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CALCIUM/CALMODULIN KINASE KINASE β HAS A MALE-SPECIFIC ROLE IN MEMORY FORMATION

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Abstract—The calcium/calmodulin (CaM) kinase cascade regulates gene transcription, which is required for long-term memory formation. Previous studies with *Camkk2* null mutant mice have shown that in males calcium/calmodulin kinase kinase β (CaMKKβ) is required for spatial memory formation and for activation of the transcription factor cyclic AMP-responsive element binding protein (CREB) in the hippocampus by spatial training. Here we show that CaMKKβ is not required for spatial memory formation in female mice as female *Camkk2* null mutants were not impaired in spatial memory formation and they had the same level of hippocampal CREB phosphorylation after spatial training as female wild-type mice. Furthermore, we show that male but not female *Camkk2* null mutants were impaired in long-term potentiation (LTP) at hippocampal CA1 synapses. Finally, a transcriptional analysis of male *Camkk2* null mutants led to the identification of a gene, glycosyl phosphatidyl-inositol anchor attachment protein 1 (GAA1), whose hippocampal mRNA expression was up-regulated by spatial and contextual training in male but not in female wild-type mice. Taken together, we conclude that CaMKKβ has a male-specific function in hippocampal memory formation and we have identified male-restricted transcription occurring during hippocampal memory formation.

Key words: calcium/calmodulin kinase kinase, hippocampus, LTP, mutant mouse, sex difference, memory.

Formation of long-term memory depends on gene transcription and de novo protein synthesis (Silva and Giese, 1994; Dudai, 2004). Activation of the transcription factor cyclic AMP-responsive element binding protein (CREB) is an important step in long-term memory formation (Silva et al., 1998; Josselyn and Nguyen, 2005). In the hippocampus Ca^{2+} signaling can activate the Ca^{2+}/calmodulin (CaM) kinase cascade to induce CREB-dependent transcription (Bito et al., 1996; Wu et al., 2001). The CaM kinase cascade consists of Ca^{2+}/calmodulin kinase kinase α (CaMKKα), Ca^{2+}/calmodulin kinase kinase β (CaMKKβ), Ca^{2+}/calmodulin Ðkinase I (CaMKI), and Ca^{2+}/calmodulin kinase IV (CaMKIV) (Tokumitsu et al., 1995; Kitani et al., 1997; Anderson et al., 1998; Corcoran and Means, 2001). CaMKKα and CaMKKβ phosphorylate CaMKI and CaMKIV to enhance the activity of these kinases, which can then activate CREB (Takemoto-Kimura et al., 2003; Chow et al., 2005). Mouse genetic studies have shown that the CaM kinase cascade is important for hippocampal memory formation. For example, CaMKIV is required for hippocampus-dependent spatial memory formation (Kang et al., 2001; but see Ho et al., 2000) and contextual fear memory formation (Wei et al., 2002), while CaMKKβ is necessary for spatial memory but not contextual fear memory in male mice (Peters et al., 2003). Furthermore, CaMKKβ has been shown to be necessary for the activation of CREB in the hippocampus by spatial training, and to contribute to late long-term potentiation (LTP) at hippocampal CA1 synapses in male mice (Peters et al., 2003). However, a role for CaMKKβ in female mice has not been reported. Here we show that female *Camkk2* null mutants [the *Camkk2* gene encodes the CaMKKβ protein] are not impaired in spatial memory formation and after spatial training have the same levels of CREB phosphorylation in the hippocampus as female wild-type (WT) mice. Furthermore, we find that male but not female *Camkk2* null mutants are impaired in LTP at hippocampal CA1 synapses. Finally, a transcriptional analysis of the *Camkk2* null mutants has identified a gene, the glycosyl phosphatidyl-inositol anchor attachment protein 1 (GAA1), whose mRNA expression is up-regulated in the hippocampus by spatial and contextual training in male but not in female WT mice.

EXPERIMENTAL PROCEDURES

Mice used for phenotypic analysis

Camkk2 null mutants (Peters et al., 2003) and *Camkk1* null mutants (Mizuno et al., 2006) in the 129B6F2-F4 genetic background were used. Heterozygotes were intercrossed to generate homozygotes and WT littermates. All mice used for behavioral and biochemical experiments were 3–6 months old, and for electrophysiological experiments were 9–15 months old. WT and mutant mice had the same appearance, and all behavioral and electrophysiological experiments were performed blind to genotype. Mice were maintained and treated according to the Animals (Scientific Procedures) Act 1986, UK. All procedures conformed to international guidelines on the ethical use of animals, and every effort was made to minimize the number of animals used and their suffering.

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Abbreviations: ANOVA, analysis of variance; CaM, Ca^{2+}/calmodulin; CaMKI, Ca^{2+}/calmodulin kinase I; CaMKIV, Ca^{2+}/calmodulin kinase IV; CaMKKα, Ca^{2+}/calmodulin kinase kinase α; CaMKKβ, Ca^{2+}/calmodulin kinase kinase β; CREB, cyclic AMP-responsive element binding protein; CS, conditioned stimulus; GAA1, glycosyl phosphatidyl-inositol anchor attachment protein 1; GPI, glycosyl phosphatidyl-inositol; LTP, long-term potentiation; qPCR, quantitative real-time PCR; TQ, target quadrant; WT, wild-type.

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Studies of spatial memory formation in the Morris water maze

The Morris water maze experiments were performed as previously described (Peters et al., 2003). Female *Camkk2* mutants and female WT littermates were tested in parallel with male *Camkk2* null mutants and male WT littermates. The results with the male mice have been published (Peters et al., 2003). We had male and female mice balanced for sex, and WT and mutants balanced for genotype in each behavioral group. The mice were handled two minutes per day for eight days before training and were then trained for six days with four trials/day. All mice were given a probe trial at the end of training to assess spatial memory formation.

