Postprint

Access to the published version may require subscription.

N.B. When citing this work, cite the original published paper.

Permanent link to this version:
http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-368137

Transglutaminase inhibition stimulates hematopoiesis and reduces aggressive behavior of crayfish,
Pacifastacus leniusculus

Kingkamon Junkunlo[1], Kenneth Söderhäll[2] and Irene Söderhäll[2*]

[1]Department of Comparative Physiology, Uppsala University, Norbyvägen 18A, 752 36 Uppsala, Sweden
[2] Science for Life Laboratory, Department of Comparative Physiology, Uppsala University, Norbyvägen 18A, 752 36 Uppsala, Sweden.

Running title: TGase inhibition affects hematopoiesis and reduces aggressive behavior

*Corresponding author: Irene Söderhäll, Science for Life Laboratory, Department of Comparative Physiology, Uppsala University, Norbyvägen 18A, 752 36 Uppsala, Sweden. Tel: +46-18-4712811; Fax: + 46-18-4716425. E-mail: irene.söderhäll@ebc.uu.se

Keywords: cystamine, transglutaminase, hematopoiesis, crustacean, blood cell, extracellular matrix, arthropod, hemocyte, hemolymph

ABSTRACT

Transglutaminase (TGase) is a Ca2+-dependent cross-linking enzyme, which has both enzymatic and non-enzymatic properties. TGase is involved in several cellular activities, including adhesion, migration, survival, apoptosis, and extracellular matrix (ECM) organization. In this study, we focused on the role of the TGase enzyme in controlling hematopoiesis in the crayfish Pacifastacus leniusculus. We hypothesized that a high TGase activity could mediate an interaction of progenitor cells with the ECM to maintain cells in an undifferentiated stage in the hematopoietic tissue (HPT) tissue. We found here that the reversible inhibitor cystamine decreases the enzymatic activity of TGase from crayfish HPT as well as from guinea pig in a concentration-dependent manner. Cystamine injection could decrease TGase activity in HPT without affecting production of reactive oxygen species (ROS). Moreover the decrease in TGase activity in the HPT increased the number of circulating hemocytes. Interestingly, the cystamine-mediated TGase inhibition reduced aggressive behavior and movement in crayfish. In conclusion, we show that cystamine-mediated TGase inhibition directly releases HPT progenitor cells from the HPT into the peripheral circulation in the hemolymph and strongly reduces aggressive behavior in crayfish.

INTRODUCTION

Transglutaminase (TGase) is a multifunctional enzyme, which participates in controlling a wide range of cellular activities (1). Dysfunction of TGase enzymes is proposed to have roles in development of many diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease (2). Nine isoforms of TGase have been identified in the human genome and these isoforms play different roles depending on their tissue distribution, subcellular localization and substrate specificity. For example TGases are involved in blood coagulation, skin formation, and signal transduction (3, 4). The catalytic activity of TGase is a Ca^{2+}-dependent posttranslational modification of proteins by generating covalent bonds between free amine groups to form an ϵ-(γ-glutamyl)lysine isopeptide bond (1). In addition to this Ca^{2+}-dependent reaction, Ca^{2+}-independent enzymatic as well as non-enzymatic activities of TGase have been described. Tissue TGase or TGase 2 is a well-studied enzyme of this family. The broad substrate specificity and the interactions with many genes or proteins by TGase 2 explain its multiple biological functions including cell adhesion, migration, growth, survival, apoptosis, differentiation, and extracellular matrix organization (4). The non-enzymatic functions are based on non-covalent interactions of TGase by acting as an adapter protein and in this way mediating adhesion/signaling at the cell surface (5).

In invertebrates TGase was first identified in horseshoe crab (*Tachypleus tridentatus*) (6) and then reported in many species such as crayfish (*Pacifastacus leniusculus*) (7), shrimp (*Litopenaeus vannamei*) (8), and the fruit fly (*Drosophila melanogaster*) (9, 10). However, the invertebrate TGases described so far, are mostly involved in coagulation or clot formation which is the same function as human factor XIIIa or plasma TGase (7, 11). In addition, the sequence of invertebrate TGase shows a high similarity with human factor XIIIa. Human factor XIIIa catalyzes the formation of ϵ-(γ-glutamyl) lysine isopeptide bonds between fibrin-fibrin strands to stabilize the fibrin clot during coagulation (12). In crayfish, clot formation is directly induced by the crosslinking activity of TGase on a clotting protein (CP) (13). In response to wounding or infection, TGase is released from hemocytes or the hematopoietic tissue (HPT), and then CP molecules are crosslinked by TGase into large aggregates (13). Recently, CP was also found to function as an extracellular matrix (ECM) component protein in crayfish HPT (14). CP functions together with collagen, TGase and the cytokine Astakine 1 (Ast1) in the regulation of HPT progenitor cell behavior (14). The important roles of TGase in hematopoietic regulation have been studied in detail in crayfish and a high extracellular TGase activity mediates an interaction of progenitor cells with the environment to maintain cells in an undifferentiated stage in the crayfish HPT (15, 16). Furthermore, crayfish TGase regulatory promoter region contains GATA binding motifs as is also found in the human factor XIIIa gene (16). GATA is a transcription factor, which plays a crucial role during hematopoietic development. GATA family proteins function in lineage specification and differentiation of hematopoietic progenitor cells (17). The presence of GATA motif in crayfish TGase genes highlights the potential roles of TGase in hematopoietic regulation like that occurring in mammals (16).

In this study, we focused on the roles of TGase enzyme in controlling hematopoiesis in crayfish *P. leniusculus*. Cystamine a small disulfide-containing molecule was used to inhibit TGase activity both in *in vivo* and *in vitro* experiments.

RESULTS

Cystamine inhibits TGase enzymatic activity - To examine the important role of TGase activity on hematopoiesis, cystamine a reversible TGase inhibitor, was used to inhibit TGase activity. First, we investigated whether cystamine could inhibit the TGase enzyme activity from crayfish HPT lysate. Cystamine at different final concentrations of 10µM, 100µM, 1mM, and 10mM were tested. The inhibitory efficiency of cystamine on crayfish TGase was compared with the inhibition of a purified commercial guinea pig liver TGase (Figure 1). By using a non-radioactive microtiter plate assay, which measured TGase-mediated 5-(biotinamido) pentylamine substrate (BPHN$_2$) incorporation, the highest concentration of cystamine at 10mM could significantly reduce (P< 0.001) the TGase activity from guinea pig compared with control (without cystamine) (Figure 1A). At the same concentration of cystamine (P<0.001) the remaining activity was 10% for TGase from HPT compared with the control (Figure 1B). The remaining activity of both TGase from guinea pig and TGase from HPT was decreased in a dose-dependent manner according to the concentration of cystamine (Figure 1A and 1B). These results indicate that cystamine was efficient in inactivating crayfish TGase as well as guinea pig TGase.

Cystamine injection has a direct inhibitory effect on TGase activity in the HPT in vivo - To investigate whether cystamine could inhibit TGase activity in HPT tissue *in vivo*. TGase activity in HPT tissue at 3h after cystamine injection was determined (Figure 2A). A significant decrease of TGase activity in the HPT was observed (P < 0.01) in animals injected with 150 µg cystamine/g compared with crayfish buffer saline (CPBS)-injected animals (Figure 2A). However cystamine at a concentration of 75 µg cystamine/g could only slightly inhibit TGase activity when compared to CPBS-injected animals (Figure 2A). It is known that cystamine may have multiple mechanisms and it has been reported to increase the glutathione production and then reduce oxidative stress-induced cell death (18). We previously showed that low ROS level induced by N-acetyl cysteine (NAC) injection could prolong the decrease in hemocyte numbers by increasing the extracellular TGase activity and thereby maintaining the cells inside the

HPT (15). Therefore, we examined the effect of cystamine on ROS production after TGase inhibition by cystamine injection. However, cystamine injection had no effect on the ROS level at 1h and 3h (Figure 2B). Thus it seems as if cystamine has a direct effect on TGase activity without interfering with ROS production.

