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RESEARCH ARTICLE

CaMKII knockdown affects both early and late phases of olfactory long-term memory in the honeybee

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ABSTRACT

Honeybees are able to solve complex learning tasks and memorize learned information for long time periods. The molecular mechanisms mediating long-term memory (LTM) in the honeybee *Apis mellifera* are, to a large part, still unknown. We approached this question by investigating the potential function of the calcium/calmodulin-dependent protein kinase II (CaMKII), an enzyme known as a 'molecular memory switch' in vertebrates. CaMKII is able to switch to a calcium-independent constitutively active state, providing a mechanism for a molecular memory and has further been shown to play an essential role in structural synaptic plasticity. Using a combination of knockdown by RNA interference and pharmacological manipulation, we disrupted the function of CaMKII during olfactory learning and memory formation. We found that learning, memory acquisition and mid-term memory were not affected, but all manipulations consistently resulted in an impaired LTM. Both early LTM (24 h after learning) and late LTM (72 h after learning) were significantly disrupted, indicating the necessity of CaMKII in two successive stages of LTM formation in the honeybee.

KEY WORDS: CaMKII, Insect, Olfactory learning, Long-term memory, Mushroom bodies

INTRODUCTION

Higher-order neuronal processes such as learning and memory are crucial for animals that need to be able to adapt to variable environmental conditions. By collecting, processing and storing information, they can use previous experiences to adjust their behavior according to specific needs. Honeybees, for example, are highly social and act as central place foragers, searching for food resources in surrounding areas and then returning to a fixed location (e.g. a nest) to provide food for their colony members (Menzel, 1999; Menzel and Giurfa, 2006; Seeley and Visscher, 1988). To maximize their intake efficiency, foragers learn to identify the most-nourishing flowers, exploit new food sources and, most importantly, remember and share this information with other members of the hive (von Frisch, 1967). To study learning and memory processes in the laboratory, learning assays – especially classical associative olfactory conditioning – have been well established (reviewed in Giurfa and Sandoz, 2012). Whereas earlier attempts to unravel molecular and biochemical pathways associated with learning and memory in the honeybee were often based on pharmacological tools (e.g. Felsenberg et al., 2011; Müller, 1996, 2000), in recent years manipulations using genetic tools such as RNA interference (RNAi) became more accessible (El Hassani et al., 2012; Farooqui et al., 2003; Louis et al., 2012; Matsumoto et al., 2014; Müssig et al., 2010). The possibilities for a combination of several manipulative approaches and the availability of robust associative learning assays make the honeybee an excellent model organism to study the link between behavioral plasticity and its molecular basis.

One protein well known as a 'memory switch' is the calcium/calmodulin-dependent kinase II (CaMKII) (Lisman, 1985). It has the ability to remain constitutively active even after the initial excitation of the neuron is no longer present (Coultrap and Bayer, 2012; Lisman et al., 2012; Malik et al., 2013; Miller and Kennedy, 1986). Because of its autophosphorylation after initial activation, the protein 'switches' to a calcium-independent constitutively active state, thereby providing a mechanism for molecular memory. In the mammalian brain the α and β forms of CaMKII occur predominantly (Miller and Kennedy, 1986) and are highly abundant in the hippocampus, a brain region that has become an important model system for understanding formation of long-term potentiation (LTP) and long-term memory (LTM) (Kerchner and Nicoll, 2008). Here, CaMKII is necessary for the induction of LTP (Giese et al., 1998; Lledo et al., 1995; Malinow et al., 1989), a process widely studied as a mechanism for learning and memory at the cellular and molecular level (e.g. Nabavi et al., 2014). Genetically manipulated mice with disrupted CaMKII function show deficits in learning tasks (Elgersma et al., 2002; Giese et al., 1998; Silva et al., 1992a,b). Together with results from pharmacological blockade of LTP by application of CaMKII inhibitors (Malenka et al., 1989; Malinow et al., 1989), this suggests an important function of CaMKII in LTP and in memory formation.

Mushroom bodies (MBs) are paired structures in the insect brain that function as multisensory integration centers and are known to be involved in associative learning and memory processes in a variety of insects, including the honeybee (Carcaud et al., 2009; Gerber et al., 2004; Heisenberg, 1998, 2003; Menzel and Giurfa, 2001). It has been suggested that MBs and the hippocampus might share a common ancestor and express functional similarities (Tomer et al., 2010; Wolff and Strausfeld, 2015). Interestingly, in accordance with the results on hippocampal distribution and function, CaMKII is highly enriched in the MB of the adult insect brain (Hodge et al., 2006; Kamikouchi et al., 2000; Pasch et al., 2011; Takamatsu et al., 2003; Wolff and Strausfeld, 2015). The first studies on the role of CaMKII in insect behavior have been performed in *Drosophila* and show the importance of CaMKII in learning and memory retention (Akalal et al., 2010; Griffith et al., 1993, 1994; Joiner and Griffith, 1999; Malik et al., 2013). In insects, recent studies also show upregulation of pCaMKII after learning (Lent et al., 2007) and inhibition of CaMKII leads to memory impairment (Matsumoto et al., 2014; Mizunami et al., 2014), which might indicate similar functions for CaMKII in memory formation in insects and vertebrates.

In the present study, we analyzed the role of CaMKII in learning and memory in the honeybee *in vivo* by using RNAi to create

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a CaMKII-knockdown phenotype. In a parallel approach and for comparability with other studies, we used pharmacological inhibition to disrupt CaMKII function. The minimally invasive manipulations were followed by olfactory conditioning and memory tests aimed to identify the potential contribution of CaMKII at various stages of memory formation in the honeybee brain.

