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Long-lasting transcription in hippocampal area CA1 after contextual fear conditioning

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Abstract

A fundamental question is how memory is stored for several weeks and even longer. A long-lasting increase in gene transcription has been suggested to mediate such long-term memory storage. Here, we used contextual fear conditioning in mice to search for lasting transcription that may contribute to long-term memory storage. Our study focussed on hippocampal area CA1, which has been suggested to have a role for at least one week in contextual fear memory. Using an unbiased microarray analysis followed by confirmatory quantitative real-time PCR, we identified an upregulation of two transcription factors, Fosl2 and Nfil3, which lasted for seven days after conditioning. To our knowledge these are the longest transcriptional changes ever detected in the hippocampus after contextual fear conditioning. Thus, our findings suggest novel transcriptional candidates for long-term memory storage.

Keywords

Hippocampus; Contextual Fear Conditioning; Transcription; Fosl2; Nfil3

Introduction

A fundamental question in brain research is how memory is stored for a lifetime. Particular emphasis has been given to the idea that memory storage could be mediated by persistent

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Credit Author Statement

Keiko Mizuno: Conceptualization, Investigation, Visualization, Formal Analysis, Writing-Original draft preparation. **Aaron R. Jeffries**: Formal Analysis. **Ted Abel**: Writing- Review & Editing. **K. Peter Giese**: Conceptualization, Writing- Review & Editing.

Declaration of interest

Authors declare no conflict of interest.

kinase activity at synapses overcoming molecular turnover (Bear et al., 2018; Crick, 1984; Lisman, 1985; 2017; Sacktor & Fenton, 2018; Smolen, Baxter, & Byrne, 2019). However, it is unknown whether such feedforward kinase signaling is sufficient for memory storage. In addition to synaptic signaling, it has been suggested that epigenetic mechanisms in the nucleus contribute to memory storage (Crick, 1984; Smolen, Baxter, & Byrne, 2019; Zovkic, Guzman-Karlsson, & Sweatt, 2013). In this case, involved epigenetic mechanisms would need to cause lasting changes in gene transcription. However, the vast majority of transcriptional studies associated with learning and memory have identified only transient transcriptional events, induced shortly after training and dissipating within 24 hours (Crick, 1984; Zovkic, Guzman-Karlsson, & Sweatt, 2013; Peixoto, & Abel, 2013; Alberini, & Kandel, 2015). Such transient transcription is essential for cellular memory consolidation, which is a prerequisite for long-term memory, but it cannot maintain long-term memory due to its short duration and molecular turnover. Nonetheless, training-induced transcription that lasts longer than the cellular consolidation window has been identified. For example, after contextual fear conditioning, a memory task in which an animal associates a novel environment with an aversive stimulus, transcription of the memory suppressor gene calcineurin is downregulated for at least 30 days in prefrontal cortex (Miller et al., 2010). At this time point also various changes in DNA methylation have been detected (Miller et al., 2010; Halder et al., 2016). Although long-term storage of contextual fear memory involves prefrontal cortex (Frankland, & Bontempi, 2005), it has emerged that the hippocampus may also involved, although this debated (Doron, & Ghoshes, 2018; Lux, Atucha, Kitsukawa, & Sauvage, 2016; Wiltgen, & Tanaka, 2013; Yonelinas, Ranganath, Ekstrom, & Wiltgen, 2019).

Here, we searched for hippocampal transcription lasting at least 7 days after contextual fear conditioning, using an unbiased microarray analysis. The 7-day time point was chosen as there is clear consensus that the hippocampus is required for contextual fear memory (Frankland & Bontempi, 2005). We focussed on hippocampal area CA1, as this region is essential for contextual fear memory (Goshen et al., 2011; Lux, Atucha, Kitsukawa, & Sauvage, 2016; Matsuo, Reijmers, & Mayford, 2008; Rampon, Tang, Goodhouse, Shimizu, Kyin, & Tsien, 2000). We detected and validated an upregulation of two transcription factors, fos-related antigen 2 (Fosl2/Fra-2) and nuclear factor interleukin-3 regulated (Nfil3/E4BP4), persisting for at least 7 days.

Materials and Methods

Subjects:

Experiments were conducted with 3–6 month-old male C57BL/6J mice (Charles River, UK), which were group-housed with food ad libitum with a 12:12 light-dark cycle. The experiments were performed in accordance with the UK Animals Scientific Procedures Act 1986.

Contextual fear conditioning:

The mice were trained as described previously (Irvine, Vernon, & Giese, 2005; Mizuno, Dempster, Mill, & Giese, 2012). Briefly, a mouse was placed in a conditioning chamber

(MedAssociates), after 148 s a mild footshock was provided (0.7 mA, 2 s). Subsequent footshocks were applied at 90 s intervals and this was repeated four times (five footshocks in total). Thirty seconds after the final footshock the mouse was returned to their home cage. The behavioral control group was tested for contextual fear memory 24 hours after conditioning, by exposing the mouse to the conditioning chamber and scoring freezing for 5 minutes.