TGase activity inhibition is involved in new hemocyte synthesis - A high extracellular TGase activity was found in less differentiated progenitor cells residing in the crayfish HPT compared to cells migrating out of the tissue (16). These results suggest the involvement of TGase activity in regulation of hematopoiesis. Since cystamine significantly reduced TGase activity and to verify whether TGase inhibition caused by cystamine could affect new hemocyte production, the differential hemocyte number of granular cells (GC), and semi-granular cells (SGC) was determined as well as the total number of circulating hemocytes (THC) after inhibition of TGase activity by cystamine injection. CPBS-injected animals served as controls (Figure 3). Two different doses of cystamine were injected into crayfish (75 μg cystamine/g and 150 μg cystamine/g) and at the high concentration of cystamine the hemocyte numbers increased significantly at 1h (P < 0.05 for GC, P < 0.001 for SGC and THC). The hemocyte numbers gradually increased from 1h to 6h and restored to a normal level at 24h (Figure 3). Injection of cystamine at 75 μg cystamine/g could similarly increase the circulating hemocyte number, but to a lesser extent at later time points compared to cystamine at 150 μg cystamine/g. The hemocyte numbers (GC, SGC, and THC) were significantly increased at 6h (P < 0.01 for GC and SGC, and P < 0.05 for THC) and recovered to normal levels after 24h (Figure 3). The inhibitory effect of cystamine on TGase activity in HPT was concentration dependent and these results support the increase of the circulating hemocyte numbers at 3h (Figure 2A and Figure 3). In addition, TGase activity in HPT was not significantly different when the number of circulating hemocytes was restored to normal level at 24h (data not shown). The results of this experiment suggest that inhibition of TGase by cystamine may stimulate the differentiation of hematopoietic progenitor cells in the HPT, and then promote the release of these cells into the peripheral circulation.

TGase activity mediated release of progenitor cells HPT into the circulation - The crosslinking function of TGase was shown to be essential in mediating the interaction between progenitor cells with ECM proteins such as collagen and CP in crayfish (14–16, 19). To determine whether inhibition of TGase activity has an effect on hematopoietic progenitor cell proliferation, 5-Bromo-2'-deoxyuridine (BrdU) incorporation was performed in HPT after cystamine injection. However, there was no clear difference in BrdU positive cell numbers in HPT after cystamine injection (data not shown), but the number of proliferating cells (BrdU labeled cells) in hemolymph was significantly increased in the 75 μg cystamine/g injected group and in the 150 μg cystamine/g injected group (Figure 4). The presence of circulating BrdU-labeled cells may indicate that newly synthesized hemocytes are released into the circulation when TGase in the HPT is inhibited. Since we could not observe a notable increase of BrdU-positive cells in the HPT, our conclusion is that inhibition of TGase activity in HPT affected the progenitor cell behavior by stimulating release of new hemocytes into the hemolymph.

Inhibition of TGase activity by cystamine induces changes in crayfish behavior - Cystamine injection clearly suppressed the mobility of the crayfish (Figure 5 and Figure 6). Normal or CPBS-injected crayfish are usually exhibiting aggressive behavior (Figure 5). This aggressive behavior included attacking, fighting over resources, raising the claw for grabbing and/or holding the object (Figure 6 and Movie S1). Cystamine at a concentration of 75 μg cystamine/g injection could suppress the frequency of movements and also induced a fear response (Figure 6 and Movie S2). A fear response is defined as a non-fighting behavior and the animal tries to escape from resources or objects. This behavior disappeared and returned to normal aggressive behavior after 24h (Figure 6). Interestingly, cystamine at a concentration of 150 μg cystamine/g injection clearly induced a non-movement behavior (Figure 6 and Movie S3). Crayfish injected with 150 μg cystamine/g were completely motionless, or moved very little with any part of the body (Figure 5 and Figure 6). The

immobility behavior was observed at 1h to 24h after 150 µg cystamine/g injection and the behavior was restored to normal active or aggressive behavior after 96 h (Figure 6). The behavior, which we have observed is corresponding to the TGase activity inhibition and also to the increase of circulating hemocytes after cystamine injection.

DISCUSSION

The balancing between self-renewal and differentiation of hematopoietic progenitor cells are tightly regulated by a variety of cells and signals, which include the microenvironment or niche (20). In crayfish, the progenitor cells are located in packed lobules or between lobules, which are surrounded by connective tissue and ECM (21). TGase is an abundant protein in HPT and hemocytes, particularly in SGC (16). The co-localization of extracellular TGase with ECM proteins for example collagen and CP in HPT has been reported (14, 15). Furthermore, the proliferating cells residing in crayfish HPT have a high extracellular TGase activity compared to the differentiated cells or in cells migrating out of the tissue (16). These results suggest the involvement of TGase enzyme activity in hematopoietic regulation.

A well-known TGase inhibitor, cystamine was used to inhibit TGase activity in crayfish HPT. Cystamine inhibits TGase by forming a mixed disulfide with the active site thiol through a thiol-disulfide interchange mechanism (22). The inhibitory effect of cystamine to TGase has been characterized in many species except crustaceans. In *Drosophila*, cystamine treatment prevents photoreceptor degeneration and enhances neurodegeneration in a spinocerebellar ataxia (SCA3) mutant (23). In human WI-38 lung cells, a reduction in TGase activity was observed in a dose-dependent manner by supplementing cystamine into the culture medium (24). In addition, cystamine at a concentration of 10mM completely reduced TGase activity in these cells (24). As in human WI-38 lung cells, the addition of cystamine completely reduced TGase activity of a commercial purified guinea pig liver TGase and decreased the activity of crayfish TGase from HPT tissue to 10% of the original activity. The inhibitory effect of cystamine on TGase activity was observed in a concentration dependent manner.

The importance of TGase in hematopoietic regulation has been first studied in crayfish (16) and later reported in other species. In black tiger shrimp, *Peneaus monodon*, the TGase isoform I (STG I) is mainly expressed in hematopoietic tissue (25) and is not involved in coagulation (26). In addition, a lipopolysaccharide (LPS) injection could stimulate the secretion of STG I and Astakine in plasma to induce the hemocytes proliferation (25, 27). In white shrimp, *Litopeneaus vannamei*, knockdown of LvTG II mRNA expression could induce an increase in hyaline cell number (28). In crayfish, knockdown of TGase mRNA transcript could promote HPT cell spreading *in vitro* (16). We therefore investigated the number of circulating hemocytes after inhibiting TGase activity by cystamine injection. Inhibition of TGase activity by 150 µg cystamine/g injection resulted in a gradual increase of the relative hemocyte numbers (GC cell, SGC cell and THC cell) from 1h to 6h and returned to normal levels after 24h in the circulation. The transient effect of cystamine may be due to that cystamine is metabolized after injection. Cystamine was shown to be quickly metabolized into cysteamine and then converted to cysteine, hypotaurine and taurine which are endogenous cellular components and commonly used as a building blocks for several proteins (29). By using HPLC to measure the level of the metabolized form of cystamine in mice plasma cysteamine could be detected at 1h to 3h after a single injection of 50 and 200 mg cystamine/kg dose and progressively diminished at 48h in mice (30). In addition to inhibit TGase activity, cystamine has antioxidant properties by increasing the L-cysteine level and enhancing the glutathione (GSH) system (31). In crayfish, cystamine injection had an effect on TGase activity in HPT at 3h whereas no effect on ROS production in the tissue was observed. A high ROS level was observed in the anterior proliferation center (APC) before the recovery of hemocyte numbers following a lipopolysaccharide (LPS) or a laminarin injection (32). APC is a small area in the middle of the anterior HPT and it is located close to the brain (32). We also showed that cystamine injection had no influence on ROS production in APC. We have reported that low ROS level by caused by a NAC injection induced the increase of extracellular TGase activity and decreased the number of circulating hemocytes

(15). Taken together, our results indicate that ROS signaling could affect the extracellular TGase activity in controlling hematopoiesis. However TGase inhibition did not interfere with the ROS level in APC or the whole HPT.

