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RESEARCH ARTICLE

Two inhibitors of the ubiquitin proteasome system enhance long-term memory formation upon olfactory conditioning in the honeybee (*Apis mellifera*)

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ABSTRACT

In honeybees (*Apis mellifera*), the proteasome inhibitor Z-Leu-Leu-Leu-CHO (MG132) enhances long-term memory (LTM) formation. Studies in vertebrates using different inhibitors of the proteasome demonstrate the opposite, namely an inhibition of memory formation. The reason for this contradiction remains unclear. MG132 is an inhibitor of the proteasome, but also blocks other proteases. Accordingly, one possible explanation might be that other proteases affected by MG132 are responsible for the enhancement of LTM formation. We test this hypothesis by comparing the effect of MG132 and the more specific proteasome inhibitor clasto-lactacystin beta-lactone (β-lactone). We show that these two inhibitors block the activity of the proteasome in honeybee brains to a similar extent, do not affect the animals' survival but do enhance LTM retention upon olfactory conditioning. Thus, the enhancement of LTM formation is not due to MG132-specific side effects, but to inhibition of a protease targeted by MG132 and β-lactone, i.e. the proteasome.

KEY WORDS: Insect, Memory, Protein degradation, Ubiquitin proteasome system

INTRODUCTION

Long-term memory (LTM) formation requires protein synthesis during a defined time window after learning in order to stabilize the new memory. In addition to protein synthesis, protein degradation and, in particular, degradation via the ubiquitin proteasome system seems to be involved in LTM formation (Jarome and Helmstetter, 2013).

We examine the mechanisms of LTM formation in classical conditioning of the honeybee (*Apis mellifera* Linnaeus 1758), an established invertebrate model organism for learning and memory research. In olfactory proboscis extension response (PER) conditioning, an initial neutral odor (conditioned stimulus; CS) is paired with a sucrose reward (unconditioned stimulus; US). When the CS precedes the presentation of a US, the CS becomes associated with the occurrence of the US. Once this association is formed, the subsequent presentation of the CS alone elicits the extension of the proboscis (the conditioned response).

In a previous study, we demonstrated that injection of the proteasome inhibitor Z-Leu-Leu-Leu-CHO (MG132) after olfactory PER conditioning and after extinction enhances LTM retention (Felsenberg et al., 2012). In contrast, studies in vertebrates using another proteasome inhibitor, i.e. clasto-lactacystin beta-lactone (β-lactone), demonstrate the opposite, namely an inhibition of memory formation (reviewed in Jarome and Helmstetter, 2013). The reason for this contradiction remains unclear. MG132 is an inhibitor of the proteasome, but also blocks other proteases (Granot et al., 2007; Ito et al., 2009; Tsubuki et al., 1996). Accordingly, one possible explanation might be that one of the other proteases affected by MG132 is responsible for the effect on LTM formation. We here test this hypothesis and compare the effect of MG132 and β-lactone on proteasome activity in honeybee brain tissue extract and on survival and memory formation upon olfactory conditioning. We demonstrate similar activity of both inhibitors in honeybee brain tissue and no effect on the animals' survival, and report similar effects of both inhibitors on LTM retention. Our results suggest that the enhancement of LTM retention by MG132 in honeybees (Felsenberg et al., 2012) is not due to the MG132-specific inhibition of non-proteasomal proteases, but can be attributed to the inhibition of a protease that is targeted by MG132 and β-lactone, i.e. the proteasome. Thus, differences between the effect of a proteasome inhibitor on LTM formation in honeybees and vertebrate model systems cannot be attributed to different proteasome inhibitors.

RESULTS

MG132 and β-lactone block chymotrypsin-like proteasome activity in honeybee brain tissue in a dose-dependent manner

We first examined the effect of MG132 and β-lactone on the proteasome in honeybee neuronal tissue following incubation with different inhibitor concentrations. We performed an *in vitro* proteasome activity assay to measure the chymotrypsin-like proteasome activity using Suc-Leu-Leu-Val-Tyr-AMC, which has been reported to be the most specific substrate to measure proteasome activity (Kisselev and Goldberg, 2005; Rodgers and Dean, 2003).

