* or glyceraldehyde 3-phosphate dehydrogenase) mRNA, and compare the test animals to the naïve group.

DNA methylation analysis

Genomic DNA (500 ng) was treated with sodium bisulfite using the EZ-96 DNA Methylation Kit (Zymo Research, Irvine, CA, USA) following the manufacturers' standard protocol. DNA methylation assays were designed using the online Sequenom EpiDesigner software (www.epidesigner.org). The oligo sequences and the location of the amplicons across which DNA methylation was assessed in this study are given in Table S2. Bisulfite-PCR amplification was conducted using Hot Star *Taq* DNA polymerase (Qiagen) and cycling conditions of 45 cycles with an annealing temperature of 56°C for all amplicons. Subsequent to bisulfite-PCR amplification, DNA methylation analysis was conducted using the Sequenom EpiTYPER system (Sequenom Inc., San Diego, CA, USA). All bisulfite-PCR reactions were performed in duplicate. Positive controls, including both artificially methylated and artificially unmethylated DNA samples were included in all experimental procedures to ensure unambiguous PCR amplification of bisulfite-treated samples. Data generated from the EpiTYPER v1.0.5 software were treated with stringent quality control analysis where CpG units with low calling rates and individuals with a high number of missing CpG units were removed.

Data analysis

Behavior data were analyzed with *t*-test and qPCR data were analyzed with one-way analysis of variance (ANOVA), Kruskal–Wallis one-way ANOVA on ranks, or two-way ANOVA, followed by Student–Newman–Keuls *post hoc* tests, if significant differences were found. Data for DNA methylation were analyzed with standard planned *t*-tests.

Results

Contextual fear conditioning

We trained mice in contextual fear conditioning in three different conditions and investigated long-term changes in *Bdnf* gene expression and DNA methylation in the hippocampus (Fig. 1a). The contextual fear conditioning protocol induced freezing 24 h after training in both sexes (males 46.3 ± 4.4%, females 29.4 ± 3.7%), with males freezing significantly more than females ($t_8 = 2.97$, $P = 0.018$) (Fig. 1b).

Nr4a1 mRNA expression in the hippocampus is regulated after contextual fear conditioning and re-exposure to the context

Nr4a1 (also known as *nur77* or *NGFI-B*) was previously identified as context–shock-specific immediate early gene (von Hertzen & Giese 2005). We tested *Nr4a1* mRNA expression using qPCR in four groups of mice (naïve, C0.5, C24.5 and R0.5; Fig. 2). Because two-way ANOVA showed that data were not normally distributed, pairwise *t*-tests or Mann–Whitney Rank Sum test were performed

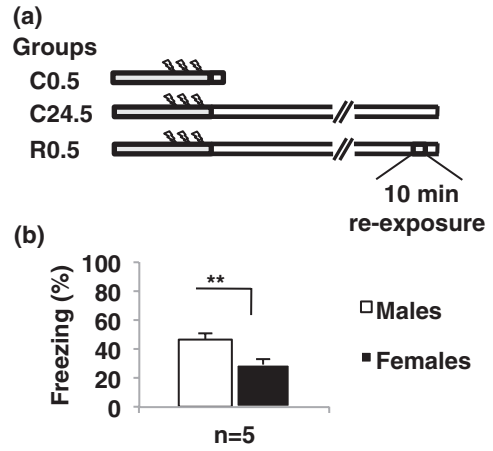

Figure 1: Experimental design and contextual memory after conditioning. (a) Groups to investigate changes in hippocampal mRNA expression and DNA methylation after contextual fear conditioning. The gray boxes indicate exposure to training context, the arrows indicate the foot shocks, and the white boxes show the time until the mice were killed. Groups were studied at: C0.5, conditioned and killed 0.5 h after conditioning; C24.5, conditioned and killed 24.5 h after conditioning; R0.5, conditioning, reexposed 24 h after conditioning and killed 0.5 h after re-exposure. (b) The freezing score during re-exposure, 24 h after the first exposure is shown. Contextual fear conditioning induced freezing to context 24 h after conditioning (R0.5) (males; $n = 5$, females; $n = 5$). Data are means ± SEM; $^{**}P < 0.01$.

