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A transcription factor glial cell missing (Gcm) in the freshwater crayfish *Pacifastacus leniusculus*☆

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ABSTRACT

The transcription factor glial cell missing, Gcm, is known to be an important protein in the determination of glial cell fate as well as embryonic plasmatocyte differentiation in *Drosophila melanogaster*. So far, no function for Gcm in crustaceans has been reported. In this study, we show the cDNA sequence of a Gcm homologue in the freshwater crayfish *Pacifastacus leniusculus*. The *P. leniusculus* Gcm transcript is expressed exclusively in brain and nervous tissue, and by *in situ* hybridization we show that the expression is restricted to a small number of large cells with morphology similar to neurosecretory cells. Furthermore, we show that the expression of Gcm coincides with the expression of a Repo homologue, that is induced in expression by Gcm in *Drosophila*. Moreover, the Gcm transcript is increased shortly and transiently after injection of cystamine, a substance that inhibits transglutaminase and also strongly affects the movement behavior of crayfish. This finding of Gcm transcripts in a subpopulation of brain cells in very low numbers may enable more detailed studies about Gcm in adult crustaceans.

1. Introduction

In *Drosophila* development, cells of the central nervous system originating from neuroblast may develop into neurons or glial cells (Hosoya et al., 1995), and their fate is determined in part by the expression of the gene *glial cell missing* (*Gcm*) (Akiyama et al., 1996; Bernardoni et al., 1997; Hosoya et al., 1995; Jones et al., 1995). The product of the *Gcm* gene, Gcm, is a transcription factor, which has DNA-binding activity in its N-terminal, and binds with high affinity to the GCM-motif in DNA, 5′-(A/G)CCCGCAT-3′ (Akiyama et al., 1996). The gene product Gcm functions as a switch between the formation of glial cells and neurons in *Drosophila* central nervous system (CNS) development (Hosoya et al., 1995; Jones et al., 1995). In addition to this role as a glial/neuron switch, the *Gcm* gene is also expressed in the early embryonic hemocytes (Bernardoni et al., 1997). These cells do first appear at embryonic stage 10, about 2 h after gastrulation and are located in the head mesoderm of the embryo (Tepass et al., 1994). These embryonic hemocytes have their main function as macrophages that phagocytose cells undergoing apoptosis in the embryo, while later in the adult fly glial cells take over this role of phagocytes within the adult nervous system (Bernardoni et al., 1997; Franc et al., 1999, 1996). The importance of *Gcm* expression in embryogenic hematopoiesis in *Drosophila* has been confirmed in several later studies (Evans et al., 2003; Lebestky et al., 2000; Meister and Lagueux, 2003), and *Gcm* is shown to specify the plasmatocyte lineage (Bataillé et al., 2005; Lebestky et al., 2000). If *Gcm* expression is downregulated, the hemocyte precursors instead develop into crystal cells rather than plasmatocytes, and this process is directed by the RUNX transcription factor Lozenge. (Bataillé et al., 2005; Lebestky et al., 2000). Later, during larval development, hemocytes are produced in a dorsally located organ named as the lymph gland (Lanot et al., 2001; Rugendorff et al., 1994; Trébuchet et al., 2019), in which plasmatocytes, crystal cells as well as lamellocytes are formed, (for review see Banerjee et al., 2019). Remaining plasmatocytes produced during the embryonic stages are circulating in the hemolymph in the larvae, and are also embedded in the so-called sessile pools in the body wall epithelium (Lanot et al., 2001; Shrestha and Gateff, 1982). These sessile plasmatocytes may be transdifferentiated into crystal cells expressing prophenoloxidase (proPO) and *lozenge* by activity of the Notch signaling pathway (Leitão and Sucena, 2015). In the lymph gland new plasmatocytes are produced, which are released upon pupation, but in contrast

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to the embryonic cells, these cells do not express the *Gcm* gene, while the GATA factor *serpent* (*srp*) is essential for all hemocyte lineages (Trébuchet et al., 2019).

We have found that the *Gcm* gene is present in several crustacean genomes, but so far, no study about its function has been published. We have earlier identified a *lozenge*-like RUNX family of transcription factor that is of importance for the differentiation of semigranular and granular hemocytes in the freshwater crayfish *Pacifastacus leniusculus* (Söderhäll et al., 2003). These cells express proPO, and as in *Drosophila* the RUNX transcription factor is essential for differentiation of proPO-expressing cells. However, in a transcriptome analysis of the hematopoietic tissue (HPT) as well as of hemocytes, we could not find any expression of a *Gcm*-like gene (Söderhäll, 2016). In the present report, we have now detected a *Gcm* sequence in *P. leniusculus* and report its characterization and localization and discuss some of its possible functions.