RNA isolation:

The mice were killed at 7 days after contextual fear conditioning. Hippocampal area CA1 was micro-dissected and fresh-frozen on dry ice and stored at −80°C. Total RNA was isolated using the AllPrep DNA/RNA/Protein mini kit (Qiagen). Total RNA (2 μg) was reverse transcribed using superscript II reverse transcriptase (Invitrogen). The obtained cDNA was diluted 1:10 and stored at −20°C.

Microarray experiment:

The quality of RNA was checked using Agilent RNA 6000 Nano Kit on Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). cRNA samples were hybridized onto Illumina mouse array chip (mouse ref 6v3, Illumina, Inc.; CA, USA) and scanned (Genome Centre; Barts and The London). The resulting data was then extracted from GenomeStudio software (Illumina) and the lumi Bioconductor package (Du, Kibbe, & Lin; 2008) used to apply variance stabilizing transformation followed by robust spline normalization.

Quantitative real-time PCR (qPCR):

qPCR was performed on the same RNA used for the microarray analysis, in triplicate on 7900HT Fast Real-Time PCR System (Applied Biosystems; ABI, US) using SYBR Green (KAPAbiosystems; Roche, Switzerland) and analysed using SDA Software 2.3 (ABI) except Ankrd35. qPCR of Ankrd35 was performed using QuantStudio 7 (ABI). Reactions were performed in 384-wells PCR Plates (Thermo Scientific, Hampshire, UK) with optical adhesive covers (ABI). The cycle conditions were 95°C for 10 min, followed by 40 amplification cycles (95°C for 15s, 60°C for 1min). Analysis of relative gene expression were using delta deltaCt (ddCt) method. The specific primers used for qPCR are listed in Supplementary Table 1.

Statistical analysis:

Normalized microarray data were analysed using significance analysis of microarrays (SAM) (Tusher, Tibshirani, & Chu, 2001) with a false discovery rate (FDR)<0.01. qPCR data freezing scores were analysed by one-way ANOVA followed by Student Newman Keuls post hoc tests. Statistical tests were carried out on SigmaPlot v13.0.

Results

To identify long-lasting transcription in hippocampus that may contribute to memory storage, we trained adult C57BL/6J mice in a strong contextual fear conditioning paradigm (Irvine, Vernon, & Giese, 2005; Mizuno, Dempster, Mill, & Giese, 2012). This training generated contextual fear memory (Fig. 1). RNA from hippocampal area CA1 was isolated

from naïve mice (N = 6) and mice killed 7 days after contextual fear conditioning (N = 6). These RNA samples were compared in a microarray analysis. This approach identified 6 candidate upregulations lasting for at least 7 days after contextual fear conditioning (Table 1). No downregulations were found. Six of these potential upregulations were studied for validation with qPCR. A long-lasting regulation of the transcription factors Fosl2 and Nfil3 was confirmed (Fig. 2), whereas expression changes for Andrd35, Pvalb, H2afj were not significant and Sdf2l1 could not be detected (Table 2). Fosl2 transcription was significantly upregulated after contextual conditioning ($F_{1,10}$=6.12; p=0.033). Nfil3 transcription was also significantly upregulated after contextual conditioning ($F_{1,10}$=12.82 p=0.005).

Discussion

We have identified long-lasting upregulation of two transcription factors, Fosl2 and Nfil3, in hippocampal area CA1 after contextual fear conditioning. The upregulation lasted for at least 7 days after conditioning. Our finding is consistent with the idea that storage of contextual fear memory involves the hippocampus for at least one week (Doron, & Ghoshes, 2018; Goshen et al., 2011; Lux, Atucha, Kitsukawa, & Sauvage, 2016; Wiltgen, & Tanaka, 2013; Yonelinas, Ranganath, Ekstrom, & Wiltgen, 2019).

Our analysis did not include a context-only group, leaving it uncertain whether the observed long-lasting transcriptional changes are specific for a context-shock association. However, we think that this is rather unlikely when considering the role of the hippocampus in contextual fear conditioning. The hippocampus stores a contextual memory, whereas the amygdala, and not the hippocampus, is thought to store the context-shock association (Rudy, Huff & Mattus-Hamat, 2005; Keeley et al., 2006). Accordingly, transcriptional changes in the hippocampus that occur after conditioning, but not in a context only group, may not indicate transcription specific for the context-shock association, instead they may simply reflect that contextual memory is stronger after a shock presentation than without shock presentation.