Contextual fear conditioning

Background contextual fear experiments were performed as previously described (Peters et al., 2003). Female *Camkk2* mutants and female WT littermates were tested in parallel with male *Camkk2* null mutants and male WT littermates. The results with the male mice have been published (Peters et al., 2003). We had male and female mice balanced for sex, and WT and mutants balanced for genotype in each behavioral group. The mice were conditioned with one tone-shock pairing and tested for contextual fear conditioning 24 h after training by scoring freezing.

Analysis of CREB phosphorylation after Morris water maze training

An immunoblot analysis was performed as described (Peters et al., 2003). Male and female mice were treated in parallel and the male data have been reported (Peters et al., 2003). WT mice and *Camkk2* null mutants were trained in the Morris water maze with four trials per day for six days followed by a probe trial. The hippocampi were dissected immediately after the probe trial. Equal hippocampal protein amounts, 15 μg on each lane, were immunoblotted and probed with antibodies against phospho-CREB (Cell Signaling, Beverly, MA, USA) and antibodies recognizing synaptotagmin I (Sigma, St. Louis, MO, USA). The protein expression was normalized to synaptotagmin I expression as the induction of hippocampal LTP does not alter synaptotagmin I levels, while it changes phospho-CREB expression levels (Cooke et al., 2006).

Hippocampal slice electrophysiology

For the submerged experiments, the method was as described (Peters et al., 2003). CA1 late LTP was induced by a stimulus of four 100 Hz, 1 s tetanizations at five minute intervals. Male and female slices were treated in parallel and the male data have been reported (Peters et al., 2003). For interface experiments, the hippocampus was cut in 450 μm slices with a tissue chopper, transferred into an interface recording chamber at 28 °C and perfused with oxygenated artificial cerebrospinal fluid at 1 ml/min. Bipolar twisted nickel-chrome electrodes (50 μm each) were used to stimulate Schaffer collaterals. Late LTP was induced by a stimulus of four 100 Hz, 1 s tetanizations at five minute intervals in male and female *Camkk2* null mutant and WT mice. The artificial cerebrospinal fluid (ACSF) contained (in mM): 124 NaCl, 5 KCl, 26 $NaHCO_3$, 1.24 KH_2PO_4, 2.4 $CaCl_2$, 1.3 $MgSO_4$, and 10 D-glucose and was bubbled with a gas mixture of 95% O_2/5% CO_2. The input–output curve and paired-pulse facilitation were analyzed for each mouse before the LTP experiment.

DNA microarray analysis

Male *Camkk2* null mutants (n=4) and WT littermates (n=4) were trained in the Morris water maze for 3 days with four trials/day. After training a 90 s probe trial was given and the hippocampi were isolated 30 min after the probe trial. Total RNA was isolated from hippocampus using Trizol (Invitrogen, Paisley, UK) and purified using an RNAeasy mini kit (Qiagen, Crawley, UK). Biotinylated cRNA probes for hybridizations were generated according to Affymetrix (Santa Clara, CA, USA) protocols. Fragmented amplified cRNAs were hybridized to a $U74A_{v2}$ microarray, which represents 12,000 genes. One microarray was used per hippocampi from one animal. Data were analyzed with Data Mining Tool (Affymetrix) and GeneSpring (Silicon Genetics, Redwood City, CA, USA) software. Genes with a transcriptional change larger than 40% ($P<0.05$) were selected for the further analyses for the confirmatory experiments.

Quantitative real-time PCR (qPCR)

After six days of training (four trials/day), hippocampi were isolated from either naïve or water maze–trained mice 30 min after a probe trial. For the expression analysis after contextual fear conditioning, hippocampi from four different groups of mice were isolated: 1) naïve mice, 2) contextually conditioned mice and killed 30 min after training; the conditioning was as described (Peters et al., 2003) [after 2 min an 80 dB/2.8 kHz tone was presented for 30 s and during the last two seconds a 0.75 mA foot shock was given], 3) box with tone control group, in which mice were exposed to the training context for three minutes in the presence of tone but in the absence of the foot shock, and killed 30 min after exposure, and 4) latent inhibition control group, in which mice were housed in the training context for 16 h, with water and food *ad libitum*, foot shocked (2 s), and killed 30 min after the foot shock. Total RNA was extracted using Trizol (Invitrogen) and purified using RNAeasy mini kit (Qiagen). cDNA was synthesized from 4 μg of RNA using superscript II reverse transcriptase (Invitrogen). PCR primers:

GAA1 forward: 5′-GGC CAA CAT TTA GCT ACT CAG CAT-3′

GAA1 reverse: 5′-GCG AGC AGC GTC AAC ACA-3′

HPRT forward: 5′-ATA CAG GCC AGA CTT TGT TGG ATT-3′

HPRT reverse: 5′-TCA CTA ATG ACA CAA ACG TGA TTC AA-3′

qPCR was performed using the ABI7000 PCR system with SYBR Green. GAA1 mRNA expressions were normalized to HPRT (hypoxanthine phosphoribosyltransferase) mRNA expression and compared with naïve mice expression level.

Data analysis

Basic synaptic transmission and PPF data were analyzed with the Student's t-test. All other data were analyzed with analysis of variance (ANOVA) followed by Student-Newman-Keuls post hoc tests.

RESULTS

Normal spatial memory formation in female *Camkk2* null mutants

We studied spatial memory formation in the hidden platform version of the Morris water maze (Morris et al., 1982). In the Morris water maze mice learn to navigate to a submerged platform by using distal cues in the room. A probe trial, during which the platform is removed, is given at the end of training to assess spatial memory as indicated by selective searching. Lesion studies have shown that spatial memory formation in our water maze setup requires the hippocampus in male and female 129B6F1 mice (Angelo et al., 2003; Irvine E. E. and Giese K. P., unpublished observations). Previously, we reported that