MATERIALS AND METHODS

Animals

For all experiments, European honeybees, *Apis mellifera carnica* Pollman 1879, were taken from the apiary of the department of Behavioral Physiology and Sociobiology at the University of Würzburg. Active foragers that were identified by carrying a pollen load and caught in front of the hive entrance were used for all experiments. In all procedures, the investigator was blind to the treatment identity of the bees.

Application of siRNA and pharmacological inhibitors

Honeybee foragers were caught the day before injections, immobilized in a refrigerator at 4°C, and harnessed in plastic holders. An acupuncture needle (Seirin, B2015) was used to poke a small hole through the median ocellus to insert a glass capillary (1B100F-3, WPI, Sarasota, FL, USA) pulled with a DMZ-Universal Puller (Zeitz Instruments, Martinsried, Germany). Using a microinjector (PV820 Pneumatic PicoPump, WPI), 300 nl diluted solution was injected through the medial ocellar tract directly in the honeybee brain. Two siRNAs (siCaMKII and siGFP, 100 µmol l^{-1}), two CaMKII inhibitors (KN-62, 0.5 mmol l^{-1} including 0.5% DMSO and KN-93, 0.5/1 mmol l^{-1}) or two control solutions (KN-92, 1 mmol l^{-1} and Ringer solution including 0.5% DMSO) were injected.

To create a CaMKII-knockdown phenotype, a specific siRNA against the enzyme (siCaMKII) with the sequence GAAUCGUGUGUCCUAUCAA (sense strand) and UUGAUAGGACACACGAUUC (antisense strand) was designed (Eurofins, Ebersberg, Germany). The siRNA sequence was blasted against the honeybee genome using the modified BLASTn search of the NCBI database. No hits showed a similarity higher than 15 nucleotides, which ensures the specificity of the siRNA and minimizes the possibility of off-target effects. Additionally, as a control for general siRNA effects, a standardized siRNA vector against GFP (siGFP) with the sequence GAAUCGUGUGUCCUAUCAATT (sense strand) and UUGAUAGGACACACGAUUCTT (antisense strand) was used (Eurofins). The siRNAs were diluted in siMAX Universal Buffer (Eurofins) to reach a final concentration of 100 µmol l^{-1}.

For pharmacological inhibition of CaMKII, the drugs KN-93 and KN-62 (Sigma-Aldrich) were used. KN-93 inhibits the phosphorylation of target proteins of CaMKII as well as the auto phosphorylation of the enzyme itself (Gao et al., 2006). KN-62 is another CaMKII inhibitor that is shown to block enzyme activity by interfering with Calmodulin binding and to inhibit learning (Tan and Liang, 1997; Tokumitsu et al., 1990). All chemicals were diluted in physiological ringer solution (130 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 4 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} $CaCl_2$, 15 mmol l^{-1} HEPES, 25 mmol l^{-1} glucose, 160 mmol l^{-1} sucrose; pH 7.2). As KN-62 is not soluble in water, 0.5% dimethyl sulfoxide (DMSO) was added. For KN-93 two different amounts were used (0.5 mmol l^{-1} and 1 mmol l^{-1}). As controls, KN-92 (Sigma-Aldrich), which is an inactive form of KN-93, or only ringer solution with 0.5% DMSO (Ringer) were injected. An additional control was not injected at all (control).

Survival rates

To test the possible influence of CaMKII inhibitors and siRNA injections on the general life expectancy of experimentally treated bees, the survival for each bee in the course of the conditioning process was recorded. Additionally, for each treatment group, 50 bees were injected and kept in a wooden box (length: 10.5 cm, width: 6.5 cm, height: 4.5 cm) and their survival rate was observed for the following 5 days. The bees were able to move freely in the boxes, and sugar solution (40% v/v sucrose) and water were always available.

Gustatory responsiveness score

To control for a similar gustatory responsiveness, we tested sucrose response thresholds using the proboscis-extension response (PER). Corresponding to the respective conditioning paradigm, gustatory responsiveness scores (GRSs) were tested during the time window when the learning experiment was performed. Thus, 1 h and 18 h after the injection of inhibitors, and 8 h after the injection of siRNA, each bee was tested for its individual GRS. Both antennae were touched with a droplet of water followed by a concentration series of 1%, 1.6%, 2.5%, 4%, 6.3%, 10%, 16%, 25% and 40% sucrose water solution (adapted from Scheiner et al., 2001) with a 10 min inter-stimulus interval to exclude sensitization effects (Menzel, 1990; Sandoz et al., 2002). A PER was scored if a bee fully extended its proboscis after the antenna was touched with one of the liquids. The sum of PERs delivered a GRS ranging between 0 (no response) and 10 (response to all solutions including water).

Validation of CaMKII knockdown

RT-PCR

To validate the knockdown of mRNA encoding CaMKII, 10–15 brains were dissected 2, 4, 6 and 24 h after siRNA injection. The ocelli, the optic and the antennal lobes were excluded. Total RNA was extracted using the peqGOLD Total RNA Kit (Peqlab). RNA (2 µg) was reverse transcribed to cDNA using a QuantiTect Reverse Transcription Kit (Qiagen). Primers specific to *CaMKII* (forward: CGTCATATGTTGCCAACTGGT, reverse: TTGAGCACGTTCAACAATGG) and to the housekeeping gene *rp49*, which is well established for adult honeybees (Laurenço et al., 2008) (forward: GACTGCATTCGAGCCAGAG, reverse: GGTGTACATGGGGATTCAGG (Sigma-Aldrich), were used.