2. Materials and methods

2.1. Experimental animals

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

2.2. Cloning of P. leniusculus glial cell missing

The partial sequences of *P. leniusculus* Gcm transcripts were obtained from Sequence Read Archive (SRA) accession: SRX768725 database. Based on these sequences, gene-specific primers with the following specific primer pairs; Gcm_GSP1_1: GATTACGCCAAGCTTCC TCTTGGCCTCCTCCAGCTCCGACGGGTA, Gcm_NGSP1_1: GATTACGC-CAAGCTTGTGACCATCCGCCCAGTCGC, Gcm_GSP2_1: GATTACGC-CAAGCTTCGATTTGCGACAAGGCCCGCAAGAAGCAGCAAGGCA, Gcm_NGSP2_1: GATTACGCCAAGCTTTCAGGGAAGCTTGAGGTCC-TACC, Gcm_GSP2_2: CAAGTCGTGTTCAGGGAAGCTTGAGGTCC-TACCCTGCCG, and Gcm_NGSP2_2: CTACCCCGTCACCCACTTCGG were designed for Gcm full-length cDNA amplification by rapid amplification of 3′ and 5′ cDNA ends (RACE). Total RNA (1 μg) from a crayfish brain was extracted using TRIzol™ LS Reagent (Thermo Fisher Scientific, USA) according to manufacturer's protocol and processed to eliminate contaminated DNA by DNase I (RNase-Free) (New England Biolabs, USA). Further the 3′ and 5′ cDNA first strand cDNA templates were synthesized and RACE-PCR was amplified using the SMARTer RACE 5'/3′ Kit (Takara, Japan) according to the manufacturer's instruction. The PCR products were purified by using GenElute™ PCR Clean-Up Kit (Sigma-Aldrich, USA) and cloned into a TOPO vector (Invitrogen, USA), and the sequences were analyzed at SciLifeLab NGI National Facility (Stockholm). The sequencing results were further characterized as described in 2.3.

2.3. Gcm sequence analysis

The obtained Gcm nucleotide sequence and predicted Gcm protein sequence were analyzed for similarity using the BLAST software at the NCBI website (http://www.ncbi.nlm.nih.gov/blast). Translation of the cDNA was performed using the SIB Bioinformatics Resource Portal (https://web.expasy.org/translate/). The ORF region of Gcm was analyzed using ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/). Prediction of a putative signal peptide was performed using the SignalP 5.0 Server software (http://www.cbs.dtu.dk/services/SignalP-5.0/), and protein domains were predicted with InterProScan software (http://www.ebi.ac.uk/interpro/) and PROSITE software (https://prosite.expasy.org/). The nuclear targeting signal (NLS) was predicted by cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi). Further, a PEST domain was identified by EMBOSS program ePEST find tool (https://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind). Multiple-sequence alignment of *P. leniusculus* Gcm with other Gcm sequences from different species was performed by using the ClustalW multiple alignment software (http://www.genome.jp/tools/clustalw/), and a phylogenetic tree was constructed using PhyML 3.0 program (http://phylogeny.lirmm.fr/phylo_cgi/one_task.cgi?task_type=phyml), based on multiple-alignment of the Gcm protein sequence from, *D. melanogaster* (AAC47808.1 and AAF74349.1), *Bombyx mori* (XP_004929375.2 *Leptinotarsa desemlineata* (XP_023017815.1), *Tribolium castaneum* (EFA04430.1), *Limulus polyphemus* (XP_022248487.1), *Litopenaeus vannamei* (XP_027216612.1), *Danio rerio* (BAD72824.1), *Gallus gallus* (NP_996863.1 and NP_001008480.1), *Rattus norwegicus* (NP_058882.1 and NP_001099575.1), and *H. sapiens* (NP_003634.2 and NP_004743.1).

2.4. Total RNA preparation and quantitative PCR

The expression levels of Gcm and Repo (Reversed polarity) homologue (MT407371) in the following tissues; HPT = Hematopoietic tissue, APC = Anterior Proliferation Center, HC = Hemocytes, B = Brain, Th-Nerve = Thoracic nerve, Ab-nerve = Abdominal nerve, muscle, heart, foregut, midgut, hindgut, gill, hepatopancreas, and green gland, were analyzed and each tissue was isolated from 3 to 4 individual crayfish and analyzed individually. To separate brain from the thin sheath that surrounds the brain (brain sheath), the brain was removed from 8 to 11 individual crayfish and put into crayfish phosphate-buffered saline (CPBS; 10 mM Na_2HPO_4, 10 mM KH_2PO_4, 150 mM NaCl, 10 mM $CaCl_2$ and 10 mM $MnCl_2$, pH 6.8), and the brain sheaths were dissected by using two pairs of very fine forceps, under a dissecting microscope. Total RNA was extracted by using Trizol LS reagent (Invitrogen, USA) according to manufacturer's protocol. To eliminate contamination of DNA, DNase I (RNase-Free) (New England Biolabs, USA) treatment was performed. The cDNA was synthesized using SuperScript™ IV First-Strand Synthesis System (Thermo Scientific, USA) for First-Strand cDNA Synthesis kits. The Gcm mRNA transcript levels were determined by RT-qPCR using QuantiTect SYBR green PCR kit (Qiagen, USA) and RT-PCR using Phusion High-Fidelity DNA Polymerase (Thermo Scientific, USA). The qPCR program used was 95 °C, 15 min, followed by 45 cycles of 94 °C for 20 s, 58 °C for 20 s, and 72 °C for 30 s. The RT-PCR program used was 98 °C, 30 s, followed by 35 cycles for Gcm, 25 and 30 cycles for Repo of 98 °C for 20 s, 58 °C for 20 s, and 72 °C for 30 s, and 30 cycles for 18S of 98 °C for 20 s, 58 °C for 20 s, and 72 °C for 30 s. The PCR products were analyzed on a 1.5% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen, USA). The transcription of a 18S ribosomal protein was used as an internal control (AF235961.1).