male *Camkk2* null mutants are impaired in spatial memory formation in the Morris water maze (Peters et al., 2003). In parallel to analyzing male mice, we studied female *Camkk2* null mutants and female WT littermates. Using the six-day training protocol in the Morris water maze, female *Camkk2* null mutants and female WT mice did not differ in latency to locate the platform as revealed by a two-way ANOVA (effect of genotype: $F_{1,31}=0.18$ $P=0.67$, training×genotype interaction: $F_{5,155}=0.94$ $P=0.46$, Fig. 1A). During the probe trial both female *Camkk2* null mutants and female WT mice searched selectively (search times in the target quadrant [TQ]: WT: 34.1±4.3%; mutants: 38.8±4.1%; selectivity: WT: $F_{3,56}=5.34$, $P<0.01$; mutants: $F_{3,68}=10.3$, $P<0.001$, Fig. 1B). Post hoc comparison showed that female *Camkk2* null mutants searched more time in TQ than in any other quadrant (TQ vs. AL: $P<0.001$; TQ vs. AR: $P<0.001$; TQ vs. OP: $P<0.001$), while female WT mice preferred TQ over one other quadrant (TQ vs. AR: $P<0.001$; TQ vs. AL: $P=0.10$; TQ vs. OP: $P=0.14$). Under these conditions the male WT mice also searched selectively in TQ but male *Camkk2* null mutants searched randomly in the probe trial (Peters et al., 2003) (Fig. 1B). A two-way ANOVA of the search times in the TQ during the probe trials of all tested mice revealed a significant sex×genotype interaction ($F_{1,63}=4.52$, $P<0.05$). Student-Newman-Keuls post hoc analysis showed a significant difference between male ($P<0.05$) but not female ($P=0.43$) genotypes. Thus, the male but not the female *Camkk2* null mutants were impaired in spatial memory formation. It should be noted that the impairment in spatial memory formation in male *Camkk2* null mutants is transient because they show normal performance in a probe trial after further training (Peters et al., 2003).

Normal regulation of CREB phosphorylation at serine 133 after spatial training in female *Camkk2* null mutants

The transcription factor CREB, which is required for the formation of hippocampus-dependent long-term memory, is activated by phosphorylation at serine 133 (Silva et al., 1998; Josselyn and Nguyen, 2005). Previously, we reported that spatial training in the Morris water maze induces CREB phosphorylation at serine 133 in the hippocampus in male WT mice but not in male *Camkk2* null mutants (Peters et al., 2003) (Fig. 1C). In parallel, we studied the regulation of hippocampal CREB phosphorylation by spatial training in female mice (Fig. 1C). Although the same amount of spatial training led to spatial memory formation in male WT mice and females of both genotypes (Fig. 1B and Peters et al., 2003), it did not induce CREB phosphorylation either in female WT mice or female *Camkk2* null mutants (one-way ANOVA between naïve and trained WT mice, $F_{1,5}=0.15$ $P=0.52$; one-way ANOVA between naïve and trained mutant mice, $F_{1,7}=4.62$ $P=0.07$) (Fig. 1C). The levels of hippocampal CREB phosphorylation after spatial training were the same in female *Camkk2* null mutants as in female WT mice (one-way ANOVA: $F_{1,9}=0.09$ $P=0.77$). Surprisingly, there was a sex difference in hippocampal CREB activation: Male but not female WT mice induced CREB phosphorylation by spatial training. The male-specific up-regulation of CREB phosphorylation required CaMKKβ (Peters et al., 2003). It is not clear why female WT mice did not induce CREB phosphorylation although they had spatial memory. However, it is possible that female activate CREB at other time points than males and this needs to be investigated in follow-up studies.

Because we observed that male but not female *Camkk2* null mutants were impaired in spatial memory formation and in the activation of CREB we tested whether female *Camkk2* null mutants lacked CaMKKβ protein (Fig. 1D). As expected, male as well as female null mutants lacked CaMKKβ protein.

Normal contextual fear conditioning in female *Camkk2* null mutants

Contextual fear conditioning is another hippocampus-dependent learning and memory task; we reported previously that male *Camkk2* null mutants have normal contextual fear conditioning although they are impaired in spatial memory formation (Peters et al., 2003). In parallel to analyzing male mice, we studied female *Camkk2* null mutants and female WT littermates (Fig. 2). We tested for contextual fear conditioning 24 h after training and two-way ANOVA of the freezing scores did not reveal a significant effect of genotype ($F_{1,31}=0.31$ $P=0.58$) and no significant genotype×sex interaction ($F_{1,31}=3.24$ $P=0.08$). Thus, female *Camkk2* null mutants were not impaired in contextual fear conditioning as male *Camkk2* null mutants.

Normal hippocampal synaptic plasticity in female *Camkk2* null mutants

LTP is a model of long-lasting synaptic plasticity that is thought to contribute to memory formation and storage (Martin et al., 2000; Pastalkova et al., 2006; Whitlock et al., 2006). Late LTP depends on transcription (Nguyen et al., 1994) and we previously reported that CaMKKβ contributes to the late-phase of LTP in hippocampal CA1 synapses in male mice using submerged slice recordings (Peters et al., 2003). At the same time we analyzed late LTP at CA1 synapses in submerged hippocampal slices from female *Camkk2* null mutants and female WT littermates (Fig. 3A). We performed a three-way ANOVA with repeated measures at the time points 30, 60, 90, 120, 150, and 180 min after tetanization in all tested hippocampal slices. The analysis revealed a significant genotype×sex×time interaction ($F_{5,90}=2.62$, $P<0.05$). Student-Newman-Keuls post hoc analysis showed that the male *Camkk2* mutants had less LTP in comparison with male WT mice ($P<0.01$), female WT mice ($P<0.01$), and female *Camkk2* null mutants ($P<0.01$) at 180 min after tetanization. Thus, male but not female *Camkk2* null mutants were impaired in late CA1 LTP.