Amplifications were carried out on a Mastercycler RealPlex² Real-Time PCR detection system (Eppendorf, Wesseling-Berzdorf, Germany) using KAPA SYBR FAST universal Master Mix (Peqlab) as per the manufacturer's instructions. The samples were measured in three technical replicates. The data were analyzed using the ΔΔCT method (Pfaffl, 2001). Additional controls included negative control samples (without enzyme) and melting curves.

Quantitative western blotting

For quantification of CaMKII protein levels, 15 honeybees were injected with either siCaMKII or siGFP and subjected to western blot analysis (1 brain/lane). The experiment was repeated three times. Eight hours after the injection the bees were anesthetized on ice, the brains quickly dissected, transferred to Laemmli buffer and frozen in liquid nitrogen. The brain samples consisted of the central brain including the MBs, but excluded the optic and the antennal lobes. The brains were homogenized, heated for 5 min at 95°C and subjected to polyacrylamide gel electrophoresis on 5% stacking gel and 12.5% separating gel (100 mA per gel, 1.5–2.5 h). After a short rinse in 0.1% TBST (10 mmol l^{-1} Tris-HCl, pH 7.9, 150 mmol l^{-1} NaCl and 0.1% Tween 20) the protein bands were blotted from the gel to a nitrocellulose membrane (230 mA, 75 min). The membrane was incubated for 1 h in 5% bovine serum albumin (BSA) in TBST and afterwards incubated with an antibody against pCaMKII (1:4000, sc-12886, Santa Cruz Biotechnology) and an antibody against actin (1:500, sc-1616, Santa Cruz Biotechnology). An antibody against the phosphorylated CaMKII (pCaMKII) was used as it showed constantly high immunoreactivity in immunostainings of brain tissue sections (Pasch et al., 2011) and corresponds to CaMKII localization in KCs in *in situ* hybridization studies (Kamikouchi et al., 2000). The membrane was rinsed in TBST (3×10 min) and incubated with the secondary antibodies IRDye 680@rabbit (1:20,000, LI-COR Biosciences) and IRDye 800@mouse (1:20,000, LI-COR Biosciences) in 5% BSA in TBST for 2 h at room temperature. After three rinses in TBST (10 min each), the membrane was analyzed with the Odyssey Infrared Imaging System (LI-COR, Bad Homburg, Germany). Prior to the experiment we ensured that both proteins were in the linear range of the fluorescence values measured for the protein concentrations used. For each lane, one value for the fluorescence intensity of the actin band and one intensity value for the four CaMKII bands was measured. As the four presumed isoforms (Pasch et al., 2011) were not easily distinguishable, one

value was measured per lane for all four pCaMKII bands together. For relative quantification, a pCaMKII/actin ratio was calculated for each sample and the obtained relative pCaMKII levels normalized to the mean pCaMKII/actin ratio for each western blot experiment.

Conditioning paradigm

Adult honeybees (foragers) were always caught the day before conditioning and harnessed in plastic holders. The bees remained in the holders for the duration of the experiments, and before and after handling they were kept in a climate chamber (25°C, 40% humidity). One hour after harnessing, the bees were fed until saturation with 40% sugar solution. To ensure that the bees were not satiated during the learning experiments, bees for the siRNA injections were fed with 30 µl sucrose solution 1 h before siRNA injection and olfactory conditioning was performed 8 h after the injection. Bees that were injected with KN-62 and KN-93 were fed with 15 µl sucrose solution 1 h before the injection and subjected to olfactory conditioning 1 h afterwards (Matsumoto et al., 2014; Mizunami et al., 2014). Additionally, bees that were injected with KN-93 and conditioned 18 h later were fed to saturation directly after injection and again with 30 µl sucrose 7 h before conditioning.

The bees were trained by olfactory conditioning of the PER (Takeda, 1961; Bitterman et al., 1983; Giurfa and Sandoz, 2012). Previous studies have shown that three (and also five) learning trials are sufficient to induce LTM lasting several days (Hourcade et al., 2010; Menzel, 1999). We used a conditioning protocol where the bees learn to associate an odor (conditioned stimulus, CS) with a sucrose reward (unconditioned stimulus, US). Before starting the conditioning process each bee was tested for an intact PER by touching the antennae with 50% sucrose solution. Only bees that showed an intact PER before the experiment and at the end of the retention tests were used for the experiments. 1-nonanol and 2-hexanol were used as the CS in an alternating manner. Bees were placed next to the conditioning setup to adapt to the surroundings 30 min before conditioning. For each bee, one trial lasted 30 s. After the bee was moved in front of an active air vent, the first 13 s were used to familiarize the bee with the conditioning setup. Afterwards, the CS was presented for 4 s followed by an overlap of the CS and US for 1 s and the presentation of the US alone for 2 s. During the first 3 s of CS presentation the occurrence of a PER was recorded. This procedure was repeated five times with an inter-trial interval of 10 min. To better compare the results with another pharmacological study (Matsumoto et al., 2014), we added three-trial olfactory conditioning after injection of KN-93 or KN-62.

After conditioning, the bees were returned to the climate chamber until memory retrieval was tested (1 h, 24 h or 72 h after the learning trials). The learned odor (CS) and the novel odor were presented for 3 s to assure a specific response to the learned CS and to rule out generalization effects. For every bee, only one single post-training test (1 h, 24 h or 72 h) was performed to exclude extinction of reconsolidation processes (Plath et al., 2012; Sandoz and Pham-Delègue, 2004; Stollhoff and Eisenhardt, 2009; Stollhoff et al., 2005). Bees that were tested for memory retention 24 and 72 h after conditioning were fed 3 times a day until saturation and then were food deprived for 5 h before their retention test.