between sexes and showed a significant sex difference in *Nr4a1* mRNA expression. *Nr4a1* mRNA expression was higher in females than in males 0.5 h after contextual fear conditioning ($t_8 = 15.04$, $P < 0.001$), but there were no significant sex differences for the other groups (naïve: $t_8 = 0.98$, $P = 0.36$; C24.5: $T_8 = 36$ $P = 0.10$; R0.5: $t_8 = 0.35$, $P = 0.74$). Therefore, the data were analyzed separately for each sex. Female data were analyzed by one-way ANOVA which showed significant differences after contextual fear conditioning ($F_{3,16} = 14.14$, $P < 0.001$). *Post hoc* analysis showed that *Nr4a1* mRNA was significantly upregulated 0.5 h after contextual fear conditioning and 0.5 h after memory reactivation compared with naïve and 24.5 h after contextual fear conditioning (naïve vs. C0.5: $P < 0.001$, naïve vs. R0.5: $P < 0.001$, naïve vs. C24.5: $P = 0.15$, C24.5 vs. C0.5: $P = 0.004$, C24.5 vs. R0.5: $P = 0.003$ and R0.5 vs. C0.5 = 0.77). Male data were analyzed by Kruskal–Wallis one-way ANOVA on ranks. Analysis showed that *Nr4a1* mRNA was significant differences after contextual fear conditioning ($H_{3,16} = 16.16$, $P < 0.05$). *Post hoc* analysis showed that Nr4a1 mRNA was significantly upregulated 0.5 h after contextual fear conditioning and 0.5 h after memory reactivation compared with naïve ($P < 0.05$). Furthermore, 24.5 h after contextual fear conditioning, *Nr4a1* mRNA expression had returned to the baseline level. *Nr4a1* mRNA expression was upregulated when the memory had been reactivated 24 h after contextual fear conditioning ($P < 0.05$) same pattern as females. Previously an upregulation of *Nr4a1* mRNA expression after reactivation of memory was not observed (von Hertzen & Giese 2005): this difference could be due to duration of re-exposure, because we re-exposured for 10 min rather than 5 min. Nr4a1 is one of three members of the NR4A family of transcription factors and is an immediate early gene induced by a variety of stimuli, and especially by the activation of transcription factor cyclic adenosine 5′-phosphate (adenosine monophosphate)-response element binding protein (Hawk & Abel 2011) and after contextual fear conditioning (von Hertzen & Giese 2005). Therefore, our *Nr4a1* mRNA expression analyses showed that our experimental conditions were suitable for the analysis of gene expression after contextual fear conditioning.

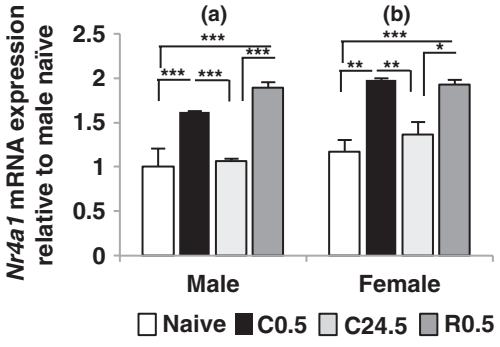

Figure 2: *Nr4a1* mRNA expression in hippocampus is upregulated after contextual fear conditioning and reconsolidation. qPCR showed that *Nr4a1* mRNA expression was upregulated in the hippocampus 0.5 h after contextual fear conditioning and 0.5 h after context-shock memory reactivation compared with the naïve and 24.5 h after contextual fear conditioning groups in males (a) and in females (b). Data are means ± SEM; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Nr4a1 DNA methylation in hippocampus after contextual fear conditioning

We quantified DNA methylation across two *Nr4a1* CpG islands located up-stream and relative to the transcription start site of exon I as shown in Fig. 3a in the hippocampus after contextual fear conditioning. None of the CpG sites showed a significant difference in DNA methylation between groups, although we observed a trend for decreased DNA methylation 0.5 and 24.5 h after contextual fear conditioning at a CpG site (CpG 7) in the promoter CpG island assay (naïve vs. C0.5: $t_8 = 2.07$, $P = 0.073$; naïve vs. C24.5: $t_7 = 2.23$, $P = 0.061$; Fig. 3b). Differences in DNA methylation at both time points do not mirror the mRNA expression changes, which occur only at 0.5 h after contextual fear conditioning.