In MC3T3 osteoblast cell culture, inhibition of TGase activity by cystamine treatment results in decrease of ECM accumulation and stimulates the differentiation of osteoblast cells (33). In crayfish, TGase activity is needed to mediate cell-ECM interactions and to provide a suitable environment for the progenitor cells (14). We hypothesize that TGase inhibition by cystamine injection could interfere with the cell-ECM interaction, and promote the differentiation of progenitor cells, and then induce cell migration out of the HPT, since we found a significant release of BrdU labeled cells in hemolymph at 3h after cystamine injection.

Cystamine has been reported to prevent the formation of huntingtin protein aggregates and exhibit a neuroprotective effect against cytotoxicity in Parkinson's disease in mice (34). In addition, cystamine could rescue the dopaminergic system by increasing the number of dopaminergic neurons. These protective effects result in prolonged life span and improvement in motor behaviors in an animal model of Parkinson's disease, R6/2 mice (35). Cystamine injection in crayfish caused a dramatic change in locomotion behavior in a dose-dependent manner. In normal or CPBS-injected crayfish, aggressive and fighting behavior including raising the claw or grabbing the object is usually easily observed. A high dose of cystamine injection at 150 μg cystamine/g, crayfish displayed a non-movement behavior with entire motionless or very slow-moving. Cystamine injection at a concentration of 200 mg cystamine/kg could induce signs of hypothermia (shivering) and drowsiness for a period of approximately 2h in mice (36). In crayfish, the impaired movement caused by cystamine injection could be observed from 1h to 24h (75 μg cystamine/g dose) and from 1h to 48h (150 μg cystamine/g dose). These changes in behavior of crayfish after cystamine injection corresponded to the inhibitory effect of cystamine on TGase activity and the increase of circulating hemocyte numbers. Furthermore, the changed behavior of crayfish had a similarity to the pharmacokinetic properties of cystamine. In adult male Sprague-Dawley rats injected with 250 mg cysteamine/kg of which is a reduced form of cystamine, resulted in tremor in neck and head and changed behavior (37). Our results indicate a new function for TGase in controlling crayfish movement. However, cystamine injection could have another effect on the nervous system in controlling behavior. Thus, the role of cystamine on TGase activity in the nervous system needs further investigation.

In conclusion, we have shown an important role of TGase in the direct regulation of hematopoiesis by mediating interactions between cell and the ECM in HPT and in addition a putative role for TGase in regulating crayfish locomotion.

EXPERIMENTAL PROCEDURES

Animals - Freshwater crayfish, *P. leniusculus*, were from Lake Erken, Sweden. The animals were maintained in aquaria with aeration at 10 °C. Healthy and intermolt male crayfish were used for the experiments.

TGase enzyme preparation - To prepare TGase, HPT was freshly dissected, as described previously (17). The tissue was then homogenized in 100 μl RIPA buffer (50 mM Tris, 150 mM NaCl, 10 mM EDTA, 1% IGEPAL, 1% SDS, pH 7.5) containing 10 times diluted protease inhibitor cocktail (Roche, Switzerland) from the recommend stock solution (10X). After centrifugation of the homogenate at $13,000 \times g$ for 15 min at 4 °C, the protein supernatant was immediately used in a TGase inhibition assay. The protein concentration was determined by using Coomassie Plus protein assay reagent (Thermo Scientific, USA). For a commercial TGase, lyophilized guinea pig liver TGase (T5398) powder (Sigma-Aldrich, USA) was dissolved in 1 ml dH$_2$O as an enzyme stock solution. One unit of commercial purified TGase was freshly diluted 1:1000 in 0.1 M Tris-HCl, pH 8.5 before immediately used in the experiments.

TGase activity assay - TGase activity was assayed by using a modified non-radioactive microtiter plate assay (38). Briefly, the microtiter plate was coated with 100 μl of N,N'-dimethylcasein (10 mg/ml) (Sigma-Aldrich, USA) at 4 °C overnight. After blocking with blocking solution (0.5% BSA in 0.1 M Tris-HCl, pH 8.5) for 30 min at room temperature, the wells were washed

three times with 0.1 M Tris-HCl, pH 8.5. The incubation mixture, which contained 5 mM CaCl$_2$, 10 mM dithiothreitol, 0.1 mM of BPHN$_2$ (Thermo Scientific, USA), 5 μg protein of whole cell lysates and 0.1 M Tris-HCl, pH 8.5, was added to obtain a total volume of 100 μl per well. After incubation at 37 °C for 30 min, the reaction was stopped by washing twice with 200 mM EDTA, followed by washing three times with 0.1 M Tris-HCl, pH 8.5. Streptavidin-horseradish peroxidase conjugate (GE Healthcare, UK) was diluted to 1:1000 in blocking solution before it was added to the wells for a 30 min incubation at room temperature. The plate was washed twice with 0.01% Triton X-100, followed by five washes with 0.1 M Tris-HCl, pH 8.5. Then, 100 μl of 3,3′,5,5′-Tetramethylbenzidine substrate solution (TMB, Sigma) was added to each well. After incubation for 5 min at room temperature, the reactions were stopped by the addition of 50 μl of 3 N HCl to each well. The TGase cross-linking activity was quantified by measuring the absorbance at 450 nm in a plate reader.

TGase inhibition assay – The inhibition experiments were performed by using a modified non-radioactive microtiter plate assay, as described above. The reaction mixture consisted of four different concentration of cystamine at final concentration of 10mM, 1mM, 0.1mM, or 10μM in the presence of a fixed amount of a commercial guinea pig TGase (1 unit of stock commercial guinea pig TGase diluted 1:1000 in 0.1 M Tris-HCl, pH 8.5) or 5 μg of the HPT lysate protein (an endogenous TGase from HPT. The optical density was quantified by measuring the absorbance at 450 nm in a plate reader.

ROS detection - To detect the ROS levels in the tissues, the HPTs were dissected and washed two times with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$ and 2 mM KH$_2$PO$_4$, pH 7.4). A stock solution of 5 mg/ml 2′,7′-dichlorofluorescin diacetate (DCF-DA) (Sigma-Aldrich, USA) was freshly diluted with PBS (1:1,000) and added to the tissues and incubated for 15 min in darkness. After washing three times with PBS, the ROS level was determined using a microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The fluorescence intensity was calculated as the fluorescence intensity of the sample minus the fluorescence intensity of PBS without tissue (serves as a baseline value). The

results were reported as fluorescence intensity per μg tissue protein. The protein concentration was determined by using Coomassie Plus protein assay reagent (Thermo Scientific, USA).