Data analysis

All statistical tests were performed with SPSS (Chicago, USA). For western blot analyses, results were tested with a Mann–Whitney *U*-test. For RT-PCR, the consistency of the housekeeping protein was tested by applying Mann–Whitney *U*-tests on *rp49* CT values. To compare the different groups, statistical analyses were performed on ΔΔCT values using one-way ANOVA, including a Tukey *post hoc* test. GRS response was analyzed using the Kruskal–Wallis test. For analysis of the behavioral paradigm, a Cochran *Q* test was used to test the learning acquisition during olfactory conditioning within each group. To compare the acquisition between the different treated groups a Mann–Whitney *U*-test (CaMKII knockdown) or a Kruskal–Wallis test (CaMKII inhibition) was applied. To test whether there was a difference in memory retention between the different treatment groups after 1 h, 24 h and 72 h, a χ^2 test was used. To compare the specific responses to the CS and those to the novel odor, a McNemar's test was used. Differences in the survival rates were tested using the Kaplan–Meier test.

RESULTS

Effect of CaMKII knockdown on survival and sugar perception

To test the influence of siRNA and inhibitor injections via the ocellar tract on the general survival of the bees, the mortality rate during conditioning trials was recorded. In the behavioral conditioning experiments no difference between all injected bees and the controls were apparent (P=0.122; Table 1). On average, 6.38% of bees died during the experiment after 24 h and 13.78% after being harnessed in plastic tubes for 72 h.

Similarly, in an additional control experiment, bees that were kept in boxes for 5 days after injections with pharmacological inhibitors, siRNA and the control injected bees did not differ significantly in their mortality rate over the observed period (P=0.578; Table 1). Notably, most of the bees that did not survive died in the first few hours after the injection, indicating that death in these cases may have been due to acute brain damage. The majority of bees that survived the first day also survived for the rest of the 5 day period and did not show any apparent changes in behavior compared with untreated control bees.

Gustatory responses and the perception of sugar solution are vital for olfactory PER conditioning. Therefore, in an additional control experiment the GRS was tested after injection of pharmacological inhibitors, siRNA and corresponding controls (adapted from Scheiner et al., 2001). All bees performed normally in perceiving the sugar concentrations and the responses did not differ between any of the groups (P=0.915; Fig. 1). This indicates that neither siRNA-mediated knockdown nor pharmacological inhibition of CaMKII affects the sucrose response thresholds during the time window when learning experiments were performed.

CaMKII mRNA and protein levels after CaMKII knockdown

To verify a successful CaMKII knockdown, mRNA and protein levels after siRNA injection were examined.

Table 1. Mortality of bees after the injection of CaMKII siRNA and inhibitors

	1 h PER		24 h PER		72 h PER		96 h box	
Treatment	*N*	Survival (%)	*N*	Survival (%)	*N*	Survival (%)	*N*	Survival (%)
Ringer	88	100	65	97.40	82	89.02	50	88
KN-62 (1 h)	73	100	93	94.62	87	86.21	50	84
KN-93 (1 h)	66	100	76	89.47	87	82.95	50	84
KN-93 (18 h)	54	100	77	85.71	71	78.87	50	88
KN-92 (18 h)	48	100	73	91.78	64	82.81	50	90
Control	62	100	60	93.33	61	78.69	50	92
siCaMKII	64	100	78	89.74	83	86.75	50	86
siGFP	77	100	77	90.91	80	85.00	50	84

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The mRNA levels significantly decreased after siCaMKII injections, reaching a minimum at 4 and 6 h compared with the control (siGFP) bees (4 h, P=0.002; 6 h, P<0.001; Fig. 2). A trend for downregulation could be seen as early as 2 h after injection (P=0.359; Fig. 2), whereas 24 h post-injection, the mRNA levels returned to the basal levels (P=0.562; Fig. 2). The CT values for the housekeeping protein rp49 were stable and did not differ between the differently treated bees (2 h, P=0.121; 4 h, P=0.142; 6 h, P=0.287; 24 h, P=0.260).

For protein quantification, western blot analysis was performed using an antibody against pCaMKII. This antibody labeled the same bands as an antibody against CaMKII (data not shown), but as the antibody against pCaMKII was more sensitive and showed a stronger signal it was used for the quantification. Control experiments included linearity curves to ensure the signals for both proteins were in a linear range. Quantitative analyses of the protein levels were performed 8 h after siCaMKII injection and control siGFP injection. The protein amount, on average, was reduced by about 50% (P<0.001; Fig. 3). Therefore, this time point (8 h) was used for further analyses and behavioral tests. As the four previously described pCaMKII bands (Pasch et al., 2011) were not always clearly distinguishable, the fluorescence signals of all bands were analyzed together. Additional evaluation of the fluorescence level separated for the distinct uppermost band and the three other bands together showed a similar downregulation effect (data not shown), indicating that siCaMKII affects all CaMKII isoforms.

Effect of CaMKII knockdown on learning and memory formation

To test whether CaMKII has an effect on memory formation, olfactory conditioning was performed after siRNA or inhibitor injection. As the learning curves of the different treatment groups (siCaMKII, siGFP, KN-92, KN-62, KN-93, control, Ringer) did not differ between the 1 h, 24 h and 72 h retention test time points, the learning acquisition data for each treatment group were pooled and only one acquisition curve is shown for each treatment (one-way ANOVA: siCaMKII, P=0.601; siGFP, P=0.788; control, P=0.738; KN-92, P=0.458; KN-93, P=0.122; Tukey HSD *post hoc* test also showed no significant differences between the different time points in any of the treatment groups; Figs 4 and 5).