Regulation of Bdnf gene expression in hippocampus during contextual fear memory conditioning

We studied whether contextual fear memory conditioning associated alterations in *Bdnf* gene expression might involve isoform-specific transcription. We quantified exon-specific

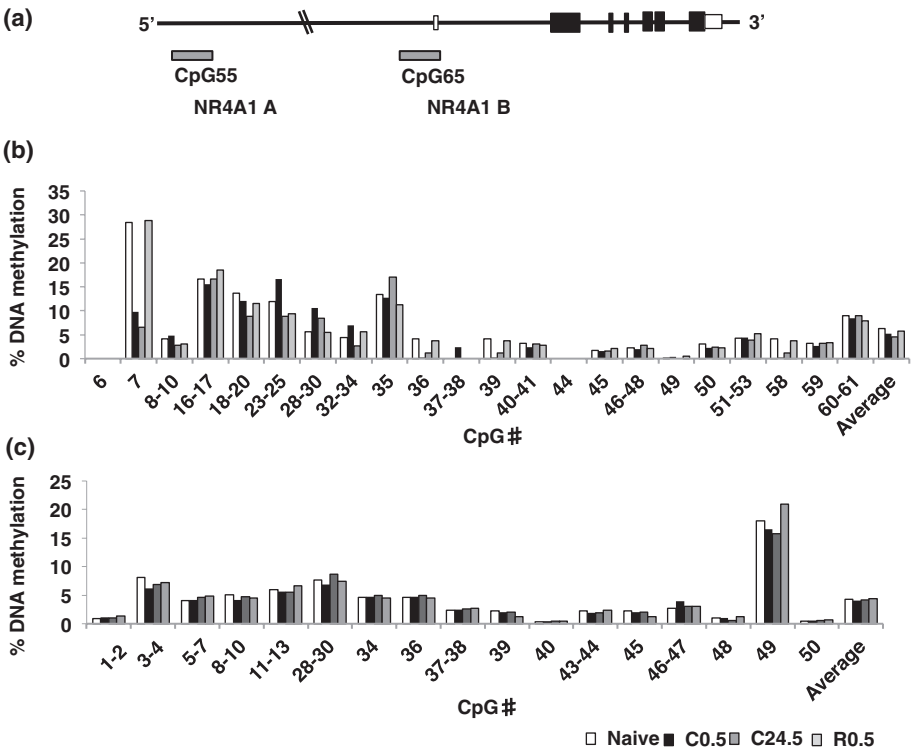

Figure 3: *Nr4a1* DNA methylation in hippocampus is altered after contextual fear conditioning. (a) *Nr4a1B* exons are indicated by boxes: the black boxes indicate the coding region of the gene. The position of two CpG islands (CpG55 and CpG65) are shown by gray boxes. Individual bisulfite-PCR amplicons are labeled NR4A1 A and NR4A1 B and shown under each CpG island. (b) Assay NR4A1A and (c) assay NR4A1B : Sequenom EpiTYPER analysis showed that CpG7 in CpG island 55 was relatively hypomethylated in the hippocampus 0.5 and 24.5 h after contextual fear conditioning compared with the naïve group shown in (b).

Bdnf mRNA using assays specific to six exons (exons I, III, IV, VI, VII and IX) in hippocampus using qPCR across four groups of mice (naïve, C0.5, C24.5 and R0.5). *Bdnf* gene transcripts and the location of the qPCR primers used in this study are illustrated in Fig. 4a.

Bdnf exon I and exon VI mRNA expression in the hippocampus is upregulated for at least 24 h after contextual fear conditioning

