



Genome assembly, gene content, and plastic gene expression responses to salinity changes in the Brackishwater Clam (*Corbicula japonica*) from a dynamic estuarine environment

Hyeonwoo Choi^{a,1}, Yun Keun An^{b,2}, Chan-Jun Lee^{c,3}, Chi-une Song^{a,4}, Eun-Jeong Kim^{a,5}, Carol Eunmi Lee^{d,6}, Sung-Jin Cho^{c,*,7}, Seong-il Eyun^{a,*,8}

^a Department of Life Science, Chung-Ang University, Seoul 06974, Korea

^b Division of Marine Technology, Chonnam National University, Yeosu 59626, Korea

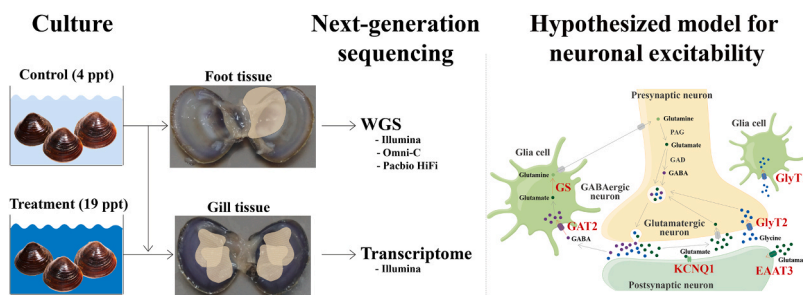
^c Department of Biological Sciences and Biotechnology, Chungbuk National University, Cheongju 28644, Korea

^d Department of Integrative Biology, University of Wisconsin, Madison, WI 53706, USA

HIGHLIGHTS

- The whole genome of *Corbicula japonica* was analyzed for the first time.
- The function of the upregulated genes from 19 ppt, compared to 4 ppt, were linked to signaling and neurotransmission.
- The *C. japonica* genome harbors genes associated with neuronal excitability, either tandemly repeated or positively selected.
- Altogether, this study provides evidence for neuronal plasticity in response to salinity stress.

GRAPHICAL ABSTRACT



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ABSTRACT

Estuaries are dynamic transition zones between marine and freshwater environments, where salinity varies greatly on spatial and temporal scales. The temporal salinity fluctuations of these habitats require organisms to rapidly regulate ionic concentrations and osmotic pressure to survive in these dynamic conditions. Understanding the extent of plasticity of euryhaline animals is vital for predicting their responses and resilience to salinity change. We generated the first high-resolution genome and transcriptome sequences of *C. japonica*. In comparison with 11 other molluscan genomes, the *C. japonica* genome displayed striking expansions of putative

* Corresponding authors.

E-mail addresses: sjchobio@chungbuk.ac.kr (S.-J. Cho), eyun@cau.ac.kr (S.-i. Eyun).

¹ ORCID: 0000-0001-9751-8372.

² ORCID: 0000-0003-2229-0312.

³ ORCID: 0000-0003-1423-1662.

⁴ ORCID: 0000-0001-9479-9818.

⁵ ORCID: 0000-0003-3322-9314.

⁶ ORCID: 0000-0001-6355-0542.

⁷ ORCID: 0000-0001-6126-6310.

⁸ ORCID: 0000-0003-4687-1066.

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neuron-related genes and gene families. The involvement of these genes in the glutamate/GABA-glutamine and glycine cycle suggests a possible contribution to the excitation of neuronal networks, particularly under high salinity conditions. This study contributes to our understanding of mechanisms underlying the rapid responses of estuarine species to changing conditions and raises many intriguing hypotheses and questions for future investigation.

1. Introduction

The estuary is a transition zone that connects freshwater and marine environments, characterized by brackishwater with a salinity range of 0.5–35 ppt [1,2,3]. In recent years, the acceleration of global temperature rise has led to drastic environmental challenges, including rising sea levels, altered precipitation patterns, and rapid changes in habitat salinity [4]. In particular, rising sea levels are leading to saltwater intrusion in many estuaries [5,6,7,8], causing rapid increases in salinity in these habitats. Euryhaline animals, which are aquatic organisms capable of surviving in such habitats, must possess the ability to tolerate a wide range of salinity. However, extreme salinity fluctuations can disrupt key processes, including organismal recruitment, feeding rates, energy demands need to maintain cell volume and prevent osmotic shock [9,10,11,12]. Therefore, understanding the resilience, plastic responses, and adaptation of estuarine animals is crucial for assessing the potential impacts of sea-level rise due to global warming.

Corbicula japonica, a brackishwater clam belonging to the family Cyrenidae, is commercially important but has lacked sufficient scientific investigation. This clam successfully inhabits estuaries in Korea and Japan [13,14] and can withstand a very broad range in salinities, from 2 to 20 ppt [15]. However, the population decline of *C. japonica* has been attributed to seawater intrusion [16]. And this issue is expected to intensify with the continued progression of global warming [17,18]. The resilience of *C. japonica* to fluctuating salinity environments positions this species as a key model for understanding genomic adaptations to environmental stressors. Unraveling these adaptive mechanisms at the genomic level could provide pivotal insights into the broader impacts of global warming on estuarine biodiversity and the adaptive capacity of estuarine species. *C. japonica* clams hyperregulate below 2 ppt, maintaining a constant hemolymph osmolality at very low salinities and are weakly hyperosmotic from 2 ppt to 32 ppt [138]. Maintaining this hemolymph osmolality would require osmotic and ionic regulation across a broad range of salinities. In the tissues of *C. japonica*, alanine constitutes the bulk of the intracellular free amino acid (FAA) pool used to maintain osmotic homeostasis across the cell membrane [19]. Moreover, concentrations of this osmolyte fluctuate in response to salinity change [20,21,22]. Rapid osmoregulatory responses to salinity change require rapid neurological responses in fish [23], nematodes [24], and bivalves [25,26]. Considering that some studies have suggested that osmolarity affects neuronal excitability [27], these osmolytes might play an important physiological role in the neural regulation of *C. japonica*.