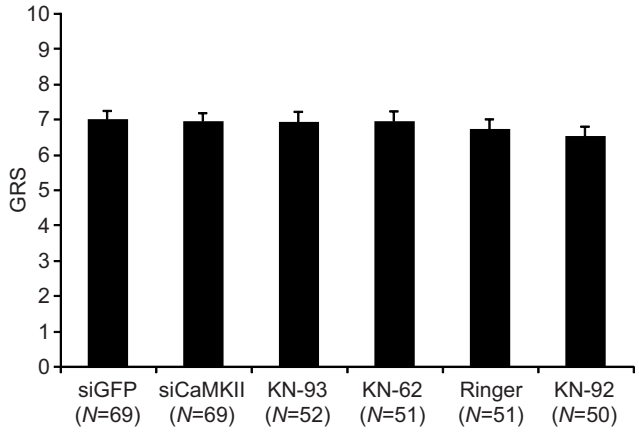

Fig. 1. Sucrose responsiveness after injection of siRNA and CaMKII inhibitors. Eight hours after the injection of siRNA against CaMKII (siCaMKII) and GFP (siGFP) and 1 h after the injection of the CaMKII inhibitors KN-93, KN-62 or Ringer solution only and KN-92 as controls, bees were tested for their gustatory response score (GRS). Error bars represent s.e.m. There was no significant difference between the different groups.

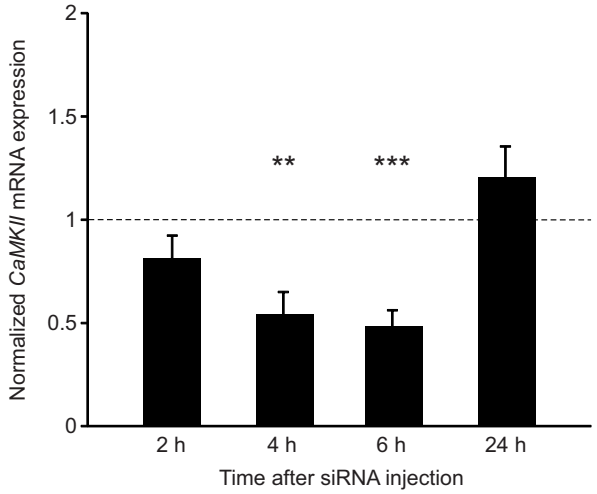

Fig. 2. Real-time quantitative PCR of *CaMKII* mRNA. Relative expression ratio of *CaMKII* mRNA in the brain after injection of siRNA against *CaMKII*. Expression was normalized to *rp49* mRNA and standardized to the control group (siGFP) at the different time points. Asterisks indicate a significant reduction of CaMKII mRNA level in siCaMKII-injected bees compared with siGFP-injected bees. Error bars represent the s.e.m. **P<0.01; ***P<0.005.

CaMKII RNAi

After injection of siRNA, learning acquisition did not differ between bees injected with siGFP or siCaMKII (P=0.104; Fig. 4A). Both groups showed typical learning curves (for comparison, see Menzel, 1999): ~75% of bees learned to associate the odor with the sucrose reward after five learning trials and showed an increase in the conditioned response (P<0.001). However, the groups did differ in the retention tests. Whereas the mid-term memory (MTM) after 1 h in siCaMKII-injected bees was not significantly different from the control bees (P=0.416; Fig. 4B), both early and late long-term memory (eLTM and lLTM) were significantly impaired in siCaMKII-injected bees (24 h, P<0.001; 72 h, P<0.001; Fig. 4B). To ensure that a specific odor memory was attained, a novel odor was presented in addition to the conditioned odor (CS). The results show that the specific response was always significantly higher than the unspecific response (P=0.015).

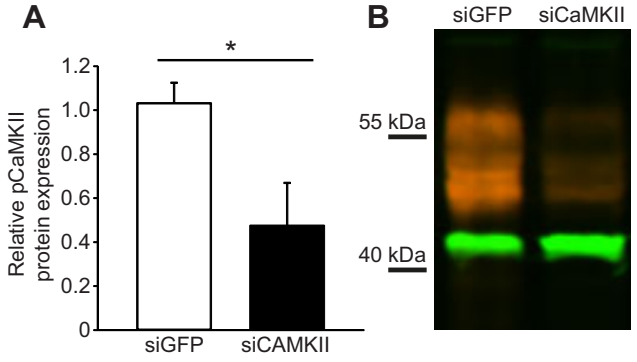

Fig. 3. Quantitative western blot analysis of pCaMKII protein levels after siRNA injection. Eight hours after the injection of siRNA against CaMKII (siCaMKII) and GFP (siGFP), protein levels in the central brain were measured using an antibody against the phosphorylated form of the CaMKII (pCaMKII). (A) pCaMKII protein levels were standardized on actin protein level and normalized to siGFP-injected control animals and show a decrease of protein level 8 h after injection. Error bars represent s.e.m. (B) Western blot with pCaMKII visualized in red and actin in green using the Odyssey imaging system. *P<0.05.