Two-way ANOVA showed that there was no significant sex difference in *Bdnf* exon I and VI mRNA expression (exon I: $F_{1,32} = 0.781$, $P = 0.384$; exon VI: $F_{1,32} = 1.11$, $P = 0.30$), so subsequent analyzes were performed on data pooled across both sexes. One-way ANOVA showed significant differences after contextual fear conditioning (exon I: $F_{3,36} = 4.42$, $P = 0.01$; exon VI: $F_{3,36} = 7.19$, $P < 0.001$), as shown in Fig. 4b,c. *Post hoc* analysis showed that *Bdnf* exon I and VI mRNA were significantly upregulated 0.5 and 24.5 h after contextual fear conditioning and 0.5 h after memory reactivation compared with naïve animals (exon I – C0.5: $P = 0.038$, C24.5: $P = 0.013$, R0.5: $P = 0.011$; exon VI – C0.5: $P = 0.049$, C24.5: $P = 0.002$, R0.5: $P = 0.001$). However, there was no significant difference in expression between 0.5 and 24.5 h after contextual fear conditioning (exon I: $P = 0.54$; exon VI: $P = 0.14$) or between 0.5 h after contextual fear conditioning and 0.5 h after memory reactivation (exon I: $P = 0.37$; exon VI: $P = 0.07$) or between 24.5 h after contextual fear conditioning and 0.5 h after memory reactivation (exon I: $P = 0.87$; exon VI: $P = 0.94$). Therefore, *Bdnf* exon I and exon VI mRNA appears to be upregulated at 0.5 h and maintained at this level for at least 24 h after contextual fear conditioning, and memory reactivation does not further upregulated the expression.

Bdnf exon IV, exon VII and exon IX mRNA expression in the hippocampus is upregulated for at least 24 h after contextual fear conditioning in females but not males

Two-way ANOVA showed significant differences in training and sex for *Bdnf* exons IV, VII and IX mRNA expression (exon IV: training $F_{3,32} = 7.36$, $P < 0.001$; sex $F_{1,32} = 10.25$, $P = 0.003$; training and sex interaction $F_{3,32} = 0.715$, $P = 0.55$; exon VII: training $F_{3,32} = 8.89$, $P < 0.001$; sex $F_{1,32} = 4.99$, $P = 0.033$; training and sex interaction $F_{3,32} = 0.94$, $P = 0.43$; exon IX: training $F_{3,32} = 9.90$, $P < 0.001$; sex $F_{1,32} = 6.41$, $P = 0.016$ and training and sex interaction $F_{3,32} = 0.97$, $P = 0.42$), as shown in Fig. 5a–c. *Post hoc* analysis showed that *Bdnf* exon IV, VII and IX mRNA were significantly upregulated 0.5 and 24.5 h after contextual fear conditioning and 0.5 h after memory reactivation compared with naïve (C0.5: exon IV, $P = 0.003$; exon VII, $P = 0.031$; exon IX, $P = 0.008$; C24.5: exon IV, $P = 0.039$; exon VII, $P = 0.005$; exon IX, $P < 0.001$ and R0.5: exon IV, $P = 0.003$; exon VII, $P < 0.001$; exon IX, $P < 0.001$). There was a significant higher expression of *Bdnf* exon IV, VII and IX mRNA at 24.5 h after contextual fear conditioning in females than in males (exon IV, $P = 0.01$; exon VII, $P = 0.034$; exon IX, $P = 0.011$). These results suggest that *Bdnf* exons IV, VII and IX mRNA is upregulated and maintained for at least 24 h after contextual fear conditioning in females, but not in males.