Our experiments reveal that both MG132 and β-lactone block the chymotrypsin-like proteasome activity in a dose-dependent manner. MG132 blocks proteasome activity at all concentrations tested, except for the lowest (2.5 nmol l^{-1}; *t*-test for single means, $P<0.025$; Fig. 1A). All concentrations of β-lactone block chymotrypsin-like proteasome activity significantly (*t*-test for single means, $P<0.025$; Fig. 1B). A comparison of MG132 and β-lactone showed that similar concentrations of inhibitors block chymotrypsin-like proteasome activity to a similar degree (ANOVA: $F_{9,118}=913$, $P<0.025$, β-lactone *post hoc*: 0.0025 versus 0.025 µmol l^{-1}, $P>0.05$; 0.025 versus 0.25 µmol l^{-1}, $P>0.05$; 0.25 versus 2.5 µmol l^{-1}, $P<0.05$; 2.5 versus 12.5 µmol l^{-1}, $P>0.05$; MG132 *post hoc*: 0.0025

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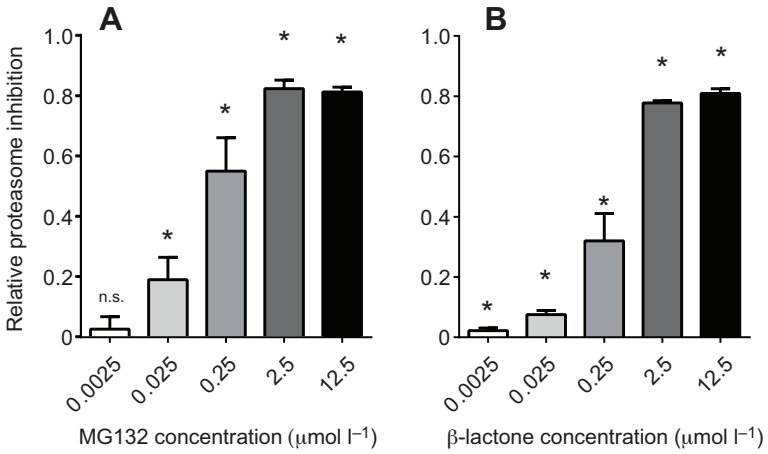

Fig. 1. MG132 and β-lactone block proteasome activity in honeybee brain tissue. (A,B) Proteasome activity of honeybee brain lysate was measured in the presence of the proteasome inhibitor clasto lactacystin beta-lactone (β-lactone) or MG132. Each sample represents the lysate of 10 pooled bee brains. (A) Relative percentage of proteasome inhibition in the presence of β-lactone at different concentrations (0.0025 μmol l^{-1}, n=18; 0.025 μmol l^{-1}, n=17; 0.25 μmol l^{-1}, n=5; 2.5 μmol l^{-1}, n=16; 12.5 μmol l^{-1}, n=8). (B) Percentage of proteasome inhibition in the presence of MG132 at different concentrations (0.0025 μmol l^{-1}, n=15; 0.025 μmol l^{-1}, n=17; 0.25 μmol l^{-1}, n=5; 2.5 μmol l^{-1}, n=15; 12.5 μmol l^{-1}, n=12). Asterisks indicate significant differences (P<0.025). Data are means ± s.e.m.

versus 0.025 μmol l^{-1}, P>0.05; 0.025 versus 0.25 μmol l^{-1}, P<0.05; 0.25 versus 2.5 μmol l^{-1}, P>0.05; 2.5 versus 12.5 μmol l^{-1}, P>0.05; β-lactone versus MG132 all concentrations, P>0.05). The inhibition reaches its asymptote at a concentration of 10 μmol l^{-1} for both inhibitors. The half maximal inhibitory concentration (IC_{50}) for MG132 is 105 nmol l^{-1} and for β-lactone is 369 nmol l^{-1}.

MG132 and β-lactone do not affect honeybee survival

Because high concentrations of MG132 and β-lactone are thought to be cytotoxic (Fenteany et al., 1994), we conducted experiments to evaluate whether the systemic injection of β-lactone or MG132 affects the survival rate of the harnessed honeybees. In all experiments we considered bees to be dead when they were motionless and did not respond to tactile stimulation at the antennae.