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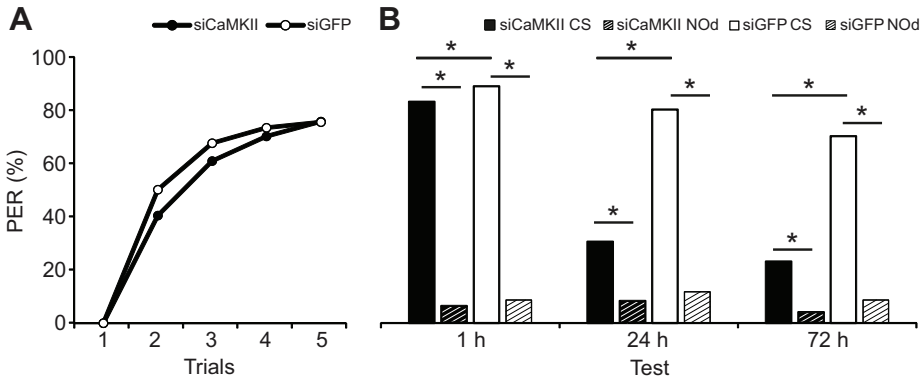

Fig. 4. Learning acquisition and memory retention after siRNA injection. Eight hours after injection of siRNA against CaMKII (siCaMKII) and against GFP as a control (siGFP) bees were conditioned in five trials to associate an odor (conditioned stimulus, CS) with a sucrose reward. After different time points (1 h, 24 h and 72 h), the responses to the CS and to a novel odor (NOd) were tested. The data for the acquisition was pooled for all subgroups, but each bee was tested only at one retention time (siCaMKII: 1 h, *N*=63; 24 h, *N*=66; 72 h, *N*=67; siGFP: 1 h, *N*=58; 24 h, *N*=68; 72 h, *N*=64).

Pharmacological inhibition of CaMKII

Similar to siRNA, the CaMKII inhibitor KN-93 was injected via the ocellar tract to inhibit CaMKII. As a control, the inactive analog KN-92 was injected and, additionally an untreated control (control) was used. Bees were subjected to five-trial learning 18 h after injection. All three groups showed a normal learning acquisition with increasing percentages of the conditioned responses during the conditioning trials ($P<0.001$) with no differences between the groups ($P=0.975$; Fig. 5A). Retention tests 1 h after training did not reveal any difference between the inhibition of CaMKII and the controls (KN-92 versus KN-93, $P=0.494$; KN-93 versus control, $P=0.966$; KN-92 versus control, $P=0.517$; Fig. 5B). But similar to the CaMKII knockdown using RNAi, in both cases both the early and late phases of LTM were disrupted after injection of the inhibitors (24 h: KN-92 versus KN-93, $P<0.001$; KN-93 versus control, $P<0.001$; KN-92 versus control, $P=0.765$; 72 h: KN-92 versus KN-93, $P=0.016$; KN-93 versus control, $P=0.001$; KN-92 versus control, $P=0.459$; Fig. 5B).

In a second experimental series, the two CaMKII inhibitors (KN-62 and KN-93) and Ringer solution (with 0.5% DMSO) as a control were injected in the same way as before, but followed by a three-trial olfactory conditioning paradigm 1 h post-injection. Again, the learning acquisition did not differ in the three groups ($P=0.572$) and the bees showed an increased percentage of the conditioned response during the three learning trials ($P<0.001$). In all groups, ~75% of the bees were able to associate the odor with a sucrose reward after three learning trials. Similar to the siRNA injections and the previous inhibitor experiment (KN-93), mid-term memory (1 h) was not affected (KN-62 versus Ringer, $P=0.622$; KN-92 versus Ringer, $P=0.614$; Fig. 5D). However, both eLTM and lLTM were significantly impaired after injection of both inhibitors compared with the control (24 h: KN-62, $P<0.001$; KN-92, $P<0.001$; 72 h: KN-62, $P<0.001$; KN-92, $P<0.001$; Fig. 5D). In comparison to the conditioning 18 h after injection (Fig. 5D), the inhibitors showed a ~20% stronger effect on the memory impairment for the bees if conditioning was performed 1 h after injection (Fig. 5B).

DISCUSSION

Using a combination of RNAi-mediated knockdown and pharmacological inhibition, the results of this study strongly