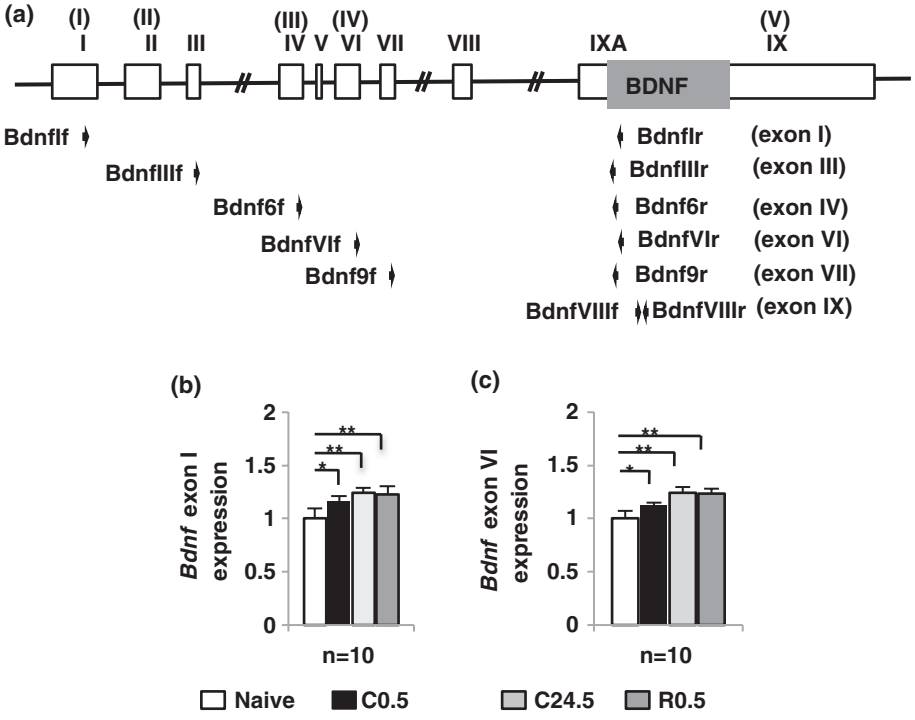

Figure 4: *Bdnf* exon I and exon VI mRNA expression in hippocampus are upregulated after contextual fear conditioning. (a) *Bdnf* exons (I to IX with older nomenclature displayed above in bracket) indicated by boxes. The gray box indicates the coding region of *Bdnf* gene. qRCR analysis was performed by using primer pairs specific for exons I, III, IV, VI, VII and IX shown by the black arrow. qPCR showed that *bdnf* exon I (b) and exon VI (c) mRNA expression were upregulated in the hippocampus 0.5 and 24.5 h after contextual fear conditioning compared the naïve group and not additionally upregulated 0.5 h after context-shock memory reactivation. Data are means ± SEM; $*P < 0.05$; $**P < 0.01$.

Bdnf exon III mRNA expression in the hippocampus is not altered after contextual fear conditioning

Two-way ANOVA identified significant differences in sex and sex/training interaction, but not in training, in *Bdnf* exon III mRNA expression (sex: $F_{1,32} = 7.96$, $P = 0.008$; training: $F_{3,32} = 1.59$, $P = 0.21$ and training and sex interaction: $F_{3,32} = 3.06$, $P = 0.042$), as shown in Fig. 5d. *Post hoc* analysis shows that *Bdnf* exon III mRNA is higher in females than in males 0.5 and 24.5 h after contextual fear conditioning (C0.5: $P = 0.022$; C24.5: $P = 0.002$). There was a significantly higher expression of *Bdnf* exon III mRNA at 24.5 h after contextual fear conditioning in females compared with the female naïve ($P = 0.024$).

Bdnf DNA methylation in the hippocampus is altered after contextual fear conditioning

We studied DNA methylation in the vicinity of four CpG islands associated with the *Bdnf* gene in the hippocampus after contextual fear conditioning. The location of these four *Bdnf* CpG islands located relative to the transcription start site of exons I, III, VI and IX are shown in Fig. 6a. Among the amplicons tested (BDNFA–BDNFI) we found significant differences at several CpG sites particularly in the vicinity of CpG island 2. In particular, CpG6 in the BDNFB amplicon (CpG island 2) was significantly hypomethylated 0.5 and 24.5 h after contextual fear conditioning compared with the naïve (Fig. 6b) (naïve vs. C0.5: $t_{17} = 2.31$, $P = 0.034$; naïve vs. C24.5: $t_{16} = 2.23$, $P = 0.04$; naïve vs. R0.5: $t_{16} = 1.30$, $P = 0.21$). Furthermore, there was a significant difference in average hypomethylation across this amplicon ($t_{17} = 2.30$, $P = 0.035$) at C0.5 compared with naive. These data suggest that specific CpG sites in *Bdnf* CpG island 2 are hypomethylated 0.5 h after contextual fear conditioning with levels maintained up to 24 h although memory reactivation seems to return DNA methylation back to the baseline levels.

Discussion

We studied whether contextual fear memory formation is associated with alterations in *Bdnf* gene expression involving isoform-specific transcription and changes in DNA methylation at relevant CpG islands. We chose contextual fear conditioning for the experiments, because the task is hippocampus-dependent and long-term memory can form by a single training session. Furthermore, *Bdnf* mRNA expression is known to be altered after contextual fear conditioning (Hall *et al*. 2000; Mizuno *et al*. 2006). We found differential transcription of *Bdnf* exons and altered DNA methylation at specific CpG sites in hippocampus after contextual fear conditioning.