In the first experiment, honeybees were systemically injected with 1 μl of PBS or remained untreated. Survival was recorded at 30 min and 1, 2, 3, 6, 24, 48 and 72 h after injection (Fig. 2A). Survival of both groups did not decay significantly over the time course of the experiment (Cochran's Q-test, untreated: Q=7, d.f.=7, P>0.05, PBS: Q=7, d.f.=7, P>0.05). In order to compare survival of both groups, we calculated a survival score counting the number of time points each animal survived after treatment (Fig. 2B). No significant differences exist between the survival scores of both groups (Mann–Whitney U-test, U=197, P>0.05). To produce stock solutions, MG132 and β-lactone have to be solved in DMSO. Thus, in the second experiment we tested the effect of different DMSO concentrations (0.1–50%) diluted in PBS (137 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, 10.1 mmol l^{-1} Na_2HPO_4 and 1.8 mmol l^{-1} KH_2PO_4 at pH 7.2) on survival. The percentage of surviving bees in all groups decayed over the time course of the experiment (Cochran's Q-test: 0% DMSO: Q=89.07, d.f.=7, P<0.05; 0.1% DMSO: Q=98.41, d.f.=7, P<0.05; 1% DMSO: Q=68.81, d.f.=7, P<0.05;

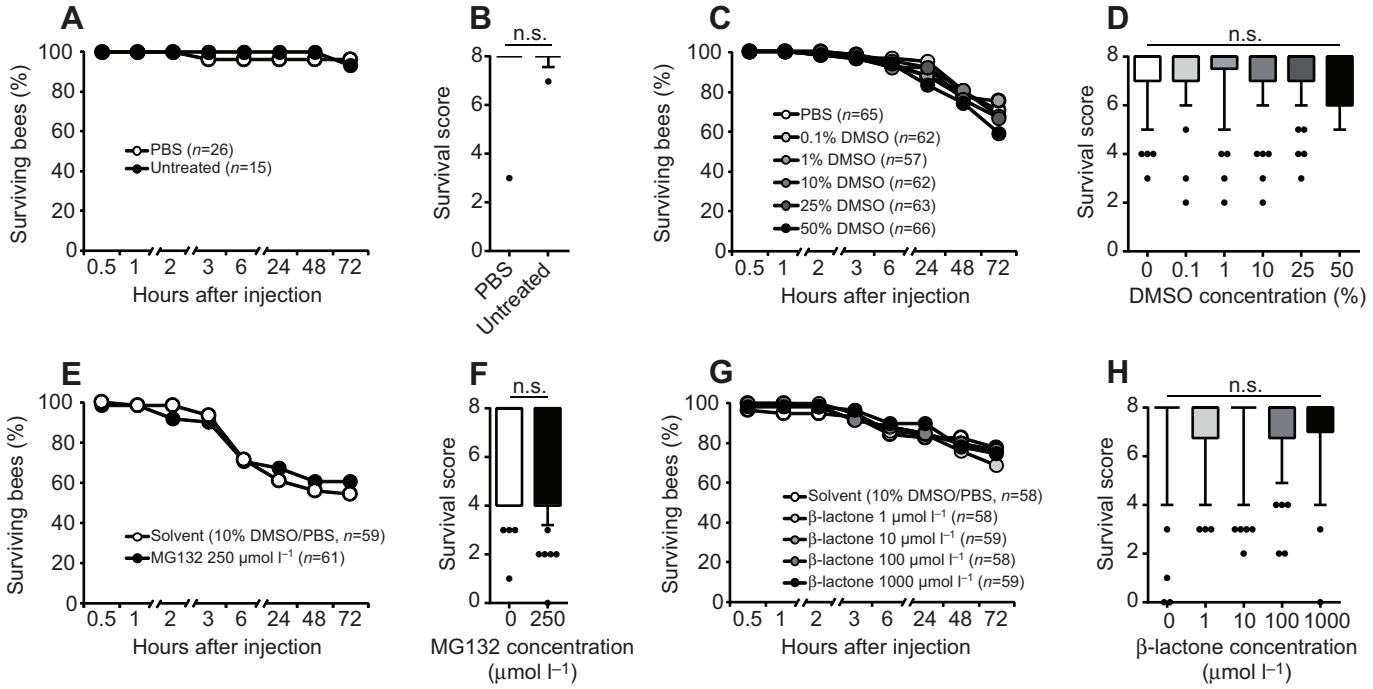

Fig. 2. MG132 and β-lactone do not alter honeybee survival. Honeybees were left untreated, received a sham lesion (A,B) or were injected with different reagents, i.e. PBS (C,D) or varying concentrations of DMSO (C,D), MG132 (E,F) or β-lactone (G,H). Panels A, C, E and G show the percentage of surviving honeybees per group at different time points after the injection. Panels B, D, F, H and J show the surviving scores of the honeybees over all time points (boxes indicate 25–75% percentiles, whiskers 10–90% percentiles, and filled circles outliers).

The Journal of Experimental Biology (2014) doi:10.1242/jeb.108142

10% DMSO: Q=85.62, d.f.=7, P<0.05; 25% DMSO: Q=104.94, d.f.=7, P<0.05; 50% DMSO: Q=124.35, d.f.=7, P<0.05; Fig. 2C). No differences were observed between the survival scores of the differently treated groups (Kruskal–Wallis test, H=3.242, d.f.=5, P>0.05; Fig. 2D). Taken together, these experiments show that animals' survival is not significantly affected by solvent injection.

Next, we injected 1 µl of 250 µmol l^{-1} MG132, the highest concentration of MG132 that was practical to produce, and observed the survival rate as in the previous experiment. In both groups, the percentage of bees surviving decays over the time points tested (Cochran's Q-test: 0 µmol l^{-1} MG132; Q=144.76, d.f.=7, P<0.05; 250 µmol l^{-1} MG132: Q=118.06, d.f.=7, P<0.05; Fig. 2E). No significant difference exists between the survival scores of MG132-injected bees and solvent-injected bees (Mann–Whitney U-test, U=832.5, P>0.05; Fig. 2F).