We also tested the role of CaMKKβ in LTP using interface recordings, which is commonly used to measure late LTP (e.g. Frey et al., 1993). Late LTP was induced by four 100 Hz trains; in WT mice the fEPSP slope increased to 229% for males and 222% for females at 30 min after

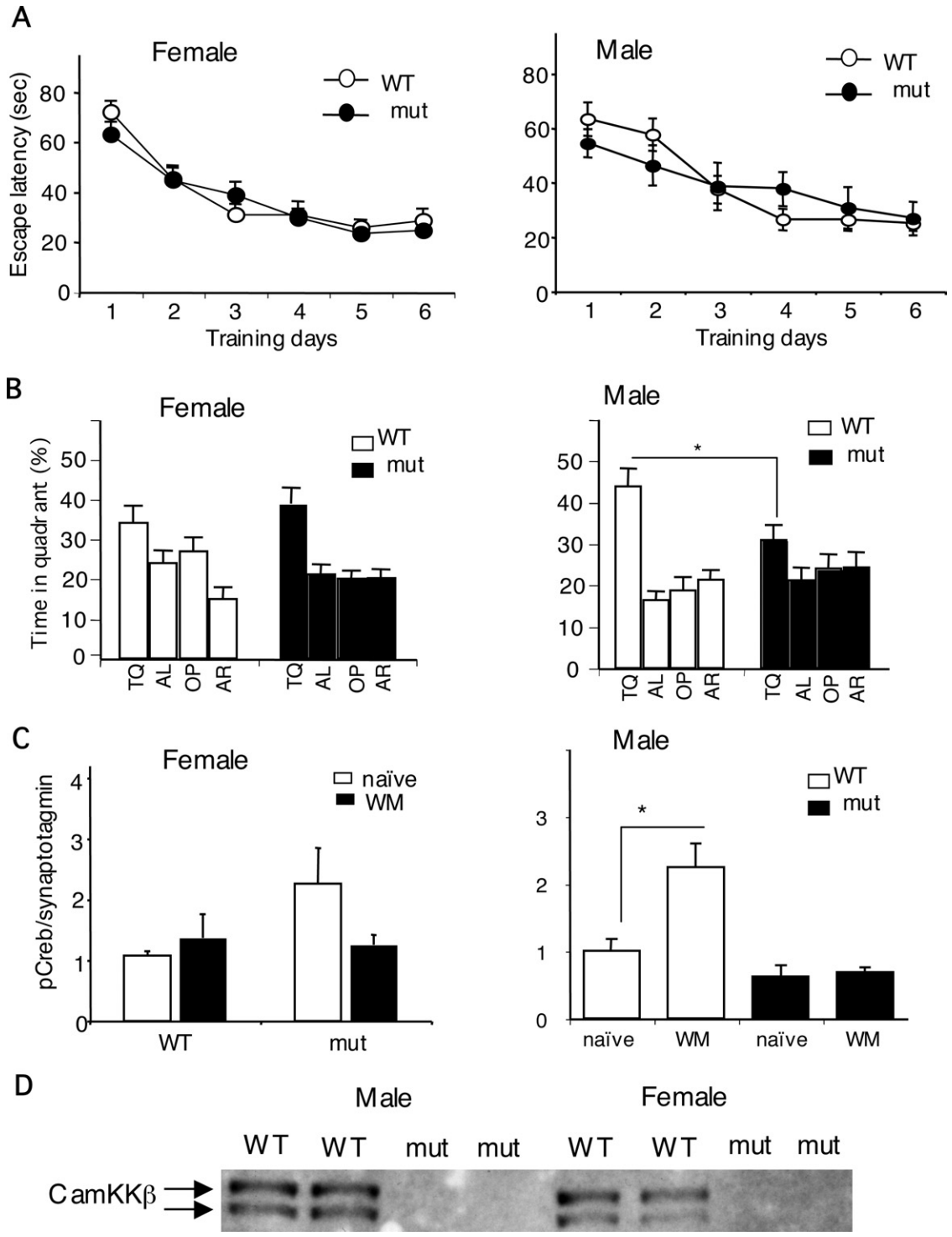

Fig. 1. Normal spatial memory formation in female *Camkk2* null mutants. Means±S.E.M. (A) Time to reach the hidden platform in the water maze did not differ between female *Camkk2* null mutants ($n=18$) and female WT mice ($n=15$). For comparison the previously published male data are shown (Peters et al., 2003). (B) Female *Camkk2* null mutants and female WT mice searched selectively in a probe trial given after training. Under these conditions male *Camkk2* null mutants search randomly in a probe trial (Peters et al., 2003). AL, OP, AR, other quadrants. (C) After spatial training the levels of CREB phosphorylation in the hippocampus were the same in female *Camkk2* null mutants and female WT mice (Naïve: WT, $n=3$; mut, $n=3$, Trained: WT, $n=4$; mut, $n=6$). Under these conditions male *Camkk2* null mutants are impaired in inducing CREB phosphorylation after spatial training (Peters et al., 2003). In naïve female WT mice and naïve female *Camkk2* null mutants the levels of CREB phosphorylation were not significantly different ($F_{1,4}=4.43$ $P=0.11$). WM, water maze-trained. (D) Female and male null mutants lack CaMKKβ protein.

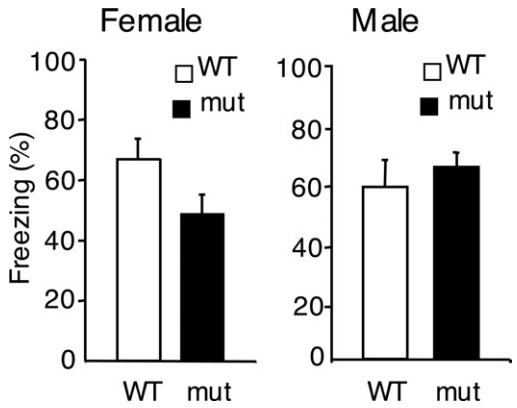

Fig. 2. Normal contextual fear conditioning in female *Camkk2* null mutants. Means±S.E.M. Female *Camkk2* null mutants (n=7) and female WT mice (n=9) froze equally to the training context 24 h after conditioning. For comparison the previously published male data are shown (Peters et al., 2003).

the tetanus ($P<0.01$, paired Student's *t*-test), and this potentiation was lasting more than three hours (Fig. 3B). However, in male but not female *Camkk2* null mutants LTP was significantly smaller in mutants than in WT mice 1, 2 and 3 h after the tetani ($P<0.05$, Student's *t*-test; 240% for male WT mice versus 197% for male mutant mice 1 h after the trains). In addition to these planned comparisons, we performed a three-way ANOVA with repeated measures at the time points 30, 60, 90, 120, 150, and 180 min after tetanization. The analysis revealed a significant sex×time interaction ($F_{5,70}=4.08$ $P<0.01$). Overall, the results of the interface recordings were in agreement with the analysis of the submerged recording data.