We measured exon-specific *Bdnf* mRNA (exons I, III, IV, VI, VII and IX) levels in the hippocampi of four group of mice, naïve, 0.5 and 24.5 h after contextual fear conditioning and 0.5 h after memory had been reactivated 24 h after contextual fear conditioning. We observed three types of expression patterns in exon-specific *Bdnf* mRNA (Table 1): (1) where mRNA expression was upregulated at 0.5 h and was maintained at least 24 h after contextual fear conditioning with no further upregulation following memory reactivation (*Bdnf* exons I and VI), (2) where mRNA expression was upregulated at 0.5 h in both sexes, and maintained at least 24 h only in females after contextual fear conditioning (*Bdnf* exons IV, VII and IX) and (3) where there was no differential

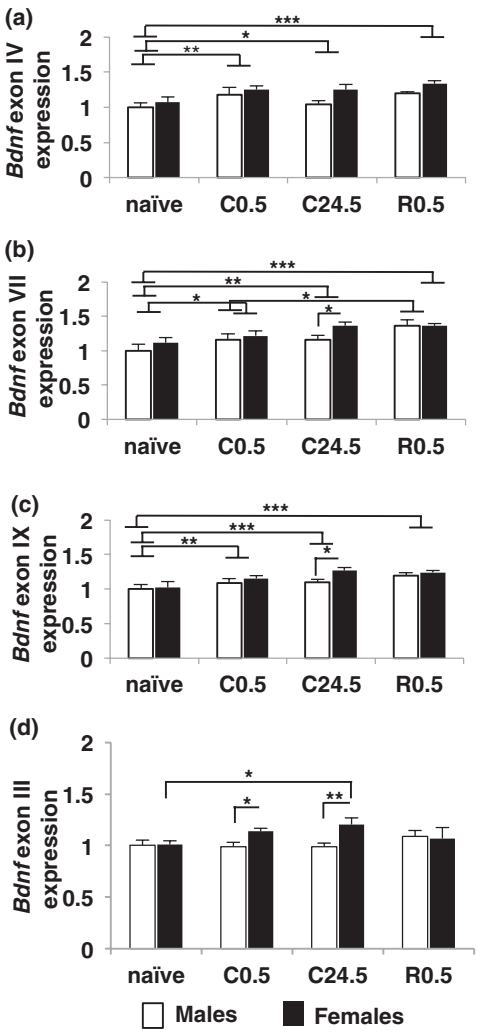

Figure 5: *Bdnf* exons IV, VII and IX mRNA expression is upregulated after contextual fear conditioning and reconsolidation, but not exon III mRNA. qPCR showed that *Bdnf* exons IV (a), VII (b) and IX (c) mRNA expression was upregulated in the hippocampus 0.5 and 24.5 h after contextual fear conditioning and 0.5 h after context–shock memory reactivation compared with the naïve group. There is a sex difference in the upregulation 24.5 h after contextual fear conditioning. *Bdnf* exon VII (b) mRNA expression was upregulated 0.5 h after context–shock memory reactivation compared with 0.5 h after contextual fear conditioning. (d) qPCR showed that *Bdnf* exon III mRNA expression was not upregulated in the hippocampus after contextual fear conditioning and context–shock memory reactivation compared with the naïve group. *Bdnf* exon III mRNA was higher in females compared with males 0.5 and 24.5 h after contextual fear conditioning. Data are means ± SEM; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

regulation of *Bdnf* by conditioning in males (*Bdnf* exon III). To our knowledge we describe for the first time transcriptional changes in the hippocampus 24 h after training. Generally, it is believed that memory consolidation is completed within 24 h (Dudai 2004). Therefore, transcriptional changes at 24 h of contextual fear conditioning might contribute to ongoing storage mechanisms of long-term memory.

Table 1: Summary of *Bdnf* exon expression after contextual fear conditioning

Type	Exon		C0.5	C24.5	R0.5
I	I, VI		↑	↑	↑
II	IV, VII, IX	M	↑	–	↑
		F	↑	↑	↑
III	III		–	–	–

F, females; M, males; ↑, upregulation compared with naïve group; – , no change compared with naïve group.