2.5. In situ hybridization of Gcm in isolated brain cells

Sense and anti-sense strand probes were generated following the manufacturer's instructions of the DIG RNA Labeling Kit (SP6/T7) (Roche, Switzerland). A cDNA template of Gcm was amplified with the following specific primer pairs; ProbeT7_GCM1_FW 5′ TAATACGACTCACTATAGGGTCACACCACTTTGACT GGGATATCAACGAT 3′, ProbeSP6_GCM4_RV ATTTAGGTGACACTATA-GAAGCCTGGAAGAATATAGCGTGATCGGT, GCM1_FW 5′ TCA-CACCACTTTGACTGGGATATCAACGAT 3′ and GCM4_RV 5′ GCCTGGAAGAATATAGCGTGATCGGT 3'. The PCR products were then purified by using the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich, USA) and were used as templates for DIG-labeled RNA synthesis. The labeling efficiency of the probes was verified before use according to the instructions of the DIG RNA Labeling Kit (SP6/T7) (Roche, Switzerland). Brains were dissected from crayfish and each brain was immediately incubated for 20 min in 800 μL of 0.1% collagenase type I and type IV (Sigma-Aldrich, USA) in CPBS at room temperature. After centrifugation at 800×*g* for 5 min, the cell pellet was washed two times with 1 mL of CPBS. The pellet of dissociated cells was then suspended in 200 μL of CPBS and seeded on SuperFrost Plus Microscope slides (Thermo

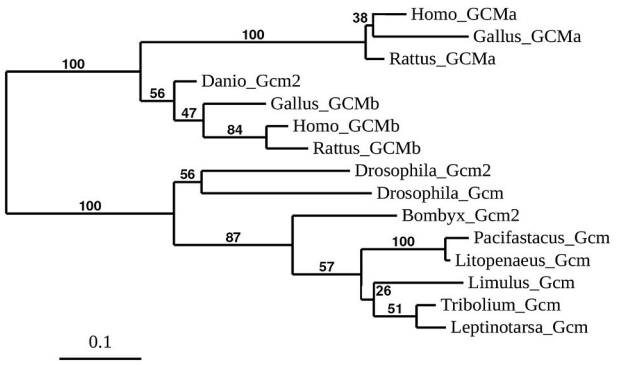

Fig. 1. Phylogenetic tree for *Pacifastacus leniusculus* Gcm. Pacifastacus_Gcm (MT407370), Tribolium_Gcm; (*Tribolium castaneum*; EFA04430.1), Leptinotarsa_Gcm (*Leptinotarsa decemlineata;* XP_023017815.1), Litopeneaus_Gcm (*Penaeus vannamei;* XP_027216612.1), Drosophila _Gcm (*Drosophila melanogaster*; AAC47808.1), Drosophila _Gcm2 (*D. melanogaster*; AAF74349.1), Homo_GCMa (*Homo sapiens;* NP_003634.2), Homo_GCMb (*Homo sapiens*; NP_004743.1), Limulus_Gcm (*Limulus polyphemus;* XP_022248487.1), Danio_Gcm2 (*Danio rerio;* BAD72824.1), Bombyx_Gcm2 (*B. mori*; XP_004929375.2), Rattus_GCMa (*Rattus norvegicus*; NP_058882.1), Rattus_GCMb (*Rattus norvegicus*; NP_001099575.1), Gallus_GCMa (*Gallus gallus*; NP_996863.1), Gallus_GCMb (*Gallus gallus*; NP_001008480.1). The phylogram was constructed by neighbor-joining method using PhyML 3.0. The numbers at each node represent bootstrap values in percentage. The bar represents 10% amino acid distance.

Scientific, USA). After fixation in 4% paraformaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.4) for 30 min at room temperature, the slides were washed three times with PBS-DEPC (PBS buffer treated with diethyl pyrocarbonate (DEPC) (Sigma-Aldrich, USA)). The cells were then pre-hybridized with Hybridization Mix (−) buffer, named HM (−) containing 50% Deionized formamide, 5X SSC, 0.1% Tween, pH 6.0 with citric acid at 60 °C for 1 h, and then incubated with Hybridization Mix (+) named HM (+) containing 50% Deionized formamide, 5X SSC, 0.1% Tween, 50 μg/mL heparin, 500 μg/mL RNase-free tRNA, (pH 6.0 adjusted with citric acid), with the addition of 100 or 150 ng of specific RNA probes at 60 or 65 °C for 16 h. Post-hybridization washes were performed two times each at 65 °C for 5 min using a serial dilution of HM (−) in 2X SSC (75% HM (−), 50% HM (−), 25% HM (−) and 100% 2X SSC), and then the cells were washed two times with 0.2X SSC at 65 °C for 30 min. The slides were then washed with a serial dilution of 0.2X SSC in PBST (0.1% Tween 20 in PBS buffer) and 100% PBST at room temperature for 5 min two times each before being incubated with blocking buffer (1x PBST, 2% horse serum, 2 mg/mL BSA) at room temperature for 1 h and incubated with blocking buffer containing 1:1000 of sheep anti digoxigenin-AP IgG (Roche, Switzerland) at 4 °C overnight. After being washed six times each, at room temperature for 15 min, the slides were equilibrated with staining buffer (100 mM Tris, pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl and 0.1 Tween 20), three times each at room temperature for 5 min. The dark purple color was developed in the dark in staining buffer containing the NBT/BCIP substrate (Roche, Switzerland). The slides were washed with stop solution (1X PBS, 1 mM EDTA and 0.1% Tween 20) three times for 5 min each and three times with PBS for 5 min each, before they were mounted with 50% glycerol in PBS.