Circulating hemocyte count after cystamine injection - The experiments were performed using 4-12 crayfish (25-30 g) in each experimental group. Prior to the injection of cystamine, one drop of hemolymph was collected and immediately fixed in 10% formalin. The hemocyte number prior to injection was counted and served as the baseline value. After baseline bleeding, the animals were allowed to rest for 48h. Subsequently, the animals were injected in the base of the fourth walking leg with 1) CPBS as a control, 2) cystamine (75 μg cystamine/g crayfish), or 3) cystamine (150 μg cystamine/g crayfish. The hemolymph concentration of cystamine after injection was estimated by using a dye dilution assay using Amaranth (Sigma-Aldrich, USA). A working solution, at 5mg/ml was then diluted to a final concentration of 0.625, 1.25, 2.5, 5, 12.5 and 25μg/ml with crayfish plasma and absorbance was measured at 520 nm for a standard curve. Then 100μl Amaranth at a concentration of 5mg/ml was injected into a walking leg of crayfish. After 2h hemolymph was withdrawn and processed to remove hemocyte by centrifugation (800 x g, 10 min 4 C). The resulting supernatant was diluted with ice-cold anticoagulant buffer 1:1 (v/v) and the absorbance analyzed at 520 nm. The concentration of dye in the hemolymph sample was examined by relative to the standard curve and the hemolymph volume was calculated by:

$$V = \frac{a(C1 - C2)}{C2}$$

V is the hemolymph volume, *a* is the injected volume, *C1* is he injected concentration of Amaranth, and *C2 is* the final concentration of Amaranth. The average hemolymph volume was estimated to 38±5% (n=4) related to body weight. The cystamine injection would then result in an approximate hemolymph concentration of 1.5 mM or 3 mM after 75 μg cystamine/g crayfish or 150 μg cystamine/g crayfish respectively.

At 1h, 3h, 6h, 24h, and 48h post-injection, hemolymph was collected and fixed in 10% formalin. The total hemocyte and differential hemocyte (granular and semi-granular) numbers after injection were subsequently counted and

reported as relative hemocyte count (hemocyte number after injection divided by the hemocyte number prior to injection).

Cell proliferation detected by BrdU incorporation – To detect the proliferating cells, crayfish were injected with 10 μl/g fresh weight of 50 mM BrdU (Sigma-Aldrich, USA) in CPBS for 24h before the animals were injected with CPBS, or cystamine (75 μg cystamine/g and 150 μg cystamine/g crayfish). At 3h post CPBS and cystamine injections, the hemocytes were collected and immediately fixed with 4% paraformaldehyde in PBS for 1h. The fixed hemocytes were treated with 2N HCl for 30 min at room temperature, and washed five times, 15 min each with PBST (0.5% Tween 20 in PBS buffer) before overnight incubation with mouse anti BrdU (1:50) (BD Biosciences, USA) in PBST at 4 °C. Subsequently, the primary antibodies were removed, and the samples were washed 5 times with PBST and incubated for 1h with FITC-conjugated anti-mouse IgG (1:300) (Life Technologies, USA) and Hoechst 33258 dye at a concentration of 1 μg/ml to stain the nuclei. After washing 5 times with PBST, the number of BrdU incorporating cells was observed under a fluorescence microscope. The number of BrdU labeled cells was calculated as a positive BrdU cell per total number of cells.

Crayfish behavior after cystamine injection – The crayfish were randomly collected from the holding tanks and separated into groups of 3 - 5 individuals, assigned to the control or test groups. The crayfish were allowed to adjust to the new surroundings for 48h before being injected with 1) CPBS as a control, 2) 75 μg cystamine/g crayfish or 3) 150 μg cystamine/g crayfish. Behavior of individual animals in each experimental group was recorded as videos for 3 min at 1h, 3h, 6h, 24h, 48h, and 96h. The activities of crayfish were analyzed based on their mobility or immobility. We focused on a specific movement and non-movement behavior at the time of data collection. Immobility was defined as that the animal remained completely not moving and with no movement of any part of the body.

Statistical analysis - The ROS levels and differential hemocyte counts are shown as the mean $\pm$ SD, and the statistical analysis was performed using one-way ANOVA followed by Duncan's new multiple range test and Tukey's test. For comparisons between two groups, a t-test was used, and statistical significance was considered at $P<0.05$.

Acknowledgments

Conflicts of Interest
The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions
KJ performed experiments, analyzed results, prepared figures and wrote the paper. KJ, KS, and IS contributed to study design, interpreted the data and participated in writing the paper. All authors reviewed the results and approved the final version of the manuscript.

Abbreviations
The abbreviations used are: TGase, transglutaminase; ROS, Reactive oxygen species; ECM, Extracellular matrix; HPT, hematopoietic tissue; CP, clotting protein; Ast1, Astakine 1; NAC, N-acetyl cysteine; GC, Granular cells; SGC, Semi-granular cells; THC, total hemocyte cells; CPBS, crayfish buffer saline; SCA3, spinocerebellar ataxia; STG I, *Peneaus monodon* TGase isoform I; LvTG II, Litopeneaus vannamei TGase isoform II; GSH, Glutathione; LPS, Lipopolysaccharide; APC, Anterior proliferation center; TMB, 3,3′,5,5′-Tetramethylbenzidine substrate solution, DCF-DA, 2',7'-dichlorofluorescin diacetate, BrdU; 5-Bromo-2'-deoxyuridine.

References

1. Eckert, R. L., Kaartinen, M. T., Nurminskaya, M., Belkin, A. M., Colak, G., Johnson, G. V. W., and Mehta, K. (2014) Transglutaminase Regulation of Cell Function. *Physiol. Rev.* **94**, 383–417

2. Martin, A., De Vivo, G., and Gentile, V. (2011) Possible Role of the Transglutaminases in the Pathogenesis of Alzheimer's Disease and Other Neurodegenerative Diseases. *Int. J. Alzheimers Dis.* 10.4061/2011/865432

3. Klöck, C., and Khosla, C. (2012) Regulation of the activities of the mammalian transglutaminase family of enzymes. *Protein Sci. Publ. Protein Soc.* **21**, 1781–1791

4. Odii, B. O., and Coussons, P. (2014) Biological functionalities of transglutaminase 2 and the possibility of its compensation by other members of the transglutaminase family. *Scientific World Journal*. **2014**, 714561

5. Nurminskaya, M. V., and Belkin, A. M. (2012) Cellular functions of tissue transglutaminase. *Int. Rev. Cell Mol. Biol.* **294**, 1–97

6. Tokunaga, F., Muta, T., Iwanaga, S., Ichinose, A., Davie, E. W., Kuma, K., and Miyata, T. (1993) Limulus hemocyte transglutaminase cDNA cloning, amino acid sequence, and tissue localization. *J. Biol. Chem.* **268**, 262–268

7. Wang, R., Lee, S. Y., Cerenius, L., and Söderhäll, K. (2001) Properties of the prophenoloxidase activating enzyme of the freshwater crayfish, *Pacifastacus leniusculus*. *Eur. J. Biochem.* **268**, 895–902

8. Yeh, M.-S., Liu, C.-H., Hung, C.-W., and Cheng, W. (2009) cDNA cloning, identification, tissue localisation, and transcription profile of a transglutaminase from white shrimp, Litopenaeus vannamei, after infection by Vibrio alginolyticus. *Fish Shellfish Immunol.* **27**, 748–756

9. Iklé, J., Elwell, J. A., Bryantsev, A. L., and Cripps, R. M. (2008) Cardiac expression of the Drosophila Transglutaminase (CG7356) gene is directly controlled by Myocyte enhancer factor-2. *Dev. Dyn. Off. Publ. Am. Assoc. Anat.* **237**, 2090–2099

10. Shibata, T., and Kawabata, S. (2015) Transglutaminase in Invertebrates. in *Transglutaminases*, pp. 117–127, Springer, Tokyo, 10.1007/978-4-431-55825-5_5