In the final survival experiment, honeybees were systemically injected with 1 µl of different concentrations of β-lactone (1 µmol l^{-1}, 10 µmol l^{-1}, 100 µmol l^{-1}, 1 mmol l^{-1}) or with the solvent (10% DMSO/PBS). Survival of individual bees was recorded at 30 min and 1, 2, 3, 6, 24, 48 and 72 h after injection (Fig. 2G). Again the percentage of surviving bees in all groups decayed over the time course of the experiment (Cochran's Q-test, d.f.=7, P<0.05 for all: 0 µmol l^{-1} β-lactone: Q=50.12; 1 µmol l^{-1} β-lactone: Q=82.53; 10 µmol l^{-1} β-lactone: Q=63.30; 100 µmol l^{-1} β-lactone: Q=79.47; 1000 µmol l^{-1} β-lactone: Q=69.56). The survival scores of all groups examined were not significantly different from PBS nor from each other (Kruskal–Wallis test, H=0.96, d.f.=4, P>0.05; Fig. 2F). Taken together, survival of animals differed between the time points tested in three of the four experiments, but no differences between the survival scores were revealed in any of these four experiments. We conclude that the survival of honeybees is neither affected by the systemic injection of the different solvents nor by the proteasome inhibitors.

MG132 and β-lactone enhance LTM retention

Next, we conducted two experiments investigating whether the proteasome inhibitors MG132 and β-lactone (Fig. 3A) have similar effects on LTM formation upon olfactory conditioning. Both inhibitors block the chymotrypsin-like activity of the proteasome in the honeybee brain lysate to a similar degree (Fig. 1); therefore, we used 1 µmol l^{-1} of β-lactone or 1 µmol l^{-1} MG132 dissolved in 0.1% DMSO/PBS, based on our previous study (Felsenberg et al., 2012). In the first experiment, we injected 1 µl MG132 (1 µmol l^{-1}) or the solvent (0.1% DMSO/PBS) as a control 1 h after olfactory conditioning with three CS–US trials (inter-trial interval of 2 min). We tested memory retention cumulatively after 24, 48 and 72 h. In addition, we counted the numbers of responses over the three trials as memory score (range=0–3). The percentage of responding bees decreased over the three cumulative tests in both groups (Cochran's Q-test, control: Q=21.74, d.f.=2, P<0.05; MG132: Q=24.5, d.f.=2, P<0.05; Fig. 3B). A repeated-measures ANOVA over the three retention tests revealed that significantly more MG132-injected bees responded compared with the PBS group (treatment effect: $F_{1,116}$=6.80, P<0.05, treatment × time: $F_{2,232}$=0.02; P>0.05, time: $F_{2,232}$=28.14, P<0.05). In line with this finding, the analysis of the memory scores shows that MG132-injected bees have higher scores than the control group (Mann–Whitney U-test, U=1002, P<0.05; Fig. 3C). The second experiment was conducted in the same manner as the previous one, but we injected 1 µl β-lactone (1 µmol l^{-1}) or the solvent (0.1% DMSO/PBS) as a control 1 h after olfactory conditioning. The percentage of bees responding with a conditioned response decayed over the consecutive tests in β-lactone-injected