2.6. Cystamine injection experiment

Our previous results (Junkunlo et al., 2019), showed that cystamine injection into crayfish has a stimulatory effect on hematopoiesis and reduces movement behavior of crayfish. To investigate the function of Gcm in crayfish, cystamine was injected. The crayfish were separated into two groups of 4–5 individuals each, in the control and test group respectively. The crayfish were allowed to adjust to the new surroundings for 48 h before being injected with 100 μL CPBS as a control or 150 μg cystamine/g crayfish in 100 μL CPBS. At 3 and 6 h post-injection, the whole brains were dissected and further analyzed for Gcm mRNA expression as described in 2.4.

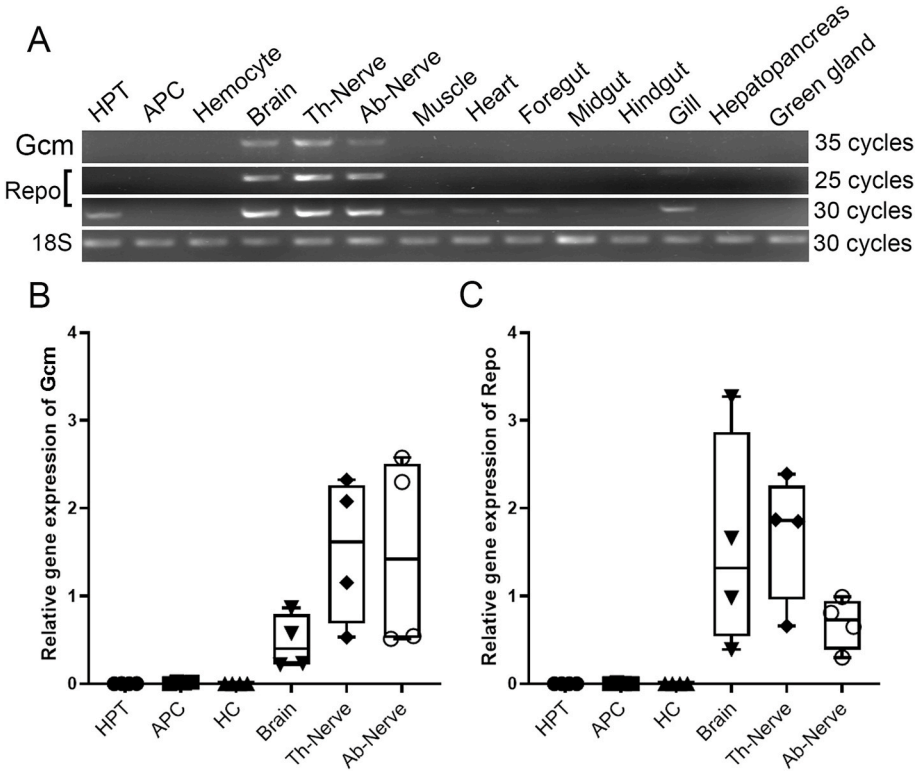

Fig. 2. The mRNA transcripts of Gcm and Repo in various tissues. A) The expression levels of Gcm and Repo by RT-PCR in different tissues. This experiment was repeated 4 times with tissues; HPT = Hematopoietic tissue, APC = Anterior Proliferation Center, HC = Hemocytes, B = Brain, Th-Nerve = Thoracic nerve, Ab-Nerve = Abdominal nerve, Muscle, Heart, Foregut, Midgut, Hindgut, Gill, Hepatopancreas and Green gland, extracted from individual animals. Expression of 18S ribosomal gene was used as an internal control. B) The expression levels of Gcm by RT-qPCR in HPT, APC, hemocytes, brain, thoracic and abdominal nerve. C) The expression levels of Repo by RT-qPCR in HPT, APC, hemocytes, brain, thoracic and abdominal nerve. Combined scatter and box plot represent data from 4 individual crayfish, and the line in the box represents median. The lower and upper bars show the minimum and maximum values.