Since LTP, induced by one 100 Hz train or theta burst stimulation in this recording system, was normal in male *Camkk2* null mutants (data not shown), we could confirm our previous finding that CaMKKβ contributes to late LTP in male mice (Peters et al., 2003). The residual late LTP in the interface recordings, is most likely due to NMDA receptor-independent LTP, which was induced in conjunction with NMDA receptor-dependent LTP in WT mice (data not shown). Basal synaptic transmission and paired-pulse facilitation were normal in both male and female *Camkk2* null mutant mice ($P>0.05$, Student's *t*-test at stimulation strength used for LTP studies, Fig. 3C, D). However, at very high stimulation strengths there seemed to be less synaptic transmission in female null mutants than in female WT mice, while there was no difference in males (Fig. 3C). The physiological relevance of a potential female-specific impairment in synaptic transmission at very high stimulation strength would not be known.

Up-regulation of hippocampal GAA1 mRNA expression after spatial training is male-specific and seems to require CaMKKβ

Our finding that female *Camkk2* null mutants were normal in spatial memory formation as well as in CREB phosphorylation after spatial training (Fig. 1), while the male *Camkk2* null mutants were impaired (Peters et al., 2003), suggested that there are sex differences in transcription during spatial memory formation. In order to identify genes that may contribute to spatial memory formation in males, we compared hippocampal gene expression between male *Camkk2* null mutants and male WT mice after water maze training using Affymetrix microarrays representing 12,000 known genes and ESTs. The candidate hits are shown in Table 1. Because microarray experiments with tissue samples have a high rate of false positive signals we performed confirmatory qPCR studies on the candidate genes with known function. qPCR confirmations had also the advantage that they could be applied for all candidate gene expressions, which would not have been possible for protein expression analysis due to the lack of relevant antibodies. The first confirmation of differential expression was for the gene encoding the glycosyl phosphatidyl-inositol (GPI) GAA1, which is one component of GPI transamidase that mediates attachment of GPI to proteins (Ikezawa, 2002). We randomly chose to study the expression of this gene in more detail. qPCR was used to test for hippocampal GAA1 mRNA expression by spatial training in male WT mice and in male *Camkk2* null mutants mice (Fig. 4A). One-way ANOVAs confirmed the microarray data: there was a significant effect of training in male WT mice ($F_{1,8}=7.49$, $P<0.05$) but not in male *Camkk2* null mutants ($F_{1,11}=0.051$ $P=0.83$) and there was no significant difference in naïve male mice ($F_{1,10}=0.71$ $P=0.42$). In contrast, a two-way ANOVA showed only a trend for the genotype×training interaction ($F_{1,19}=3.19$ $P=0.09$). Thus, some, but not all evidences suggest that CaMKKβ contributes to the up-regulation of GAA1 mRNA expression in the hippocampus by spatial training in male mice.

Because male, but not female, *Camkk2* null mutants were impaired in spatial memory formation (Fig. 1) and because male *Camkk2* null mutants did not up-regulate hippocampal GAA1 mRNA expression (Fig. 4A) it was conceivable that females do not up-regulate hippocampal GAA1 expression by spatial training. We tested this idea in WT mice. We trained both male and female WT mice in the Morris water maze (Fig. 4B). Two-way ANOVA showed a significant sex×training interaction regarding hippocampal GAA1 mRNA expression ($F_{1,22}=9.67$; $P<0.01$). Student-Newman-Keuls post hoc analysis revealed a significant effect of training for male WT mice ($P<0.001$) but not for female WT mice ($P=0.72$), even though a spatial memory was formed to the same degree as for trained male WT mice (search times in TQ: males; 46.8±4.0%; females; 41.3±2.0%). Furthermore, the GAA1 mRNA expression in naïve WT mice was significantly lower in females than in males ($P<0.001$). Thus, spatial training up-regulated GAA1 mRNA expression in the hippocampus in male but not in female WT mice.

Up-regulation of hippocampal GAA1 mRNA expression after contextual fear conditioning is male-specific and does not require CaMKKα

Our studies on the regulation of hippocampal GAA1 mRNA expression by spatial training could not determine whether the male-specific up-regulation occurs during spatial mem-