Previous analyses of transcription after contextual fear conditioning have identified up- and downregulations that terminate within one day in the hippocampus. This is consistent with the widely held view that cellular consolidation is completed within one day. However, it has emerged that beyond this window still cellular consolidation processes occur (Bambah-Mukku, Travaglia, Chen, Pollobibi, & Alberini, 2014; Katche et a., 2010; Mizuno, Dempster, Mill, & Giese, 2012; Ryan et al., 2012). Therefore, in principle even 7-day transcriptional changes may be part of a late consolidation process. Alternatively, such late transcriptions may represent mechanisms of memory storage.

Fosl2 is a member of the c-Fos family of transcription factors (Nishina, Sato, Suzuki, Sato, & Iba, 1990). Phosphorylation of the transcription factor CREB and the CREB-regulated NR4A transcription factors, which are required for memory consolidation (Alberini, & Kandel, 2015), stimulate Fosl2 transcription (Hawk et al., 2012; Spessert, Rapp, Jastrow, Karabul, Blum, & Vollrath, 2000). In turn, Fosl2 transcription increases expression of the transcription factor C/EBPβ (Chang, Rewari, Centrella, & McCarthy, 2004), an effector of memory consolidation (Alberini, & Kandel, 2015; Bambah-Mukku, Travaglia, Chen,

Pollobibi, & Alberini, 2014). Therefore, we suggest that Fosl2 upregulation may execute transcription needed for generation and maintenance of memory lasting for weeks.

Nfil3 is transcriptional repressor, which is activated by CREB and competes with CREB and C/EBP isoforms for binding to target genes (MacGillavry et al., 2009; 2011). It has been suggested that this results in feedforward loops, which seems important for dynamic control of target gene expression. Therefore, we propose that Nfil3 upregulation may also execute transcription required for generation and maintenance of long-lasting memory.

Finally, there may be more long-lasting transcription changes after contextual fear conditioning, which our approach could not detect. For example, a recent study identified 7-day long upregulation of delta-isoform of calcium/calmodulin kinase II (CaMKIIδ) transcription in hippocampus after object location training (Zalcman et al., 2019). Our microarray analysis did not detect a significant CaMKIIδ upregulation 7 days after contextual fear conditioning. This discrepancy could be due to the fact that our analysis focussed on hippocampal area CA1, whereas the object location memory study considered the entire hippocampus. In future, sophisticated approaches, such single-cell RNA-sequencing, may succeed to identify more comprehensively long-lasting transcriptions after contextual fear conditioning.

In conclusion, we have identified for the first time transcription in hippocampal area CA1 that lasts for at least 7 days after contextual fear conditioning. This is an unexpected result, as most transcriptional studies of learning and memory assume that in the hippocampus training-induced transcriptional dissipate within one or two days. Our findings suggeststhat lasting alterations in transcription in hippocampus contribute to contextual long-term memory storage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Unbiased microarray analysis of gene transcription in hippocampal area CA1 7 days after contextual fear conditioning
- Fosl2 expression is upregulated for 7 days after conditioning.
- Nfil3 expression is upregulated for 7 days after conditioning.