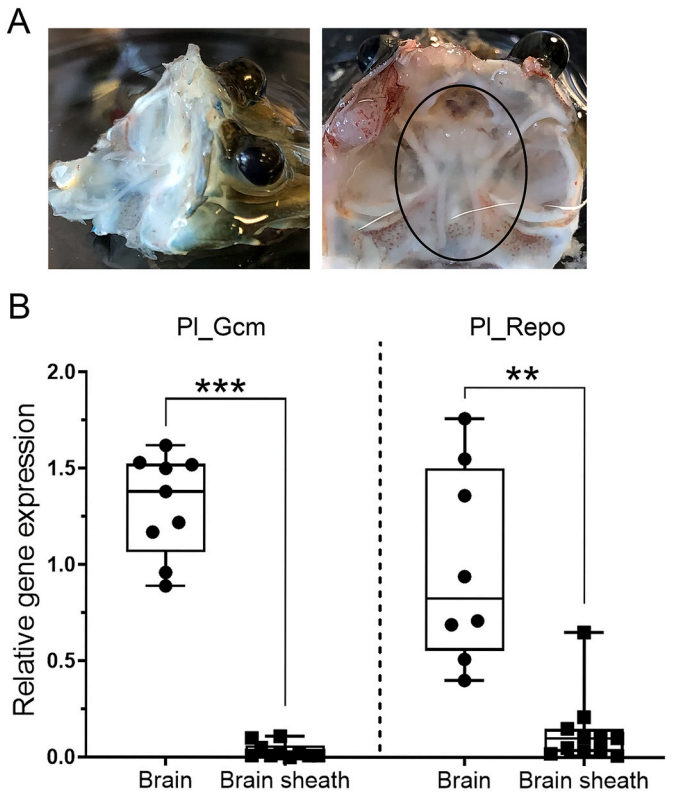

Fig. 3. Relative mRNA expression of Gcm and Repo in brain and brain sheath analyzed by RT-qPCR. A) Localization of the crayfish brain in cephalothorax. The brain is covered with a transparent sheath under carapace and is shown in a circle. B) The expression levels of Gcm and Repo mRNA in brain and brain sheath by RT-qPCR. A combined box and scatter plot represent data from 9 to 11 individual crayfish, and the line across the box represents median. The lower and upper bars represent the minimum and maximum values. $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate a significant difference between samples.

2.7. Statistical analysis

The relative mRNA expression levels were shown as a combined scatter and box plot graphs and 4–11 individual crayfish were used in each experimental group. 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 $P < 0.05$.

3. Results

3.1. Characterization of P. leniusculus Gcm sequence

We used the protein sequence of *D. melanogaster* GCM (AAC47808.1) for search in our transcriptome database from *P. leniusculus* (Sequence Read Archive (SRA) accession: SRX768725) and detected a number of partial cDNA sequences with high similarity to Gcm. These fragmented sequences were used as templates for further searches for a *P. leniusculus* Gcm sequence. We then obtained 813 bp of a Gcm sequence, with a complete Gcm domain, consisting of an open reading frame of 639 bp, with a deduced amino acid sequence of 213 amino acids (Supplementary Fig. S1). The sequence was deposited in GenBank with the accession number MT407370. The deduced protein sequence contains a typical GCM motif (pfam 03615) spanning from the YDVF motif at amino acid 41 to the EARR motif ending at amino acid 180 (Supplementary Fig. S1), and contains seven conserved cysteine residues and four histidine residues (Supplementary Fig. S2). The three stretches of 9–10 amino acids which are highly conserved in *D. melanogaster* and *Homo sapiens* GCM

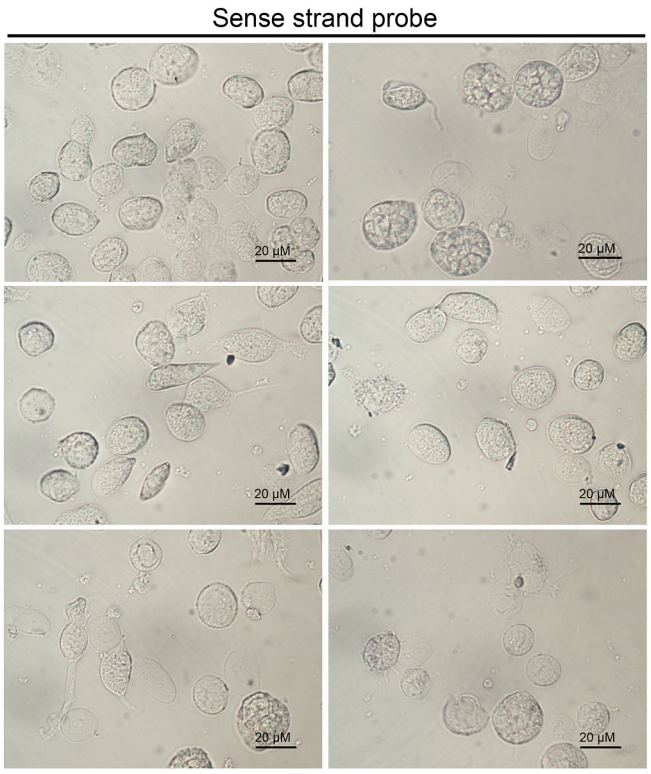

Fig. 4. Different morphology of cells in crayfish brain. Sense strand DIG-labeling RNA probe detected mRNA localization of Gcm in isolated brain cells by *in situ* hybridization, developed in staining buffer containing the NBT/BCIP as substrate.

domain are detected in *P. leniusculus* Gcm (Supplementary Fig. S2). Moreover, the sequence contains a nuclear localization signal (NLS), and a PEST-like sequence within the GCM domain is found in the N-terminal (Supplementary Fig. S1).

Next, we compared the *P. leniusculus* Gcm sequence with similar sequences from a number of arthropods that we could detect in GenBank, and also with a selected number of vertebrate Gcm sequences. Based on a multiple sequence alignment by ClustalW we constructed a phylogenetic tree showing a clear split of vertebrate and arthropod Gcm into two separate groups (Fig. 1). *P. leniusculus* Gcm showed closest similarity with Gcm from *L. vannamei,* and the crayfish sequence was closer related to Gcm sequences from Coleopteran species and *B. mori* compared to the two fly sequences of *D. melanogaster* Gcm and Gcm2 (Fig. 1).