Our findings are in agreement with recent studies examining the differential usage of *Bdnf* exons in hippocampus and amygdala during consolidation of fear learning in male rats (Lubin *et al*. 2008; Rattiner *et al*. 2004; Ou & Gean 2007). We show that *Bdnf* exons I and VI are upregulated by contextual fear conditioning. Our experimental design could not determine whether this regulation is specific for the context–shock association or whether it is due to context exposure *per se*. Consistent with our findings, Lubin *et al*. (2008) found that *Bdnf* exons I and VI are upregulated in the contextual fear conditioning task. Their control experiment determined that this alteration is due to learning a novel environment rather than associative learning between context and shock, as these exons are upregulated after context exposure alone and not context–shock association. Additionally, Lubin *et al*. found that *Bdnf* exon IV mRNA is upregulated in hippocampus 2 h after training in male rats. Consistently, our data show that *Bdnf* exon IV mRNA is upregulated at 30 min after training in male mice. Lubin *et al*. did not find a regulation at this time point for total *Bdnf* exon IX transcripts in male rats, which we also did not find in male mice at this time point. However, we investigated *Bdnf* regulation 24 h after training in more detail than previous studies. We found that in male mice *Bdnf* exons I and VI expression is still upregulated 24 h after conditioning. We also show a long-lasting regulation of these two *Bdnf* transcripts in female mice. Additionally, in female mice *Bdnf* exons IV and IX mRNA expression is still upregulated 24 h after conditioning. In the current experiments we could not specify what causes this long-lasting upregulation in females, although we hypothesize it could be either due to novelty or the learned association between context and shock, or other factors such as fear (Dalla & Shors 2009). Another limitation of this study is that we were only able to specifically assess the transcription of six of the nine *Bdnf* exons. However, the data presented here highlight that some, but not all, *Bdnf* exons are regulated in a long-lasting manner. Future analyses will build upon these data, and include longer time points and the analysis of additional exons.

Previously, we found sex differences in *Bdnf* exon IX expression after contextual fear conditioning, showing that *Bdnf* mRNA was upregulated 0.5 h after training in males, but not females (Mizuno *et al*. 2006). This is in contrast with the

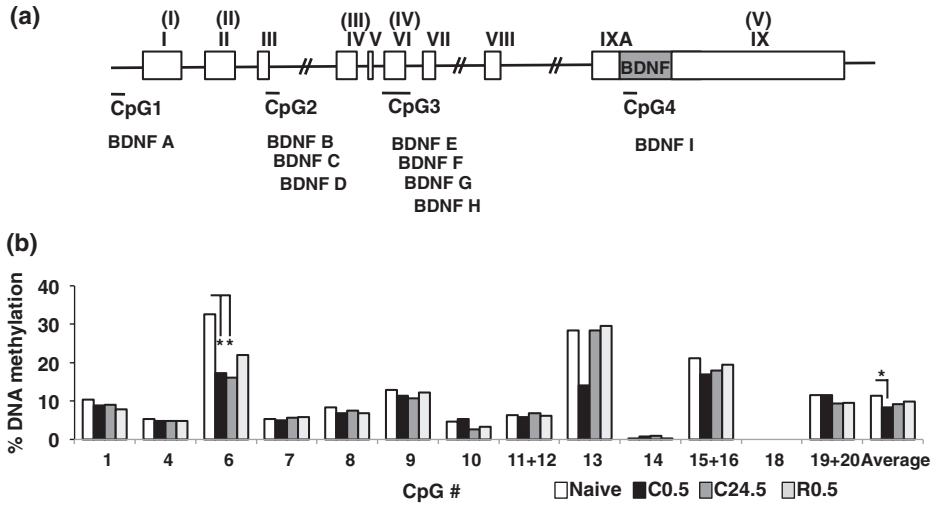

Figure 6: *Bdnf* DNA methylation in hippocampus is altered after contextual fear conditioning. (a) *Bdnf* exons (I to IX with older nomenclature displayed above in bracket) are indicated by boxes; the gray box indicates the coding region of the gene. The position of four *Bdnf* CpG islands are shown by solid lines and indicated relative to transcription site of exons I, III, VI and IX. Individual bisulfite-PCR amplicons are labeled BDNFA to BDNFI and shown under each CpG islands. (b) Assay BDNFB: Sequenom EpiTYPER analysis showed that CpG6 in CpG island 2 was relatively hypomethylated in the hippocampus 0.5 and 24.5 h after contextual fear conditioning compared with the naïve group. $*P < 0.05$.

results for the females presented here. Various parameters differed between these two experiments, including the strain of mice (hybrid vs. C57BL/6J), training paradigm (one shock vs. three shocks) and handling (without handling vs. with handling). These differences can potentially account for different results, although further investigations are warranted.