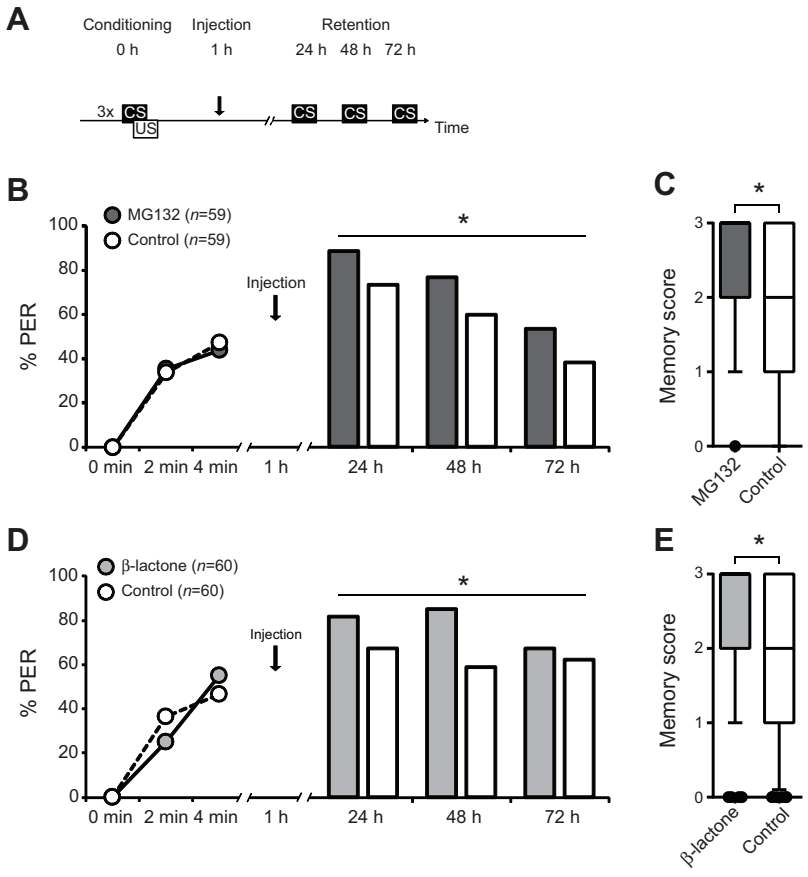

Fig. 3. Proteasome inhibitor injection enhances long-term memory formation. (A) Honeybees were conditioned with three CS-US trials presented with an inter-trial interval of 2 min (CS, conditioned stimulus; US, unconditioned stimulus). One hour after conditioning, honeybees were injected (arrow) with 1 µmol l^{-1} of either β-lactone (dark grey) or MG132 (light grey) or the solvent (control; white). Memory was tested cumulatively at 24, 48 and 72 h after conditioning. (B) Performance of olfactory conditioned honeybees injected with MG132 and the respective control during acquisition (circles) and memory retention (bars) and (C) their memory scores (boxes indicate 25–75% percentiles, whiskers 10–90% percentiles, and filled circles outliers). (D) Performance of olfactory conditioned honeybees injected with β-lactone and the respective control during acquisition (circles) and the memory retention (bars) and (E) their memory scores. Asterisks indicate significant differences (P<0.05).