11. Cerenius, L., and Söderhäll, K. (2011) Coagulation in invertebrates. *J. Innate Immun.* **3**, 3–8

12. Nikolajsen, C. L., Dyrlund, T. F., Poulsen, E. T., Enghild, J. J., and Scavenius, C. (2014) Coagulation Factor XIIIa Substrates in Human Plasma. *J. Biol. Chem.* **289**, 6526–6534

13. Hall, M., Wang, R., van Antwerpen, R., Sottrup-Jensen, L., and Söderhäll, K. (1999) The crayfish plasma clotting protein: A vitellogenin-related protein responsible for clot formation in crustacean blood. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 1965–1970

14. Junkunlo, K., Söderhäll, K., and Söderhäll, I. (2018) Clotting protein - An extracellular matrix (ECM) protein involved in crustacean hematopoiesis. *Dev. Comp. Immunol.* **78**, 132–140

15. Junkunlo, K., Söderhäll, K., Söderhäll, I., and Noonin, C. (2016) Reactive Oxygen Species Affect Transglutaminase Activity and Regulate Hematopoiesis in a Crustacean. *J. Biol. Chem.* **291**, 17593–17601

16. Lin, X., Söderhäll, K., and Söderhäll, I. (2008) Transglutaminase activity in the hematopoietic tissue of a crustacean, Pacifastacus leniusculus, importance in hemocyte homeostasis. *BMC Immunol.* **9**, 58

17. Gao, J., Chen, Y.-H., and Peterson, L. C. (2015) GATA family transcriptional factors: emerging suspects in hematologic disorders. *Exp. Hematol. Oncol.* **4**, 28

18. Siegel, M., and Khosla, C. (2007) Transglutaminase 2 Inhibitors and their Therapeutic Role in Disease States. *Pharmacol. Ther.* **115**, 232–245

19. Junkunlo, K., Söderhäll, K., Noonin, C., and Söderhäll, I. (2017) PDGF/VEGF-Related Receptor Affects Transglutaminase Activity to Control Cell Migration During Crustacean Hematopoiesis. *Stem Cells Dev.* **26**, 1449–1459

20. Hoffman, C. M., and Calvi, L. M. (2014) Minireview: Complexity of Hematopoietic Stem Cell Regulation in the Bone Marrow Microenvironment. *Mol. Endocrinol.* **28**, 1592–1601

21. Chaga, O., Söderhäll, K., and Lignell, M. (1995) The haemopoietic cells of the freshwater crayfish, Pacifastacus leniusculus. *Anim Biol.* **4**, 50–70

22. Lorand, L., and Conrad, S. M. (1984) Transglutaminases. *Mol. Cell. Biochem.* **58**, 9–35

23. Lin, Y., He, H., Luo, Y., Zhu, T., and Duan, R. (2015) Inhibition of transglutaminase exacerbates polyglutamine-induced neurotoxicity by increasing the aggregation of mutant ataxin-3 in an SCA3 Drosophila model. *Neurotox. Res.* **27**, 259–267

24. Birckbichler, P. J., Orr, G. R., Patterson, M. K., Conway, E., and Carter, H. A. (1981) Increase in proliferative markers after inhibition of transglutaminase. *Proc. Natl. Acad. Sci. U. S. A.* **78**, 5005–5008

25. Huang, C.-C., Sritunyalucksana, K., Söderhäll, K., and Song, Y.-L. (2004) Molecular cloning and characterization of tiger shrimp (Penaeus monodon) transglutaminase. *Dev. Comp. Immunol.* **28**, 279–294

26. Chen, M.-Y., Hu, K.-Y., Huang, C.-C., and Song, Y.-L. (2005) More than one type of transglutaminase in invertebrates? A second type of transglutaminase is involved in shrimp coagulation. *Dev. Comp. Immunol.* **29**, 1003–1016

27. Chang, Y.-T., Lin, C.-Y., Tsai, C.-Y., Siva, V. S., Chu, C.-Y., Tsai, H.-J., and Song, Y.-L. (2013) The New Face of the Old Molecules: Crustin Pm4 and Transglutaminase Type I Serving as RNPs Down-Regulate Astakine-Mediated Hematopoiesis. *PLoS ONE*. 10.1371/journal.pone.0072793

28. Chen, Y., Chen, W.-C., and Cheng, W. (2014) The second type of transglutaminase regulates immune and stress responses in white shrimp, Litopenaeus vannamei. *Fish Shellfish Immunol.* **37**, 30–37

29. Gibrat, C., and Cicchetti, F. (2011) Potential of cystamine and cysteamine in the treatment of neurodegenerative diseases. *Prog. Neuropsychopharmacol. Biol. Psychiatry*. **35**, 380–389

30. Bousquet, M., Gibrat, C., Ouellet, M., Rouillard, C., Calon, F., and Cicchetti, F. (2010) Cystamine metabolism and brain transport properties: clinical implications for neurodegenerative diseases. *J. Neurochem.* **114**, 1651–1658

31. Borrell-Pagès, M., Canals, J. M., Cordelières, F. P., Parker, J. A., Pineda, J. R., Grange, G., Bryson, E. A., Guillermier, M., Hirsch, E., Hantraye, P., Cheetham, M. E., Néri, C., Alberch, J., Brouillet, E., Saudou, F., and Humbert, S. (2006) Cystamine and cysteamine increase brain levels of BDNF in Huntington disease via HSJ1b and transglutaminase. *J. Clin. Invest.* **116**, 1410–1424

32. Noonin, C., Lin, X., Jiravanichpaisal, P., Söderhäll, K., and Söderhäll, I. (2012) Invertebrate hematopoiesis: an anterior proliferation center as a link between the hematopoietic tissue and the brain. *Stem Cells Dev.* **21**, 3173–3186

33. Al-Jallad, H. F., Nakano, Y., Chen, J. L. Y., McMillan, E., Lefebvre, C., and Kaartinen, M. T. (2006) Transglutaminase activity regulates osteoblast differentiation and matrix mineralization in MC3T3-E1 osteoblast cultures. *Matrix Biol. J. Int. Soc. Matrix Biol.* **25**, 135–148

34. Min, B., and Chung, K. C. (2018) New insight into transglutaminase 2 and link to neurodegenerative diseases. *BMB Rep.* **51**, 5–13

35. Cisbani, G., Drouin-Ouellet, J., Gibrat, C., Saint-Pierre, M., Lagacé, M., Badrinarayanan, S., Lavallée-Bourget, M. H., Charest, J., Chabrat, A., Boivin, L., Lebel, M., Bousquet, M., Lévesque, M., and Cicchetti, F. (2015) Cystamine/cysteamine rescues the dopaminergic system and shows neurorestorative properties in an animal model of Parkinson's disease. *Neurobiol. Dis.* **82**, 430–444

36. Gibrat, C., Bousquet, M., Saint-Pierre, M., Lévesque, D., Calon, F., Rouillard, C., and Cicchetti, F. (2010) Cystamine prevents MPTP-induced toxicity in young adult mice via the up-regulation of the brain-derived neurotrophic factor. *Prog. Neuropsychopharmacol. Biol. Psychiatry*. **34**, 193–203

37. Haroutunian, V., Mantin, R., Campbell, G. A., Tsuboyama, G. K., and Davis, K. L. (1987) Cysteamine-induced depletion of central somatostatin-like immunoactivity: effects on behavior, learning, memory and brain neurochemistry. *Brain Res*. **403**, 234–242

38. Slaughter, T. F., Achyuthan, K. E., Lai, T.-S., and Greenberg, C. S. (1992) A microtiter plate transglutaminase assay utilizing 5-(biotinamido) pentylamine as substrate. *Anal. Biochem.* **205**, 166–171

Figure Legends

Figure 1. Inhibition by cystamine on TGase activity *in vitro*.