A previous study suggested that BDNF mRNA expression is upregulated during reconsolidation (Kirtley & Thomas 2010), although such transcription may not be essential for maintaining the long-term memory (Lee *et al*. 2004). Therefore, we also investigated BDNF mRNA expression after reactivation of long-term memory. We found that BDNF mRNA expression is not regulated by memory reactivation in either males or females. Our finding concurs with the notion that BDNF has a role in consolidation but not reconsolidation, as proposed by Lee *et al*. (2004).

We found that DNA methylation is altered at specific CpG sites in the *Bdnf* gene after contextual fear conditioning. In particular, we identified a significant and persistent DNA hypomethylation at a CpG site in the vicinity of CpG island 2, which is located at the end of exon III. This hypomethylation was detected 0.5 h and maintained up to 24 h after contextual fear conditioning. It is notable that the sites of differential DNA methylation appear to be quite restricted in this area, because we did not detect significant change of DNA methylation in the other nearby CpGs. This hypomethylation up to 24 h after contextual fear conditioning corresponds with the increase in exons I and VI mRNA expression in both sexes and IV, VII and IX mRNA expression in females. Although, we have no mechanistic insight about how reduced DNA methylation up-stream of exon IV influences the expression of these exons, the observation suggests that demethylation of CpG sites within CpG islands might promote transcription of exon-specific *Bdnf* gene after contextual fear conditioning. Previously, it was shown that contextual fear conditioning decreases DNA methylation within the *Bdnf* exon IV promoter in rat hippocampus CA1, and that this is correlated with the upregulation of contextual learning specific *Bdnf* exon IV mRNA expression (Lubin *et al*. 2008). Our findings, although preliminary, represent the first demonstration of training-induced hypomethylation related to altered transcription in the mouse *Bdnf* gene.

We found that several *Bdnf* transcripts are upregulated after contextual fear conditioning, although all encode the same protein. Recently, the functional consequence of multiple transcripts of BDNF is suggested in the 'spatial code hypothesis of *Bdnf* transcripts' (Tongiorgi 2008). In this model, different transcripts represent a spatial code used by neurons to selectively target the effects of BDNF to distinct dendritic compartments (Baj *et al*. 2011). Thus, BDNF might have spatially restricted effects upon dendritic complexity after contextual fear conditioning.

Previously, it was reported that hippocampal DNA methylation is associated with memory formation, however, these hippocampal changes are transient, returning to basal levels within 24 h after conditioning (Miller & Sweatt 2007). Instead, persistent changes in cortical DNA methylation were proposed to underlie long-lasting memory formation (Miller *et al*. 2010). However, our study suggests that there may be longer lasting alterations to DNA methylation in the hippocampus. As contextual fear memory has been suggested to become hippocampus-independent within 4 weeks after training (Frankland & Bontempi 2005), future studies will need to establish how long any dynamic changes to DNA methylation in the *Bdnf* gene lasts.

The hippocampus plays an important role in acquisition and protein synthesis-dependent consolidation of new memories into long-term memory. For the longer-term storage of memory, so called system consolidation, the memory trace is believed to be transiently stored in the hippocampus and transferred to other brain areas such as cortical structures (Frankland & Bontempi 2005). However, it is not clear whether the hippocampus has a temporary or a permanent role in the storage and retrieval of memory (Sutherland & Lehmann 2011). It was shown that 12 h after contextual fear conditioning a novel protein synthesis-dependent phase and BDNF activity in the hippocampus is necessary for memory persistence but not for memory formation (Bekinschtein *et al*. 2007, 2008a, 2010). Here, we show that transcriptional changes in the *Bdnf* gene can persist at least 24 h after conditioning. This suggests that BDNF is required for the establishment of long-lasting memory storage.

In summary, our findings support the recent idea that long-term memories are established and maintained in the hippocampus, in parallel with multiple extra hippocampus networks (Sutherland & Lehmann 2011).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1: Primer sequences for qPCR.

Table S2: Primer sequence for Sequenom.

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