bees (Cochran's Q-test: β-lactone: Q=10.84, d.f.=2, P<0.05), but not in the solvent-injected control animals (control: Q=1.23, d.f.=2, P>0.05; Fig. 3D). The repeated-measures ANOVA revealed that the β-lactone-injected bees showed a significantly increased response compared with the PBS control group over the three retention tests (treatment effect: $F_{1,118}$=6.84, P<0.05; treatment × time: $F_{2,236}$=2.60; P>0.05; time: $F_{2,236}$=2.39, P>0.05). This result is confirmed by the analysis of the memory score, which shows higher scores in the β-lactone-injected group than in the control group (Mann–Whitney U-test, U=1326, P<0.05; Fig. 3E).

Taken together, these experiments demonstrate that the injection of both inhibitors increases performance during the retention tests upon olfactory conditioning.

Dilution of substances systemically injected into honeybees

MG132 and β-lactone have been reported to block proteases other than the proteasome in vertebrates, but overlapping side effects have only been reported for the ATP-dependent Lon protease (Granot et al., 2007; Ostrowska et al., 2000; Tsubuki et al., 1996). In order to estimate whether the concentration of MG132 or β-lactone systemically injected into honeybees would correspond to a concentration that has been reported to affect Lon proteases in vertebrates, we next estimated the dilution factor when injecting drugs systemically into the honeybee hemolymph. We weighed honeybees before and after drying them at high temperature until their mass did not change anymore. We calculated differences between the mass of animals before and after drying, thereby estimating the animal's body water. We calculated the mean body water volume of a forager bee to be ~70 µl (71.08±0.91 µl, n=35). Thus, the dilution factor of a substance systemically injected into a honeybee is maximally 1:70. Given this dilution factor, the effective amount of inhibitor we injected in the behavioral experiments (1 µmol l^{-1}:70, which is equal to 14 nmol l^{-1}) was lower than the doses of MG132 and β-lactone reported to effectively inhibit the ATP-dependent Lon protease, which has an IC_{50} of 20 µmol l^{-1} for MG132 and an IC_{50} of 3 µmol l^{-1} for β-lactone as well as other non-proteasomal proteases reported to be affected by either MG132 or β-lactone (Granot et al., 2007; Ito et al., 2009; Ostrowska et al., 2000; Tsubuki et al., 1996).

Taken together, we conclude that the enhancement of LTM formation is not due to MG132-specific side effects on non-proteasomal proteases, but to the inhibition of a protease targeted by MG132 and β-lactone. We suggest that this protease is the proteasome.

DISCUSSION

In honeybees (*A. mellifera*), the proteasome inhibitor MG132 enhances LTM formation. Studies in vertebrates using different inhibitors of the proteasome demonstrate the opposite, namely, an inhibition of memory formation. The reason for this contradiction remains unclear. Because MG132 is an inhibitor of the proteasome, but also blocks other proteases, we hypothesized that other proteases affected by MG132 are responsible for the enhancement of LTM formation. We tested this hypothesis by comparing the effect of MG132 and β-lactone. We demonstrate that MG132 and β-lactone block the activity of the proteasome in honeybee brains to a similar extent, and do not affect the animals' survival but do enhance LTM retention upon olfactory conditioning.

The IC_{50} values of MG132 (105 nmol l^{-1}) and β-lactone (369 nmol l^{-1}) in honeybee brain tissue found in this study are comparable to IC_{50} values reported from studies in vertebrates (MG132: 850 nmol l^{-1}; β-lactone: 1 µmol l^{-1}) (Dick et al., 1997; Tsubuki et al., 1996). Moreover, we report 80% inhibition when using 10 µmol l^{-1} MG132 or β-lactone. This result is comparable to that found in vertebrates, where in different cell types 75–95% of chymotrypsin-like activity is inhibited within 1 h of application with >20 µmol l^{-1} β-lactone (Craiu et al., 1997). Taken together, we conclude that the enhancement of LTM formation is not due to MG132-specific side effects, but to inhibition of a protease targeted by MG132 and β-lactone.