A) Inhibition of commercial TGase from guinea pig liver by different concentrations of cystamine. B) Inhibition of TGase activity from HPT lysate. Each symbol represents individual crayfish. The columns represent the mean from four to six crayfish and the error bar represents the SD value. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ indicate a significant difference compared to the control.

Figure 2. Effect of cystamine on *in vivo* TGase activity in HPT and ROS production *in vivo*.

A) TGase activity in HPT at 3 h after injection of cystamine (75 μg cystamine/g, 150 μg cystamine/g) or CPBS as a control. The columns represent the mean from four to five crayfish and the error bar represents the SD value. Each symbol represents individual crayfish. ** $P < 0.01$ indicates a significant difference compared to the control. B) ROS production in HPT at 1h and 3h post-injection of cystamine (75 μg cystamine/g, 150 μg cystamine/g) or CPBS as a control. The ROS level was calculated per microgram of tissue protein of each individual. Six crayfish were used in each experimental group. The middle lines represent the mean from six crayfish and the error bar represents the SD value. Each symbol represents individual crayfish.

Figure 3. *In vivo* effect of cystamine injection on the circulating hemocyte number.

A) Relative granular cell count (GC). B) Relative semi-granular cell count (SGC), and C) Relative total hemocyte count (THC) at 1h, 3h, 6h, 24h, and 48h post-injection of cystamine (75 μg cystamine/g (■), 150 μg cystamine/g (▲)) or CPBS (●) as a control. Four to twelve crayfish were used in each experimental

group, the data points represent the mean from four to twelve crayfish and the error bar represents the SD value. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ indicate a significant difference compared to the control.

Figure 4. BrdU positive cells in the circulation 3 hours after injection of cystamine.
A) BrdU positive cells (green) in CPBS injected group, B) BrdU positive cells (green) in the 75 μg cystamine/g injected group, C) BrdU positive cells (green) in the 150 μg cystamine/g injected group. D) Percentage of BrdU positive cells relative to total number of hemocytes after cystamine injection at 3h. Seven crayfish were used in each experimental group. Each symbol represents individual crayfish. The middle lines represent the mean from seven crayfish and the error bar represents the SD value. *** $P < 0.001$ indicate a significant difference compared to the control.

Figure 5. Characteristics of crayfish behavior after cystamine injection. Three hours after CPBS-injection; a front and a left side views show a resting position of normal behavior or an aggressive behavior. 3 hours after 150 μg cystamine/g injection; a front and a left side views show a non-movement behavior.

Figure 6. The effect of cystamine on crayfish behavior. After injection, behavior of the individual crayfish in each group was recorded as videos for 3 min at 3h, 6h, 24h, 48h, and 96h. The pictures show in still frames from a video used to determine the different activities of crayfish after cystamine injection. The activities of crayfish in each group were compared with CPBS injected crayfish as a control. The crayfish behavior was analyzed based on their mobility, immobility, aggressiveness, and activities. 20 crayfish were used in these experiments.
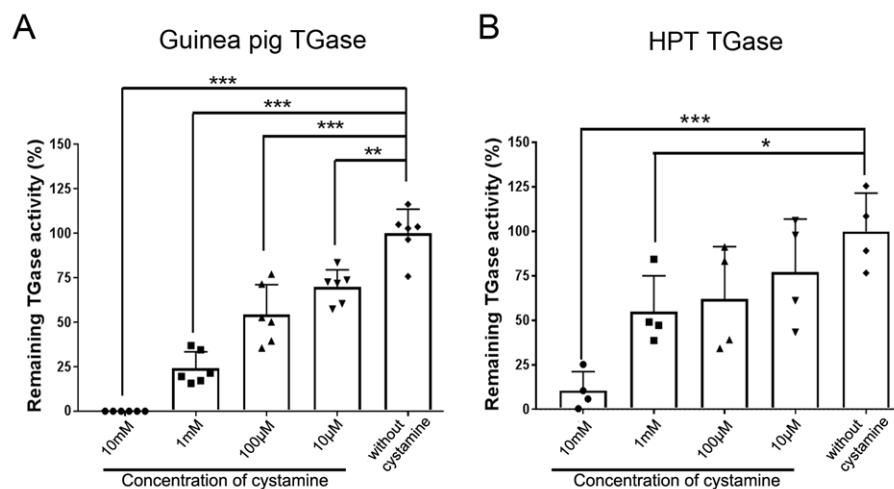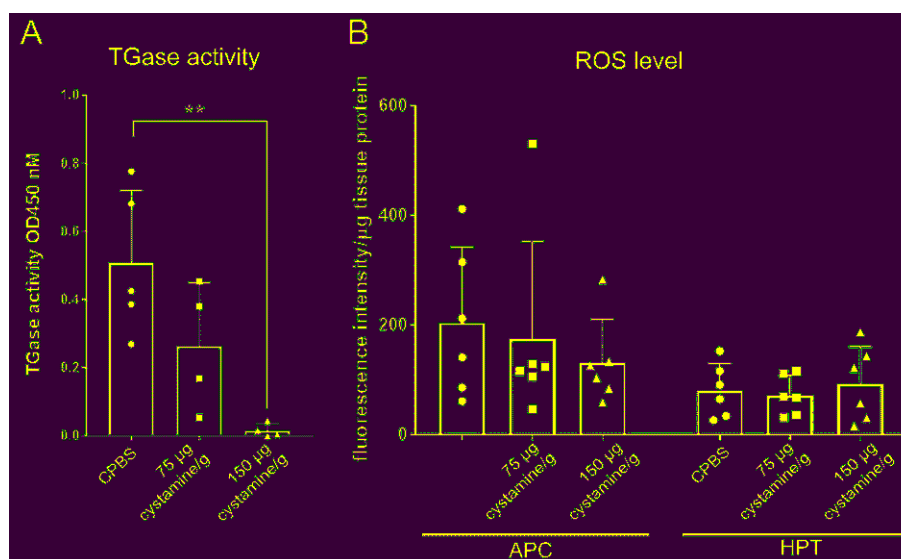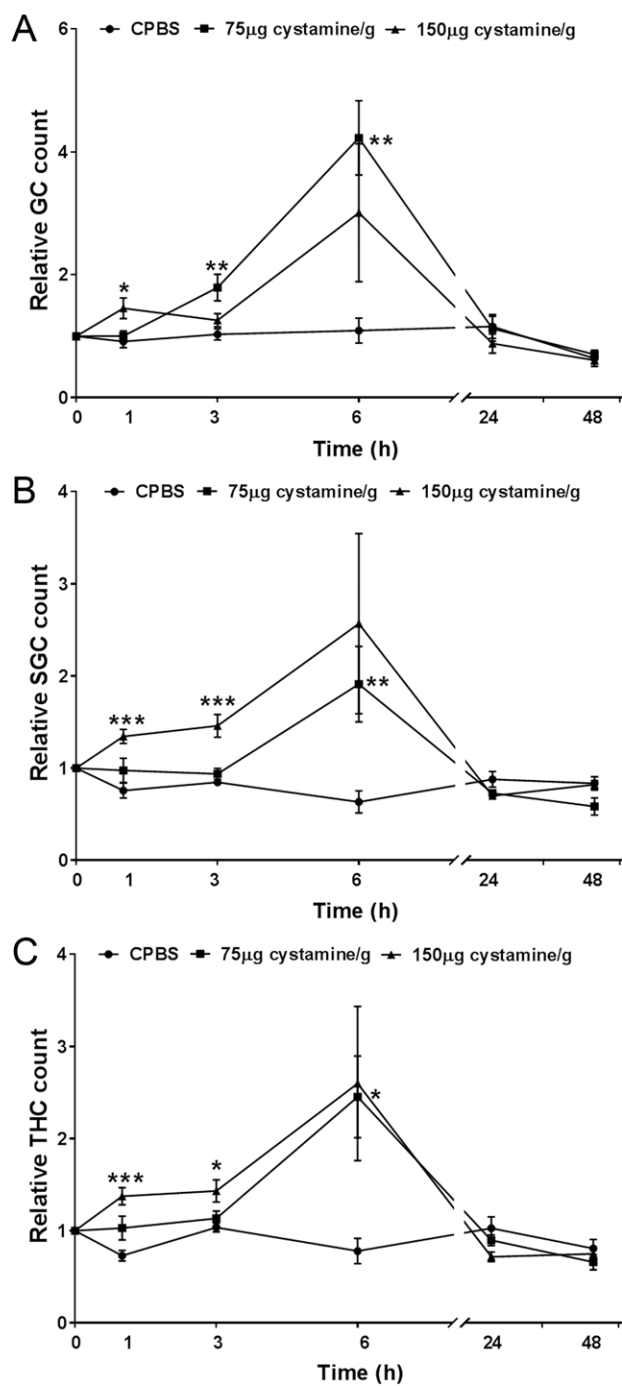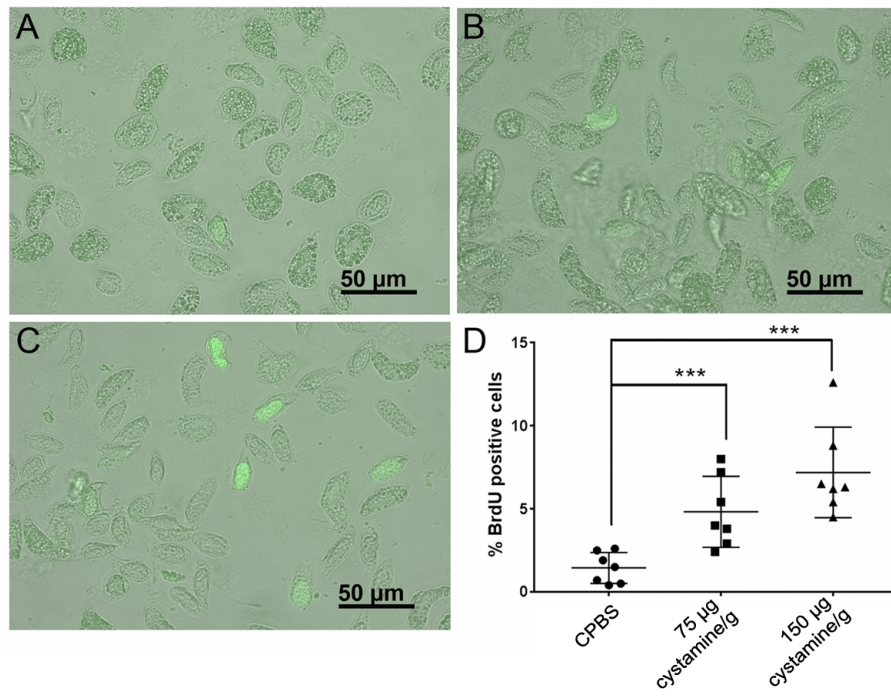
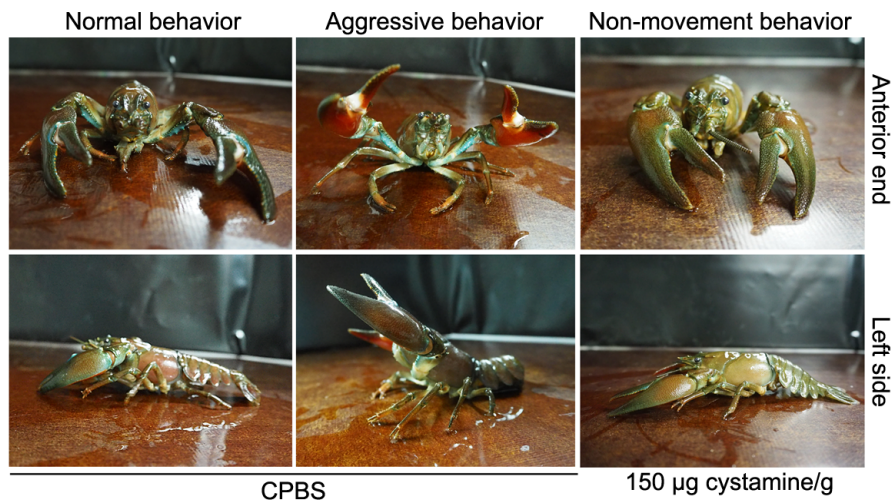Figure. 1