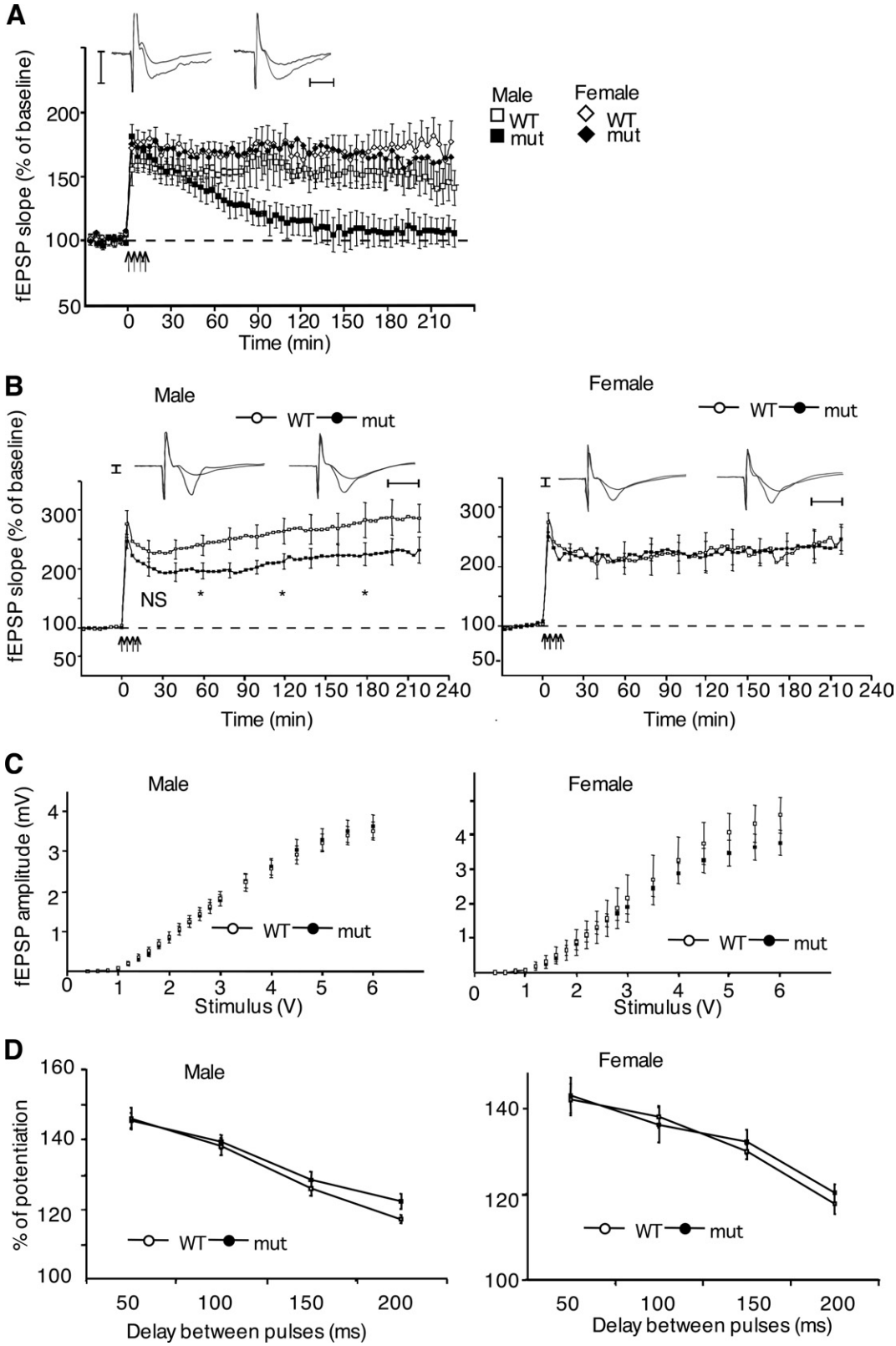

Fig. 3. Normal synaptic plasticity in hippocampal area CA1 in female, but not male, *Camkk2* null mutants. Means±S.E.M.; * $P<0.05$. (A) In submerged slice recordings late LTP was normal in female *Camkk2* null mutants (WT $n=5$ slices, five mice, mut $n=5$ slices, five mice). Under these conditions male *Camkk2* null mutants are impaired in late LTP (Peters et al., 2003). Representative recording traces are shown for WT (left) and mutant mice (right) before and after the induction of LTP. Vertical graduation=1 mV, and horizontal graduation=5 ms. (B) In interface recordings late

Table 1. Genes found at different expression levels in the hippocampus between male *Camkk2* null mutants and male WT mice, which have been trained in the Morris water maze

Gene	Expression level difference: Mut/WT	
	Affy DMT	GeneSpring
Serpina 3a (serine protease inhibitor)	50%	38%
AMPA-1 alpha 1 GluR1	70%	67%
Star (steroidogenic acute regulatory protein 1)	50%	60%
IQGAP1 (IQ motif containing GTPase activating protein 1)	n.d.	67%
GPI-anchor attachment protein 1 (GAA1)	n.d.	67%
Bcl-2-related ovarian killer protein	n.d.	61%
Splicing factor arginine/serine rich 3 (SRp20)	n.d.	56%
PTB2 (polypirimidine tract binding protein 2)	n.d.	160%
Arih1 (ariadne-like E3 ubiquitin ligase)	n.d.	300%
ATP binding cassette subfamily ALD PMP70	n.d.	200%
Riken cDNA 6330403k07, FMS-like tyrosine kinase	100%	150%
Riken cDNA 5730414c17 similar to hippocampal transcript	120%	140%
Synaptotagmin 4	125%	n.d.
Pre B-cell leukemia transcription factor 3 (Pbx3)	n.d.	150%
Neuropeptide Y receptor Y2	n.d.	173%
U2 small nuclear ribonuclear protein (U2AF)	n.d.	149%
Splicing factor praline/glutamine rich (PTB associated) (PSF)	n.d.	190%

(n.d. = Not detected); qPCR confirmed the expression change for GAA1.

ory formation or is induced by other factors such as swimming activity. To determine whether the up-regulation of hippocampal GAA1 mRNA expression is specific for memory formation we investigated the expression after contextual fear conditioning, a more simple hippocampus-dependent learning and memory task, for which control experiments can be easily performed (e.g. von Hertzen and Giese, 2005). First, we compared the hippocampal GAA1 mRNA expression in naïve male WT mice, conditioned stimulus (CS)-control WT males (Box+Tone), and contextually conditioned WT males (Fig. 5A). A one-way ANOVA revealed a significant difference between the groups ($F_{2,18}$=4.34; P<0.05). Student-Newman-Keuls post hoc analysis showed that there was a specific up-regulation for contextual fear conditioning (P<0.05 for both conditioned vs. naïve and conditioned vs. CS control). Furthermore, we investigated whether the unconditioned stimulus (US) alone could have caused the up-regulation in the male