3.2. Localization of P. leniusculus Gcm transcript

In order to find out the function for Gcm in crayfish we first isolated RNA from different tissues from 3 to 8 animals and analyzed mRNA expression of Gcm individually by semi-quantitative RT-PCR. As shown in Fig. 2A, we could only detect Gcm mRNA expression in the brain and the nervous tissues tested. Since we know that Gcm appears to be involved in hematopoiesis, we further analyzed the tissues within the hematopoietic lineage by RT-qPCR. Then, we concluded that no Gcm transcript was detectable in the hematopoietic lineage, neither in the APC or HPT nor in the hemocytes, and therefore Gcm seems to be exclusively expressed in nervous tissues (Fig. 2A and B). This is in contrast to that found in *D. melanogaster* where Gcm is present in the hematopoietic lineage. The brain is covered by thin sheaths and these sheaths may include some hemocytes. Therefore, we further separated the brains from their sheaths and analyzed Gcm transcripts within these tissues and the localization of which is shown in Fig. 3A. As shown in

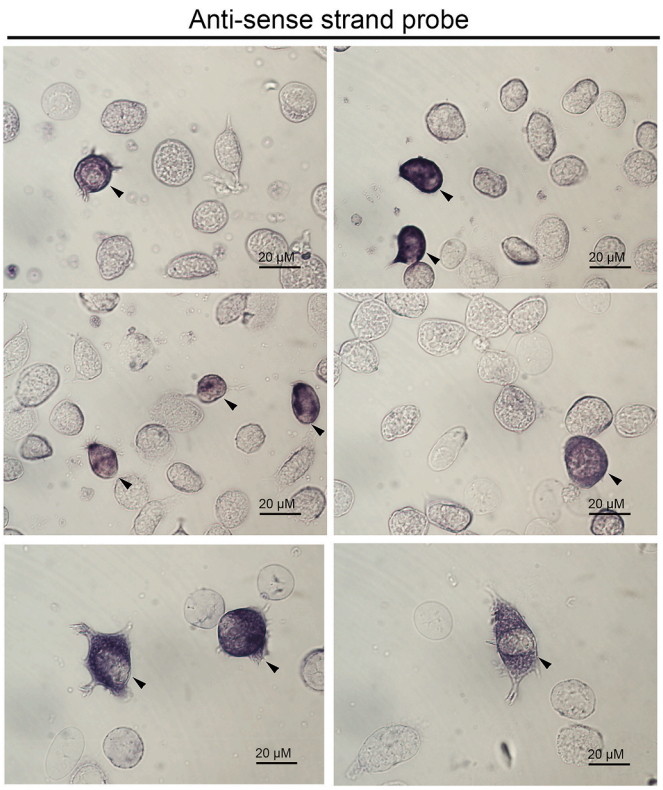

Fig. 5. Specific cell types expressing Gcm in crayfish brain. Anti-sense strand DIG-labeling RNA probe detected mRNA localization of Gcm in isolated brain cells by *in situ* hybridization, developed in a staining buffer containing the NBT/BCIP as substrate.

Fig. 3B no Gcm transcripts were detected in the brain sheaths, and Gcm transcripts were solely found in the real brain tissue (Fig. 3B), again confirming that hemocytes do not contain the Gcm transcript.

Then, we decided to investigate in which area of the brain, and in what cell types Gcm expression was localized. First, we tried to detect Gcm expression by *in situ* hybridization in whole mount brains. However, no cells could be detected with a clear positive signal (data not shown). Therefore, we decided to isolate single cells from the brain and optimized a digestion method for dissociation of brain cells. The dissociation worked nicely with a combination of collagenase I and collagenase IV, and after dissociation into single cells we could perform *in situ* hybridization on slides. We produced 416 bp long DIG-labeled sense and antisense RNA probes covering the cDNA sequence from nucleotide 67 to nucleotide 482 which covers the GCM motif. The sense probe served as a control, and did not give any signal as shown in Fig. 4. However, with the antisense probe we could detect clear Gcm expression in a small subpopulation of the dissociated brain cells (Fig. 5). The proportion of Gcm positive cells in the isolated brain cell population was very low, about 20–40 cells out of 20,000–30,000 cells, i.e. about 0.1%. In Fig. 6, a number of Gcm positive cells are presented, and the figure indicates that the morphology of the cells is similar to one another (Fig. 6). All cells are large and contain what appears to be secretory granules.

3.3. Expression of Repo

In order to compare the role of Gcm in directing glial cell and hemocyte differentiation, we searched the *P. leniusculus* transcriptome for a sequence similar to *Drosophila* Repo, a transcription factor dependent on Gcm expression (Trébuchet et al., 2019). We could detect one full length sequence of a transcript with high similarity to *Drosophila* Repo (Accession number GBYW01023909.1) as shown in Supplementary Fig. S3. Furthermore, the expression of *P. leniusculus* Repo was high in brain and nervous tissue and coincides quite well with how Gcm is expressed in different tissues (Fig. 2B and C). An interesting observation though, is that very low expression could be detected in the HPT (Fig. 2A, 30 PCR cycles) as well as in brain sheaths from some individual animals (Fig. 3B)).