Figure. 2

Figure. 3
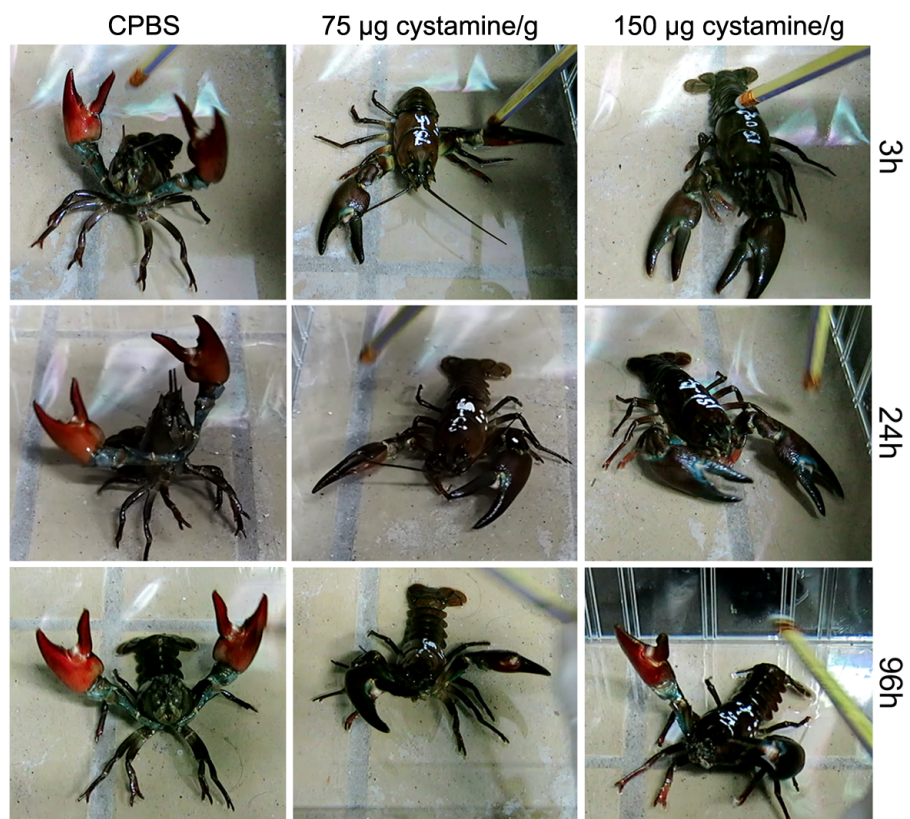
Figure. 4

Figure. 5

Figure. 6

License to Publish

The Journal of Biological Chemistry

This Agreement (the "Agreement") is made and entered into effective as of <u>November 25</u>, 2018 (the "Effective Date"), between the American Society for Biochemistry and Molecular Biology, Inc. (ASBMB), on the one hand, and <u>Kingkamon Junkunlo, Kenr</u>, and <u>Irene Söderhäll</u> "the Authors" and on the other hand. ASBMB and the Authors are referred to collectively as the "Parties."