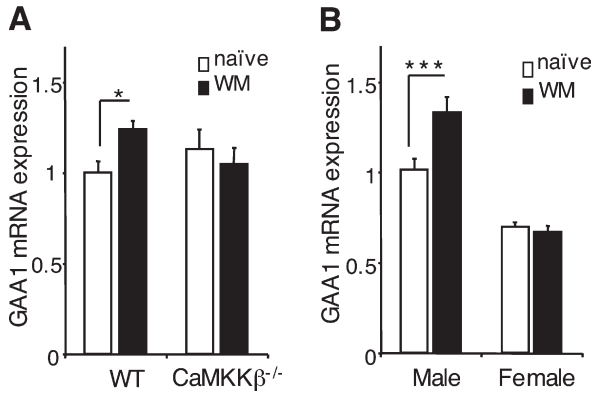

Fig. 4. Up-regulation of hippocampal GAA1 mRNA expression after spatial training is male-specific and seems to require CaMKKβ. Means±S.E.M.; * P<0.05, *** P<0.001. (A) One-way ANOVAs confirmed the microarray analysis that hippocampal GAA1 mRNA expression is up-regulated after spatial training in male WT mice but not in male *Camkk2* null mutants. Basal level expression was not different between the genotypes (WT: naïve n=6, WM n=4, mut: naïve n=6, WM n=7). WM, water maze-trained. (B) Hippocampal GAA1 mRNA expression was up-regulated after spatial training in male, but not in female, WT mice (males: naïve n=8, WM=5, females: naïve n=7, WM n=6).

hippocampus by comparing the expression between naïve males and a latent inhibition control group that is commonly used to control for shock-related expression changes (von Hertzen and Giese, 2005) (Fig. 5A). One-way ANOVA showed no significant effect of latent inhibition (P=0.41). Thus, contextual fear conditioning induced an up-regulation of hippocampal GAA1 mRNA expression in male WT mice that was specific for the learned context-shock association, indicating that the up-regulation occurs during context-fear memory formation.

Next, we tested whether hippocampal GAA1 mRNA expression is also up-regulated by contextual fear conditioning in female WT mice (Fig. 5B). In contrast with male WT mice there was no significant up-regulation of hippocampal GAA1 mRNA expression of conditioned female WT mice ($F_{1,9}$=1.54, P=0.25). Two-way ANOVA comparing hippocampal GAA1 mRNA expression in naïve and contextually conditioned WT mice of both sexes revealed a significant sex×training interaction ($F_{1,22}$=6.32; P<0.05). Student-Newman-Keuls post hoc analysis showed a significant effect of training for male WT mice (P<0.01) but not for female WT mice (P=0.51). Thus, hippocampal GAA1 mRNA expression was up-regulated during contextual fear memory formation in male but not in female WT mice.

Regarding the regulation of hippocampal GAA1 mRNA expression, it was surprising that the expression was up-regulated after contextual fear conditioning because

LTP was reduced in male, but not in female, *Camkk2* null mutants (males: WT n=5 slices, five mice, mut n=5 slices, five mice, females: WT n=4 slices, four mice, mut n=4 slices, four mice). Representative recording traces are shown for WT (left) and *Camkk2* null mutants (right) before and after the induction of LTP. Vertical graduation=1 mV, and horizontal graduation=5 ms. (C) Input–output curve of fEPSP amplitude (mV) versus stimulus (V) at the Schaeffer collateral pathway did not differ significantly between genotypes (males: WT n=20 slices, 10 mice, mut n=20 slices, 10 mice, females: WT n=8 slices, five mice, mut n=8 slices, five mice). (D) Paired-pulse facilitation did not differ between genotypes (males: WT: n=19 slices, 10 mice, mut: n=19 slices, 10 mice, females: WT n=8 slices, five mice, mut n=8 slices, five mice).

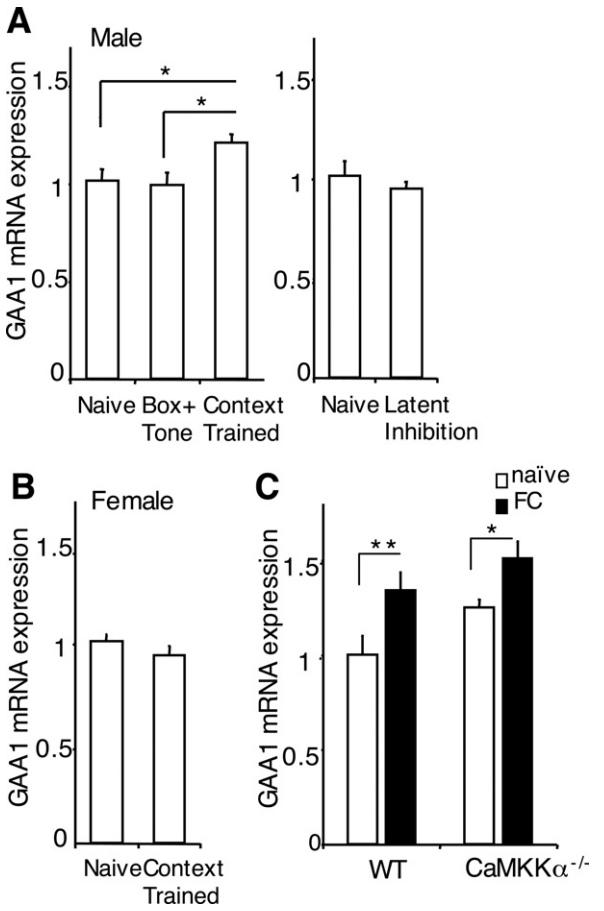

Fig. 5. Up-regulation of hippocampal GAA1 mRNA expression after contextual fear conditioning is male-specific and does not require CaMKKα. Means±S.E.M.; * $P<0.05$, ** $P<0.01$. (A) Hippocampal GAA1 mRNA expression was up-regulated after contextual fear conditioning in male WT mice and the up-regulation was specific for the learned context-shock association (left: naïve $n=8$, box+tone $n=6$, context-trained $n=7$; right: naïve $n=4$, latent inhibition $n=5$). (B) Hippocampal GAA1 mRNA expression was not regulated by contextual fear conditioning in female WT mice (naïve $n=6$, context-trained $n=5$). (C) Hippocampal GAA1 mRNA expression was up-regulated after contextual fear conditioning in male *Camkk1* null mutants demonstrating that CaMKKα is not required for the up-regulation (WT: naïve $n=4$, FC $n=5$, Mut: naïve $n=4$, FC $n=5$).