Lon protease is the only protease that is inhibited by both inhibitors besides the proteasome. It has an IC_{50} in vertebrates that is three (β-lactone) to 20 (MG132) times higher than the inhibitor amount systemically injected in our behavioral experiment and eight (β-lactone) to 190 (MG132) times higher than the IC_{50} we report for honeybee brain tissue (see above) (Granot et al., 2007). Thus, we conclude that most likely the protease targeted by MG132 and β-lactone is not the Lon protease but the proteasome.

Accordingly, differences between the effect of MG132 on LTM formation in honeybees and other proteasome inhibitors in vertebrate model systems cannot be attributed to side effects of MG132 in honeybees. Several alternative explanations for this difference are possible.

Differences of the ubiquitin proteasome system of honeybees and vertebrates might cause differences in memory formation. β-lactone, for example, inhibits all three protein-degrading activities of the proteasome, the chymotrypsin-like, the trypsin-like and the peptidylglutamylpeptide-hydrolyzing activity, although with different affinities and reaction rates (Fenteany et al., 1995). Thus, β-lactone and MG132 might inhibit these three protein-degrading activities in honeybees and vertebrates to a different extent. However, the effect of MG132 and β-lactone for the honeybee chymotrypsin-like activity is comparable to that of vertebrates (see above). Therefore, it seems unlikely that the observed difference is due to differences in the proteasomal site mediating chymotrypsin-like activity. Rather, sites for trypsin-like and peptidylglutamylpeptide-hydrolyzing activity might differ between insects and vertebrates.

Moreover, proteasome target proteins might differ between insects and vertebrates, causing differences in LTM formation. One example is the transcription factor NF-κB, which plays a role in synaptic plasticity and LTM formation (reviewed in Alberini, 2009). NF-κB is activated by the proteasome, which degrades NF-κB precursor protein following learning (Merlo and Romano, 2007). Thus, structural differences between insect and vertebrate precursor proteins could result in differences in proteasomal activation following learning and thus LTM formation.

In addition, different time points of inhibitor application in honeybee and vertebrate model organisms might have different effects on LTM formation. A study on long-term plasticity (late-phase long-term potentiation, L-LTP) suggested that the proteasome inhibits the induction but facilitates the maintenance of synaptic plasticity (Dong et al., 2008). During an early phase of L-LTP, β-lactone stabilizes translational activators, whereas during a late phase of L-LTP, β-lactone stabilizes translational repressors (Dong et al., 2014). Accordingly, degradation of translational activators by the proteasome might play a role in setting the threshold for induction of L-LTP, whereas degradation of translational repressors during later stages of L-LTP prolongs translation, thereby supporting maintenance of L-LTP (Dong et al., 2014). In line with these findings, in the sea hare *Aplysia californica*, an MG132-dependent enhancement of the synaptic strength has been described (Zhao et al., 2003), and in vertebrates, MG132 injected into the amygdala enhanced memory formation in the fear potentiated startle paradigm, suggesting that MG132-sensitive protein degradation constrains the

formation of a memory trace after acquisition (Yeh et al., 2006). However, several results contradict the hypothesis that the proteasome inhibits the induction but facilitates the maintenance of long-term plasticity and LTM. In an inhibitory avoidance task, injection of the inhibitor 1, 4 and 7 h following training results in an inhibition of 24 h memory (Lopez-Salon et al., 2001). Also, injecting the inhibitor immediately and 3 h after training into the hippocampus inhibits 24 h memory for a spatial learning task in the Morris water maze (Artinian et al., 2008). Moreover, injecting the inhibitor immediately after fear conditioning into the amygdala inhibits LTM formation (Jarome et al., 2011). Thus, the contradiction between our results and the results of other studies might not be fully explained by different time points of injection.

Alternative explanations are the use of an appetitive paradigm in our studies in contrast to aversive paradigms and spatial learning tasks used in most vertebrate studies, and the fact that we injected the inhibitor systemically and not into specific regions of the brain. Thus, the reason for differences in the role of the proteasome in LTM formation of the honeybee and vertebrate model organisms for learning and memory formation needs to be clarified in future studies. However, it seems clear that side effects of MG132 on non-proteasomal proteases are not the reason for an enhancement of LTM by MG132.

MATERIALS AND METHODS

Honeybees were caught at bee hives at the Freie Universität Berlin. Handling, conditioning, systemic injection and statistical analysis were carried out as described previously (Felsenberg et al., 2011; Felsenberg et al., 2012).