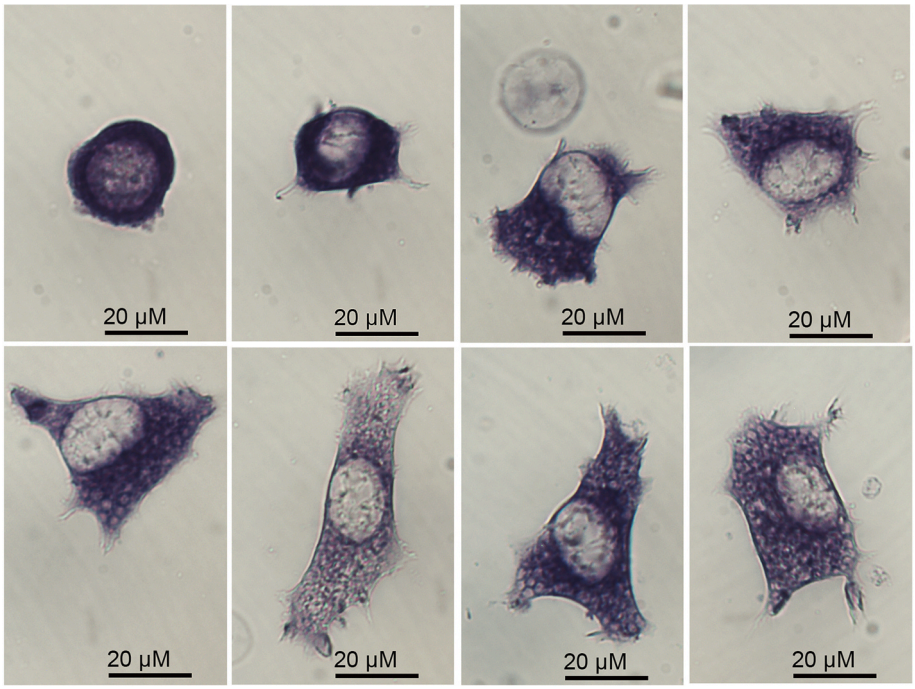

Fig. 6. Different size and morphology of Gcm expressing cells. Anti-sense strand DIG-labeling RNA probe detected mRNA localization of Gcm in isolated brain cells by *in situ* hybridization, developed in a staining buffer containing the NBT/BCIP as substrate.

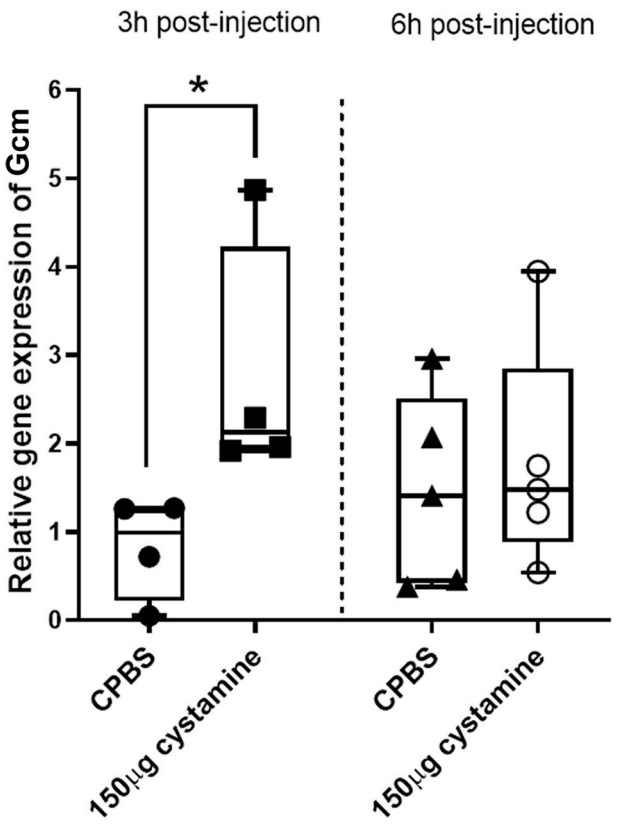

Fig. 7. ***In vivo*** **effect of cystamine injection on the transcription level of Gcm in brain by RT-qPCR**. Cystamine was injected at a concentration of 150 μg of cystamine/g of crayfish in a volume of 100 μL and CPBS in a volume of 100 μL was injected as a control. At 3 and 6 h post-injection, the brains were collected for performing RT-qPCR. A combined box and scatter plot represent data from 4 to 5 individual crayfish, and the line across the box represents median. Each dot represent the expression in one individual animal. The lower and upper bars represent the minimum and maximum values. *$p < 0.05$ indicates a significant different between samples.

3.4. Gcm expression after cystamine treatment

Due to that Gcm is expressed in a minor population of brain cells, we hypothesized that these cells may be involved in brain cell repair or glia cell functions, such as removing infectious agents and/or damaged cell debris. Therefore, we tested whether Gcm expression was affected in the brain after injection of cystamine which we know has a transient inhibitory effect of crayfish moving behavior (Junkunlo et al., 2019). As shown in Fig. 7, the level of Gcm mRNA increased significantly at 3 h after cystamine injection and returned to a normal level already after 6 h.