CaMKKβ is not required for contextual fear conditioning in male mice (Peters et al., 2003). However, CaMKKα is required for contextual fear conditioning in male but not female mice (Mizuno et al., 2006). Therefore, we tested the idea that CaMKKα is required for the up-regulation of hippocampal GAA1 mRNA expression after contextual fear conditioning by studying the expression in male *Camkk1* null mutant mice and in male WT mice (Fig. 5C). Two-way ANOVA revealed a significant effect of training ($F_{1,14}=15.7$ $P=0.001$) and no significant genotype× training interaction ($F_{1,14}=0.71$ $P=0.41$). Student-Newman-Keuls post hoc analysis showed a significant effect of training for male WT mice ($P<0.01$) and male *Camkk1* null mutants ($P<0.05$). Thus, CaMKKα is not required for the up-regulation of hippocampal GAA1 mRNA expression by contextual fear conditioning. Taking into consideration the contextual fear conditioning impairment of the male *Camkk1* null mutants (Mizuno et al., 2006), we conclude that the GAA1 mRNA up-regulation in these mutants is not sufficient for contextual fear memory formation.

DISCUSSION

We have shown that in female mice CaMKKβ is not required for spatial memory formation and late LTP, while in male mice the kinase is required for spatial memory formation and late LTP (Peters et al., 2003). Importantly, a combined statistical analysis confirmed that CaMKKβ is required for spatial memory formation and late LTP in male but not in female mice. Furthermore, a transcriptional analysis of male *Camkk2* null mutants led to the identification of a gene, GAA1, whose hippocampal mRNA expression is up-regulated after spatial and contextual training in male but not in female WT mice. In agreement with a sex-specific regulation of hippocampal GAA1 mRNA expression we found that GAA1 mRNA expression was lower in naïve female than in naïve male WT mice.

The existence of sex differences in spatial memory formation in the hidden-platform version of the Morris water maze in WT rodents has been debated (Jonasson, 2005; Cahill, 2006). However, it is important to note that the inability to detect sex differences in standard behavioral performance in WT rodents does not preclude the possibility of mechanistic differences. Our finding that male but not female *Camkk2* null mutants are impaired in spatial memory formation and LTP at hippocampal CA1 synapses is a genetic dissection of sex-specific mechanisms of hippocampal memory formation. Further genetic evidence for sex-specific mechanisms of hippocampal memory formation is provided by experiments with transgenic mice expressing low-levels of p25; female but not male p25 mutants have improved spatial memory formation and enhanced LTP at hippocampal CA1 synapses (Ris et al., 2005). Additional evidence for sex-specific mechanisms of spatial memory formation is given by our finding that identical spatial training up-regulates hippocampal CREB phosphorylation and induces an up-regulation of hippocampal GAA1 mRNA expression only in male WT mice. However, it should be noted that the observed sex differences in hippocampal CREB phosphorylation after water maze training could also have resulted from sex differences in stress caused by the training (Shors, 2006). In contrast, the sex difference in the up-regulation of hippocampal GAA1 mRNA expression is very likely to be specific for spatial memory formation as our contextual fear conditioning experiments have shown that the up-regulation of hippocampal mRNA expression in males is specific for a learned association. Thus, there are evidences for sex-specific mechanisms of spatial memory formation; next to them there are also sex-independent mechanisms of spatial memory formation, such as autophosphorylation of the α-isoform of Ca^{2+}/calmodulin-dependent kinase II, which is essential for spatial memory formation in males and females (Giese et al., 1998; Need and Giese, 2003).

It has been suggested that sex differences in spatial learning in rodents result from distinct spatial learning strategies, making use of either geometric or landmark cues, which has also been shown for humans in virtual reality experiments (Maguire et al., 1999; Sandstrom et al., 1998; Roof and Stein, 1999). In principle different spatial learning strategies could induce distinct transcriptions during spatial memory formation. However, our results are not conclusive as to whether the impairment in spatial memory formation in male *Camkk2* null mutants resulted from a deficient learning strategy, which was not used by female *Camkk2* null mutants. Our contextual fear conditioning studies have shown that male and female *Camkk2* null mutants can equally solve a contextual learning task, suggesting that male and female mutants use similar hippocampal learning strategies. Nonetheless follow-up investigations are needed to establish whether male and female mutants use the same spatial learning strategies.

To our knowledge only one study has identified male-specific processes of hippocampal memory formation (Kudo et al., 2004). In this study, CREB phosphorylation was examined after contextual fear conditioning and passive avoidance training; a male-specific up-regulation of hippocampal CREB phosphorylation was identified. However, as CREB phosphorylation is not sufficient to activate transcription (Silva et al., 1998) it remains unclear whether male-specific phosphorylation of CREB in the hippocampus leads to male-specific transcription. Here we have shown that there is male-specific transcription during hippocampal memory formation; contextual fear conditioning induces an up-regulation of hippocampal GAA1 mRNA in male but not in female WT mice. The up-regulation of GAA1 mRNA expression is specific for the learned context-shock association, because the context alone or the shock alone is not sufficient for the up-regulation of GAA1 mRNA expression. GAA1 is involved in GPI anchoring of proteins, suggesting that an up-regulation of GAA1 expression would result in increased GPI anchoring of proteins, which might contribute to memory formation. One of the relevant GPI-anchored molecules might be contactin, a cell adhesion molecule that contributes to hippocampal synaptic plasticity (Murai et al., 2002). However, the function of GAA1 in memory formation will need to be determined in follow-up studies.

CONCLUSION

We conclude that CaMKKβ has an unexpected male-specific function in hippocampal memory formation and we have identified male-restricted transcription occurring during hippocampal memory formation for the first time. Our results suggest that the mechanisms of hippocampal memory consolidation differ between the sexes.

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