In vitro proteasome activity assay

The *in vitro* proteasome activity assay using the fluorogenic peptide Suc-LLVY-AMC (Calbiochem, Darmstadt) was performed as described previously (Kloß et al., 2009; Dahlmann et al., 2000).

Ten honeybee brains were pooled in a 100 µl TSDG buffer [10 mmol l^{-1} Tris/HCl, 1.1 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} NaCl, 0.1 mmol l^{-1} EDTA, 1 mmol l^{-1} NaN_3, 1 mmol l^{-1} DTT, 2 mmol l^{-1} ATP, 10% (v/v) glycerol, pH 7.0] and homogenized in three repeated freeze–thaw cycles with liquid nitrogen. The homogenates were centrifuged at 20,000 ***g*** for 40 min and supernatants were used as tissue extracts for an *in vitro* proteasome activity assay. The fluorogenic peptide substrate succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-AMC; Calbiochem, Darmstadt, Germany) was diluted from a stock solution of 10 mmol l^{-1} Suc-LLVY-AMC/100% DMSO with TEAD buffer (20 mmol l^{-1} Tris/HCl, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} DTT, 1 mmol l^{-1} NaN_3, pH 7.2) to 200 µmol l^{-1} Suc-LLVY-AMC in TEAD/2% DMSO. Suc-LLVY-AMC was used to measure the chymotrypsin-like activity at a final concentration of 100 µmol l^{-1}. The inhibitors MG132 (Calbiochem, Darmstadt) and β-lactone (Sigma-Aldrich, Munich) were dissolved in 100% DMSO and diluted to different concentrations with PBS (137 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, 10.1 mmol l^{-1} Na_2HPO_4, 1.8 mmol l^{-1} KH_2PO_4 at pH 7.2) from 50 µmol l^{-1} dissolved in 5% DMSO/PBS to 10 nmol l^{-1} dissolved in 0.001% DMSO. Five microliters of these MG132 or β-lactone solutions were added to 5 µl honeybee brain lysate (10 brains in 100 µl TSDG puffer) and 10 µl of 200 µmol l^{-1} Suc-LLVY-AMC solution. After 60 min of incubation at 37°C, the released AMC was measured fluorimetrically. The percentage of remaining proteasome activity following inhibition was calculated by normalizing the chymotrypsin-like proteasome activity of samples containing the inhibitor to the proteasomal activity of parallel processed control samples containing 1.25% DMSO/H_2O instead of the inhibitor.

Measuring honeybee body water

To determine the bees' average body water, harnessed honeybees were caught and fed to satiation. The following day, bees were weighed before they were incubated at a high temperature. During incubation, individual bees were repeatedly weighed (roughly every hour) until no further mass loss could be observed. The difference between the first and the final mass measurement was taken as the amount of body water.

Data and statistical analysis

Analyses were carried out using the software Prism 6 (GraphPad Software, La Jolla, CA, USA) and Statistica (StatSoft, Hamburg, Germany).

Inhibition of chymotrypsin-like activity

Differences between the control with no inhibition (zero inhibition) and the mean relative inhibition of chymotrypsin-like activity by different concentrations of MG132 and β-lactone were tested with a *t*-test for single means against zero.

Honeybee survival

We used Cochran's *Q*-test to analyze the survival of bees within a group over the time of the experiment. To test for differences between the survival scores of the groups, we used either a Mann–Whitney *U*-test in the case of comparison between two groups or a Kruskal–Wallis test in the case of multiple group comparisons. IC_{50} values were calculated with non-linear fit analysis in Prism 6.

Behavioral experiments

To analyze PER performance within a group over the repetitive memory tests, we used Cochran's *Q*-test. The differences in the performance during the memory tests were analyzed with a repeated-measures ANOVA. To analyze the memory score we used a Mann–Whitney *U*-test.

Competing interests

The authors declare no competing financial interests.

Author contributions

D.E. and J.F. conceptualized the study; D.E., J.F., A.K., B.D. and P.-M.K. designed the experiments; J.F., Y.D. and A.K. carried out the experiments and analyzed the data; D.E. and J.F. wrote the manuscript.

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