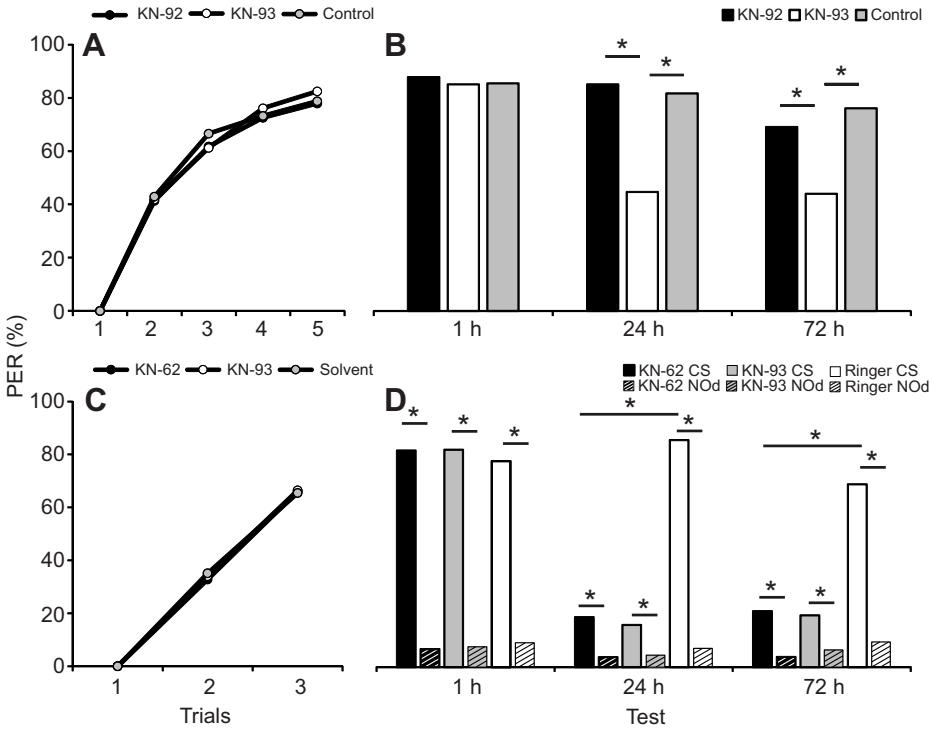

Fig. 5. Learning acquisition and memory retention after injection of CaMKII inhibitors. (A) PER in bees injected with KN-93, its inactive form KN-92 and a control that was not injected at all after five-trial olfactory conditioning 18 h after injection. (B) PER in bees tested 1 h, 24 h and 72 h later (KN-93: 1 h, *N*=54; 24 h, *N*=66; 72 h, *N*=56; KN-92: 1 h, *N* =48; 24 h, *N*=67; 72 h, *N*=53; control: 1 h, *N*=62; 24 h, *N*=60; 72 h, *N*=61). (C) Three-trial olfactory conditioning to associate an odor (conditioned stimulus=CS) with a sucrose reward. PER 1 h after CaMKII inhibitors KN-62 and KN-93 were injected. As a control, Ringer solution including 0.5% DMSO was injected and bees conditioned 1 h afterwards. (D) PER in bees tested 1 h, 24 h and 72 h later with the CS and a novel odor (NOd) (KN-62: 1 h, *N*=70; 24 h, *N*=87; 72 h, *N*=73; KN-93: 1 h, *N*=62; 24 h, *N*=67; 72 h, *N*=69; Ringer: 1 h, *N*=85; 24 h, *N*=60; 72 h, *N*=70). **P*<0.05.

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suggest that CaMKII plays an essential role in the formation of LTM in the honeybee. RNAi has been used more abundantly in recent years to study the function of proteins regarding their role in honeybee behavior (El Hassani et al., 2012; Leboulle et al., 2013; Louis et al., 2012; Müssig et al., 2010). Gene knockdown using RNAi is a particularly powerful experimental approach in a social insect model organism like the honeybee, where transgenic manipulations are not readily available. Pharmacological manipulations are widely used, but often have the disadvantage of unwanted effects on other proteins next to the original target. The CaMKII inhibitors KN-62 and KN-93, for example, were shown to act on L-type Ca^{2+} and voltage-gated K^{+} and Ca^{2+} channels, as well as inhibiting the CaMKII (Gao et al., 2006; Ledoux et al., 1999; Li et al., 1992). For these reasons, and for reasons of comparability with other studies (see below), we chose a dual approach – using pharmacological inhibition and specific RNAi in parallel.

We found that olfactory conditioning led to a normal acquisition phase and an intact MTM (1 h retention) for all drug and RNAi manipulations of CaMKII. The memory phases in honeybees can be subdivided into short-term memory (STM), lasting only seconds, MTM, lasting for several hours, eLTM, lasting 1–3 days and lLTM, which can be retrieved 3 or more days after conditioning (reviewed in Menzel, 2012). In our experiments, both eLTM (24 h retention) and lLTM (72 h retention) were significantly impaired after drug (KN-62 and KN-93) and siCaMKII injection compared with the corresponding controls (Ringer, siGFP, control). This is in agreement with a related approach in crickets showing an intact MTM (1 h retention) and an impaired eLTM (24 h retention) after a three-trial olfactory learning assay and inhibition by KN-93 and KN-62 (Mizunami et al., 2014). Similarly, indirect and direct manipulations that decrease the amount of autophosphorylated CaMKII in the MB in *Drosophila* disrupted MTM as well as LTM (Malik et al., 2013).

Although the CaMKII turnover rate in mice is 3.02 days (Cohen et al., 2013), we observed protein downregulation and a behavioral effect as early as 8 h after CaMKII knockdown in the honeybee. lLTM requires *de novo* transcription and translation in the honeybee (Hourcade et al., 2009; Menzel, 1999; Schwärzel and Müller, 2006) and disrupting CaMKIIα translation in mice causes impaired LTM in three different behavioral assays, suggesting the necessity for replenishing active CaMKII by translation to maintain late LTP and lLTM (Miller et al., 2002). Interestingly, LTP and LTM in mice are dependent on local protein translation from pre-existing mRNA pools in the post-synapse (reviewed in Steward and Schuman, 2001) and it was shown that dendritic-specific translation of CaMKII is necessary for late LTP (Giovannini et al., 2001; Ouyang et al., 1999). Therefore, we suggest that knockdown of CaMKII might also preferentially deplete the local dendritic pools of *CaMKII* mRNA in the honeybee, leading to the impaired memory-retention phenotype.

A recent study on the function of CaMKII in honeybees used KN-62 as an inhibitor and showed an impaired 72 h retention, but in contrast to our present study, did not detect an effect after 24 h (Matsumoto et al., 2014). Therefore, we extended our experiments using two CaMKII inhibitors KN-62 and KN-93 within the same learning assay which has been used by Matsumoto et al. (2014) (three-trial learning with same odor, novel odor). The results clearly confirmed our results of the RNAi approach, showing significant memory retention impairment after 24 (eLTM) and 72 h (lLTM). One major difference between the study by Matsumoto et al. (2014) and our present study was the drug injection site (thorax versus medial ocellar tract in our study). Matsumoto et al. (2014) injected the inhibitor in the thorax and olfactory conditioning started only 20 min later. The rather remote injection site may not have given the drug enough time to interfere with early LTM mechanisms.