4. Discussion

Transcriptional regulation of hematopoiesis as well as nervous system development have been studied in depth in the fruit fly *D. melanogaster* (Graveley et al., 2011; Hilu-Dadia and Kurant, 2020; Lanot et al., 2001; Meister and Lagueux, 2003). The knowledge about transcription factors which regulate embryonic nervous system development as well as hematopoiesis is considerable less in crustaceans (Fabritius-Vilpoux et al., 2008), although detailed studies about embryonic development have been conducted in the small amphipod *Parhyale hawaiensis* (Alwes et al., 2011; Extavour, 2005; Gerberding, 2002; Price et al., 2010; Rehm et al., 2009). We have now identified the sequence of a Gcm homologue in the freshwater crayfish *P. leniusculus* and when searching available databases, we did find similar sequences in the genome of the decapod *L. vannamei* (XM_027360811.1), in the amphipod *Hyalella azteca* (XM_018156780.1), in one maxillopodan, *Eurytemora affinis* (XM_023483031.1) and in the branchiopod *Daphnia magna* (XM_032921109.1). This widespread distribution of Gcm indicates that this gene is conserved within several arthropod phyla, although up until now no functional studies of the gene has been reported in any crustacean. The Gcm sequence in *P. leniusculus* showed very high similarity with that in *L. vannamei*, as well as with coleopteran and lepidopteran insects, whereas the *Drosophila* sequence was a bit more distant, even if the conserved domains were similar.

In *Drosophila*, Gcm has been shown to act as a glial cell fate determinant in embryonic development (Cattenoz and Giangrande, 2016), and it is also needed for the formation of embryonic plasmatocytes (Jacques et al., 2009; Trébuchet et al., 2019), whereas during larval hematopoiesis plasmatocytes develop without expressing the Gcm transcription factor (Banerjee et al., 2019; Lanot et al., 2001). In our present study, we used adult *P. leniusculus,* and we could detect Gcm mRNA expression solely in nervous tissue and not in the hematopoietic lineage as is the case in *Drosophila.* To confirm this pattern of tissue expression, we also dissected the brain sheaths where hemocytes may reside, and analyzed the expression of Gcm but the results were clearly negative and this confirms that this Gcm is exclusive for nervous tissue.

We further identified a sequence in the *P. leniusculus* transcriptome database (SRX768725 database) with high similarity to Repo, and therefore we decided to analyze the expression of putative Repo mRNA in different tissues. Repo is a homeodomain transcription factor, and in the fly, Gcm directs the expression of Repo and as a result glial cell fate is induced. In the head mesoderm on the other hand, Repo is repressed by the expression of Twist and miR-1, and as a result hemocyte markers are triggered and embryonic plasmatocytes are developed (Trébuchet et al., 2019). Interestingly, we could not detect any significant Repo expression in the hematopoietic lineage, whereas high expression was found in brain and nervous tissue. Although, this result is still preliminary and does not reveal a clear link between Repo and Gcm, it is an indication that there might be a similar role of Repo in directing cells into glial cells and block hemocyte differentiation as in *Drosophila* (Trébuchet et al., 2019).

Furthermore, in order to find a function for Gcm in crayfish nervous system, we tried to find out what cell type is expressing this transcript. We could reveal that the number of Gcm positive cells was very low, and therefore we failed to find any positive signal when performing *in situ* hybridization on whole mount brain samples. However, after dissociation of whole brains, we could detect cells with high expression of Gcm and these cells were large (>20 μm) and most of them contained numerous granules. The structure of glial cells in crustacean is not very well studied, but in general these glial cells are reported to be much smaller than the Gcm positive cells detected in this study (Allodi et al., 1999; Silva et al., 2001; Zhang et al., 2016). The Gcm positive cells in *P. leniusculus* brain, showed a morphology more similar to neurons, or neurosecretory cells. Large neuronal stem cells with size between 20 and 25 μm have earlier been described in crayfish (Sandeman and Sandeman, 2003). Moreover, in *Drosophila*, studies have shown that Gcm expression occurs in both a few postembryonic neuronal lineages as well as in glial cell lineages (Chotard et al., 2005; Colonques et al., 2007; Soustelle and Giangrande, 2007).

In an earlier study, we have shown that the injection of the transglutaminase inhibitor cystamine into crayfish transiently affected the number of circulating hemocytes as well as the movement behavior in the animals. Cystamine injection resulted in a rapid increase in total hemocyte counts, and the crayfish was more or less paralyzed for about 96 h (Junkunlo et al., 2019). A neuroprotective role of cystamine has been shown in mammals (Cisbani et al., 2015; Li et al., 2015), and also the opposite role for cystamine in blocking transglutaminase dependent phagocytosis of amyloid β by astrocytes in Alzheimer's disease was shown (Kawabe et al., 2018, 2017). Here, we show that an injection of cystamine into crayfish that results in transient movement behavior

changes, also resulted in a rapid increase in brain Gcm mRNA expression. This increase was evident after 3 h but then the expression returned to normal levels, indicating a putative role for Gcm in this temporal behavioral effect such as to function as a scavenger for cells having been negatively affected by the cystamine treatment. The rapid transient increase in expression of Gcm after an injection of cystamine could mean that Gcm in this case in turn induces expression of other genes that are important for restoring homeostasis in the parts of the brain that regulate movement.

In conclusion, we believe that our finding may be of high importance for developmental studies in model crustaceans such as *P. hawaiensis* in which a genome is already available (Kao et al., 2016) and the brain morphology has been deciphered in detail (Wittfoth et al., 2019). In addition, our finding of Gcm transcripts in a small subpopulation of brain cells may lead to more detailed studies about the functional role for Gcm in adult crustaceans.

Author contributions

KJ, KS and IS designed the experiments, interpreted the data and participated in writing the paper. KJ performed the experiments and KJ, KS and IS analyzed the corresponding results.

All authors reviewed the results and approved the final version of the manuscript.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2020.103782.

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