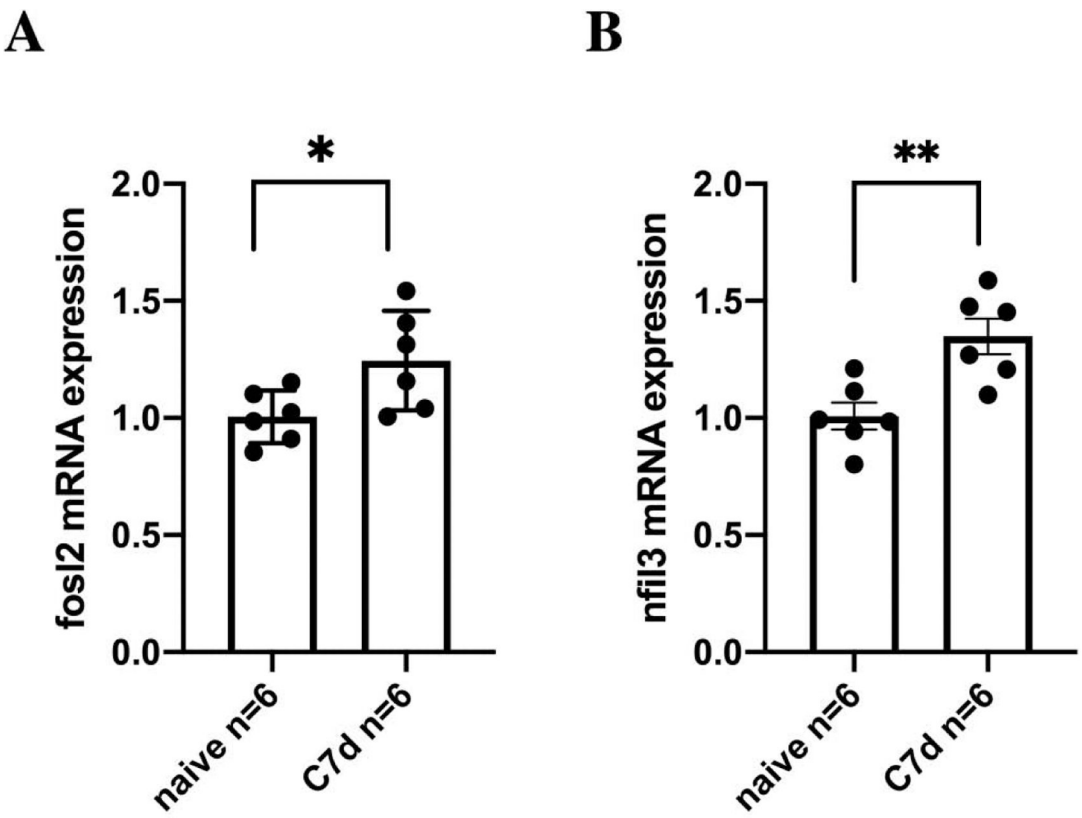

Figure 1. Contextual fear conditioning paradigm generated robust contextual fear memory.
(A) Mice were conditioned after placed in the conditioning chamber for 148s and a mild footshock was provided and followed 4 footshocks at 90s intervals. The mouse was returned to the home cage 30s after the final shock (footshocks are indicated in red arrows). (B) Mice underwent contextual fear conditioning and their 1-day memory was tested by scoring freezing to the trained context. N=6 Means ± sem are indicated.

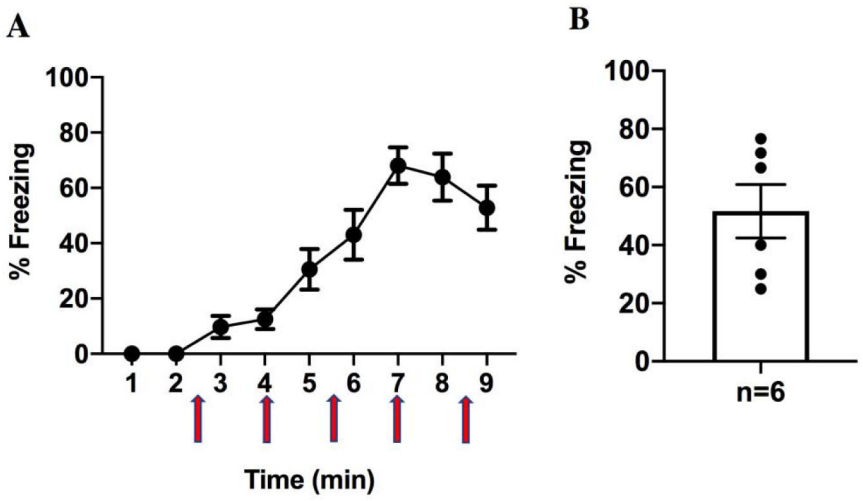

Figure 2. Regulation of Fosl2 and Nfil3 transcription in hippocampal area CA1 after contextual fear conditioning.

Results from a qPCR analysis of Fosl2 (A) and Nfil3 (B) in hippocampal CA1 of naïve mice (N=6) and 7 days (N=6) after contextual fear conditioning. Means ± sem are indicated. ** ($p<0.01$), * ($p<0.05$).

Table 1

Candidate genes upregulated 7d after tear conditioning in microarray analysis.

Gene ID	Gene Name	Score(d)	Numerator(r)	Denominator(s + s0)	Fold change	q-value (%)	
H2afj	770240	4.859465621	0.27775047	0.057156587	1.21230312	< 0.01	ILMN_1222767
Fosl2	7400044	4.079573912	0.328429365	0.080505801	1.255645632	< 0.01	ILMN_1252481
Ankrd 35	5220754	4.004673635	0.251250379	0.06273929	1.190238244	< 0.01	ILMN_3157692
Pvalb	5340064	3.721709584	0.301169958	0.080922477	1.232143219	< 0.01	ILMN_1218223
Sdf2l1	3360010	3.713768409	0.292996474	0.078894654	1.225182338	< 0.01	ILMN_1221943
Nfil3	3840521	3.709728092	0.395717585	0.10667024	1.315596965	< 0.01	ILMN_2595732

Table2.

Summary of confirmational qPCR 7d after fear conditioning

Gene ID	p value naive vs 7dFC
Fosl2	p<0.05
Nfil3	p<0.01
Ankrd35	p>0.05
Pvalb	p>0.05
H2afj	p>0.05
Sdf2l1	not detected