In addition, different injection sites were shown to influence different memory phases in chicken (Zhao et al., 1999). In this case, it was postulated that distinct brain regions are responsible for STM, MTM and LTM because injection of CaMKII inhibitor in specific brain areas led to the impairment of separate memory phases and pCaMKII was upregulated in different brain centers during STM or LTM (Zhao et al., 1999).

In *Drosophila*, memory traces were also attributed to specific brain locations, specifically to distinguish Kenyon cell (KC) types (Akalal et al., 2010; Malik et al., 2013; Yamagata et al., 2015). Moreover, changing the levels of autophosphorylated CaMKII in *Drosophila* in those KCs blocked MTM and LTM (Malik et al., 2013).

The application of two pharmacological inhibitors and specific RNAi led to disrupted eLTM and lLTM in our study. Combined with the observations in crickets and in *Drosophila*, we assume that the divergence with the findings by Matsumoto et al. (2014) is probably due to the injection method and resulting differences in diffusion time, degradation and internal concentrations.

As we showed that in the honeybee different memory phases (eLTM and lLTM) were affected by CaMKII manipulations, we conclude that they could be transmitted by specific neuronal and molecular pathways and potentially also by specific KC populations based on the observations in *Drosophila*.

In vertebrates, eLTM was shown to be induced by CaMKII affecting f-actin remodeling (Okamoto et al., 2007) and AMPA phosphorylation (Barria et al., 1997a,b; Derkach et al., 1999; Lisman et al., 2002), which are both eLTM-related processes. Disrupting the dendritic translation of CaMKIIβ in mice severely impairs eLTM in contextual fear conditioning (24 h retention) and synaptic plasticity in the hippocampus, indicating a structural role of CaMKIIβ during LTM (Borgesius et al., 2011). In honeybees, CaMKII protein was shown to be colocalized with f-actin in the postsynaptic dendritic spines of subpopulations of KCs in the MB calyx (Pasch et al., 2011) giving further support to potential molecular interactions of CaMKII with the actin cytoskeleton, which, in turn, could affect spine motility and shape (Okamoto et al., 2009). CaMKII was also shown to play a role in short-term synaptic plasticity in the presynapse. CaMKII-knockout mice show a disrupted short-term plasticity that is independent of the kinase activity, but is due to a structural role influencing presynaptic transmitter release (Chapman et al., 1995; Hinds et al., 2003). In this context, CaMKII may also function in presynaptic plasticity in honeybees. Immunolabeling of pCaMKII showed a strong immunoreactivity in the MB vertical and horizontal lobes (Pasch et al., 2011), which represent the presynaptic regions of KCs (Strausfeld, 2002). However, not much is known about the role of CaMKII in molecular pathways leading to processes which maintain LTP, synaptic strength and thereby potential lLTM. In vertebrates, LTM is thought to be mediated by strengthening the post-synapse through interaction of CaMKII with AMPA and NMDA receptors (NMDARs). CaMKII can phosphorylate existing AMPA channels, bind to AMPA to increase their conductance and modulate vesicle transport to include new AMPA channels into the membrane, which are all processes that increase the conductance of the synapse (reviewed in Lisman et al., 2002). In particular, the formation of the CaMKII–NMDAR complex plays a key role in LTP induction and learning (Lisman et al., 2012). Further LTP maintenance is thought to be transmitted by the CaMKII–NMDAR

complex (Sanhueza et al., 2011), as these complexes are persistent once they are formed (Bayer et al., 2006). In honeybees, three genes encode NMDAR subunits, and the mRNA and protein of the neurotransmitter glutamate are found throughout the brain (Bicker et al., 1988; Zannat et al., 2006; Zachepilo et al., 2008). Pharmacological inhibition of NMDAR transmission in the honeybee does not impair 1 h memory, but it disrupts 24 h memory (Si et al., 2004). Knockdown of NR1 (honeybee subunit homolog of NMDAR) also causes impairment of eLTM (Müssig et al., 2010). *NR1* mRNA shows a similar distribution to *CaMKII* mRNA in the KC somata (Kamikouchi et al., 2000; Zannat et al., 2006), indicating a possible interaction of these two proteins in the honeybee too. The fact that only eLTM and not lLTM was impaired by the knockdown of NR1, is probably due to the specific knockdown of only one subunit and future investigations are needed to clarify the role of NMDAR–CaMKII complexes in honeybee memory formation.

The data presented in this study suggest that CaMKII is not necessary for learning acquisition itself and MTM, but represents an important mediator of both early and late phases of LTM formation in the honeybee. The fact that two different memory stages (eLTM and lLTM) were affected suggests that this kinase plays a role in the formation of distinct memory stages associated with different locations and interacts at different levels of the olfactory pathway (e.g. antennal lobe, MB lobes, MB calyx). As we knocked down all isoforms of CaMKII, it would be interesting to use a more-specific approach to look at the differential functions and distributions of the different isoforms, which might contribute to different memory stages.

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Competing interests
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Author contributions
Study design: C.S., W.R., T.S.M. Data collection: C.S., N.K. Data analysis and evaluation: C.S. Discussion of results and manuscript writing: C.S., W.R.

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