Recitals
A. The Authors are the authors of an article entitled <u>Transglutaminase inhibition stimulates hematopoiesis and reduces aggressive beh</u> ("the Article").
B. The Authors wish for the ASBMB to consider publication of the Article and the ASBMB desires the same.

Agreement
NOW, THEREFORE, in consideration of the promises and mutual agreements contained herein, and for other good and valuable consideration, the receipt and sufficiency of which is hereby acknowledged, the Parties agree as follows:

1. In consideration of publication in *Journal of Biological Chemistry* of the work currently titled <u>Transglutaminase inhibition stimulates hematopoiesis and reduces aggressive beh</u> <u>Transglutaminase inhibition stimulates hematopoiesis and reduces aggress</u> and all associated supplemental materials, data and/or video files (the "Work") and authored by <u>Kingkamon Junkunlo, Kenneth Söderhäll and Irene Söderhäll</u> ("Authors"), the sole and exclusive, irrevocable right is hereby granted to ASBMB to publish, reproduce, distribute, transmit, display, store, translate, create derivative works from and otherwise use the Work in any form, manner, format, or medium, whether now known or hereafter developed, throughout the world and in any language, for the entire duration of any such right and any renewal or extension thereof and to permit/sublicense others to do any or all of the foregoing as well.

2. Ownership of the copyright in the Work shall remain with the Authors, provided that the Authors credit first publication of the Work in the *Journal of Biological Chemistry* when reproducing the Work or extracts from it. The Authors retain the following nonexclusive rights:
   a. The Authors reserve the right after the publication of the Work by the *Journal of Biological Chemistry,* to use all or part of the Work in compilations or other publications of the Authors' own commercial and noncommercial works (includes theses/dissertations), to use figures, photos, and tables created by them and contained in the Work, to present the work orally in its entirety, and to make copies of all or part of the Work for the Authors' use for lectures, classroom instruction or similar uses. If the author is employed by an academic institution, that institution also may reproduce the article for teaching purposes.
   b. The Authors reserve the right, after publication in the *Journal of Biological Chemistry*, to post the accepted manuscript version of the Work, the "Paper in Press," on the author's personal web page, their personal or institutional repository, or their funding body's archive or designated noncommercial repository, provided that a link to the article in the *Journal of Biological Chemistry* is included.
   c. The Authors reserve the right to post a manuscript version of the Work on not-for-profit preprint servers provided that the Authors retain distribution rights to the Work, that ASBMB formatted final files are not posted, and that a link to the article in the *Journal of Biological Chemistry* is included.
   d. The Authors reserve the right twelve months after publication of the Work in the *Journal of Biological Chemistry* to post the final edited PDFs, created by ASBMB, to their own departmental/university websites, provided that a link to the article in the *Journal of Biological Chemistry* is included.

   Reuse of JBC content must include the following: This research was originally published in the Journal of Biological Chemistry. Author(s). Title. J. Biol. Chem. Year; Vol:pp-pp. © the Author(s).

3. **Inurement:** The Parties agree that this Agreement shall inure to the benefit of, and be binding upon, each of the Parties and their respective affiliates, predecessors, successors and assigns, and the agents and other authorized representatives, shareholders, officers, directors, employees, insurers, heirs, executors, trustees, partners, and joint venturers of any of the foregoing. As used herein, "affiliates" means any person or entity, who/which directly, or indirectly through one or more intermediaries, controls, is controlled by, or is under the common control with, either of the Parties hereto.

4. **Governing Law and Venue; Future Disputes:** This Agreement shall be deemed executed and delivered in the State of Maryland, and, unless and except to the extent governed by the Federal laws of the United States, shall be construed and governed solely by the internal laws of the State of Maryland, without regard to Maryland's conflict of laws provisions. The Parties submit to the personal jurisdiction and venue of the Federal and State courts of Montgomery County, Maryland, for resolution of any dispute arising out of or related to this Agreement.

5. **Arbitration:** The Parties further agree that any controversy or claim arising out of or relating to this Agreement, or the breach thereof, shall be settled by arbitration administered by the American Arbitration Association under its Commercial Arbitration Rules. Judgment on the award rendered by the arbitrator(s) may be entered in any court having jurisdiction thereof.

6. **Severability:** It is the belief of the Parties that this Agreement does not contain any provision contrary to law. However, if any part of this Agreement shall be determined to be illegal, invalid, or unenforceable: (a) that part shall nevertheless be enforced to the extent permissible in order to effect the intent of the Parties; and (b) the remaining parts shall be deemed valid and enforceable, so long as the remaining parts continue to fulfill the original intent of the Parties.

7. **No Waiver:** No delay or failure by either Party to exercise its rights under this Agreement shall be construed to be a waiver thereof, unless memorialized by written instrument signed by both Parties. The agreed waiver of any covenant, condition, or agreement to be performed hereunder shall not be construed to be a continuing waiver of the same covenant, condition or agreement or the waiver of a different covenant, condition or agreement. Furthermore, the agreed waiver of any breach of this Agreement shall not be considered to be the agreed waiver of a different or subsequent such breach.

8. **Integration:** This Agreement, including the Judgments/Injunctions incorporated herein, constitutes the entire agreement between the Parties, and supersedes any and all prior or contemporaneous agreements, promises, representations, or understandings, written or oral, between them relating to the subject matter of this Agreement. No other agreements, promises, representations, or understandings shall be binding upon the Parties with respect to this subject matter unless contained in this Agreement, or separately agreed to in writing and signed by an authorized representative of each of the Parties.

9. **Amendment:** No amendment, modification, or addition to this Agreement shall be valid unless it is in a writing executed by the Parties.

10. **Representations:** The Parties each represent that: (a) this Agreement is freely and voluntarily entered into, and that each of the Parties has had an opportunity to consult with counsel with respect to the advisability of entering into this Agreement; (b) no promise, inducement, or agreement not contained in this Agreement has been made on any subject in connection with this Agreement; (c) each Party has made such investigation of the facts pertaining to this Agreement and of all the matters pertaining thereto as it deems necessary; (d) each Party's signatory to this Agreement is fully authorized to execute this Agreement on its behalf; and (e) the Parties jointly participated in the drafting of this Agreement, with the result that any ambiguity contained therein shall not be interpreted or construed against either Party as the drafter thereof. The Authors separately represent that they are the original authors of the Article and that the Article does not infringe the intellectual property (including but not limited to copyrights, trademarks, and/or trade secrets) of any other party.

11. **Notices and Service of Process:** Any and all notices, demands or requests required or permitted to be given under this Agreement shall be given in writing and sent by registered or certified mail, return receipt requested, or by hand or overnight delivery, with a copy sent via email, to the addresses below. The Parties agree that service of process and service of a summons related to any disputes between the Parties can be served via these notice provisions.

If to the ASBMB:
11200 Rockville Pike, Suite 302, Rockville, MD 20852
jbc@asbmb.org

If to: Irene Söderhäll   :
        Dep. Comparative Pysiology, Uppsala University, Norbyv. 18A SE 75236 Uppsala, Sweden Irene.Sode •

If to: _____ :
        _____ :

12. **Counterparts:** This Agreement may be executed in counterparts, each of which shall be deemed to be an original, but all of which, taken together, shall constitute one and the same Agreement. For purposes of execution, facsimile signatures and signature pages sent through electronic mail shall be considered effective and binding.

U.S. GOVERNMENT EMPLOYEES: This work was done in my capacity as a U.S. government employee; the above assignment applies only to the extent allowable by law.

[IS] Type your initials here to confirm that all Authors know of and concur with the signing of this license and have granted the corresponding author the authority to sign this license on their behalf

Corresponding author on behalf of all authors: IS _____

Corresponding author name: Irene Söderhäll _____

Corresponding author signature: _____

Date: November 25 2018 _____