Residing within dynamic estuarine environments with fluctuating salinities requires rapid physiological responses, including rapid neural responses. The regulation of ion regulatory homeostasis is achieved through interactions between neuronal excitation and inhibition [28]. Also, invertebrate glia cells share both morphological and physiological characteristics with vertebrates [29]. Thus, considering the integral role of the nervous system in maintaining homeostasis, uncovering the neural mechanisms could provide valuable insights into how bivalves respond to rapid fluctuations in external stimuli. Thus, it is important to explore the dynamics of neurotransmitters and neural network plasticity in bivalves in response to salinity change.

In this study, we present a high-quality genome assembly of the brackishwater clam *C. japonica*, along with patterns of gene family expansions in this genome. In addition, we conducted comparative genomic and transcriptomic analyses to identify potential genes associated with neural plasticity and osmotic and ionic regulation in

response to changes in salinity. Our findings suggest the potential role of neural plasticity in contributing to the resilience of estuarine animals to salinity stress.

2. Material and methods

2.1. Sample collection and experimental design

Brackishwater clams (*Corbicula japonica*) were collected from the Seomjingang River (35° 3'29" N, 127° 44'59" E, Figs. 1a and 2b) in South Korea in April 2021, using a Korean traditional clam rake (called *Georangye*). At the time of collection, salinity was 4 ppt and temperature was 18 °C. Detailed information on the water quality of the Seomjingang River can be found in Table S1. This site typically experiences salinity ranges of 0 to 22.8 ppt [30]. Sampled clams were then transported to the laboratory and cultured in aerated 10 L plastic tanks at a water temperature of 20 °C and a salinity of 4 ppt for a period of two weeks to allow the clams to acclimate to laboratory conditions.

After the acclimation period, ten healthy clams with a shell length of 1.5–2.5 cm were selected and divided into two groups. One group, serving as the control, was kept in water with a salt concentration of 4 ppt, the optimal salt concentration (LSG) [19]. The other group was subjected to high salinity-exposure treatment (HSG). The salinity in this group was gradually increased by 3 ppt per day until it reached 19 ppt, after which the clams were allowed to acclimatize for an additional two days. To prevent the potential influence of starvation-driven effects on our results, we provided cultivated unicellular microalgae, *Isochrysis galbana*, as a food source at 10:00 am daily. Throughout the experiment, to prevent water contamination, 8 L (80 %) of the water was replaced with sterilized water once a day at 4:00 pm, and salinity levels were measured before and after the water replacement. Also, clams from both LSG and HSG were maintained in aerated tanks. In the salinity exposure experiment, water was not poured all at once; instead, it was slowly added by dropping water droplets to expose the samples to a salt concentration that increased by 3 ppt within 30 min. All environmental factors (temperature, pH), except for the salinity level, were controlled for both groups. No clam mortality was observed throughout the experiment, indicating the healthy condition of the specimens used.

2.2. Genome and transcriptome sequencing and assembly

Genomic DNA was extracted from the foot tissue of brackishwater clams. The extraction involved pooling tissue samples from different numbers of individuals in the control group: one for Illumina, 20 for HiFi, and eight for Omni-C. A total of 58.5 Gb of PacBio HiFi reads, 86 Gb of Illumina reads, and 75 Gb of Omni-C reads were sequenced (Table S2 and Table S3). Using trimmed data, we obtained a *k*-mer size of 21 by jellyfish (ver. 2.3.0) [31] which were subsequently utilized by GenomeScope (ver. 2.0) [32] to estimate the size of the genome (Fig. S2). To generate a high-resolution haplotype genome, NextDenovo (ver. 2.5.0) was used to integrate HiFi sequencing data [33,34]. Then, identification and removal of both haplotigs and heterozygous overlaps were processed using purge_dups (ver. 1.2.5) [35]. To increase contiguity, Omni-C raw Illumina reads were utilized to produce scaffolds using SALSA2 [36]. Next, metazoan BUSCO (ver. 5.2.2) [37] analysis was performed to evaluate the completeness of the genome assembly. We mapped Illumina and HiFi sequencing reads onto the brackishwater clam genome using BWA (ver. 0.7.17) [38]. Furthermore, Quast (ver.

5.2.0) [39] was employed to examine the genome quality. For detailed information, please refer to Table S4.

From the salinity acclimation experiment, three surviving individuals from each group were selected. Gill tissues from these individuals were collected and immediately immersed in RNAlater solution. The samples were then frozen at -80°C and stored in a refrigerator until RNA extraction. Total RNA was extracted from six-gill samples (three from LSG and three from HSG) using a TruSeq Stranded

mRNA sample preparation kit following the manufacturer's protocol. The complementary DNA (cDNA) libraries were constructed and sequenced using the Illumina NovaSeq 6000 platform, generating approximately 5.1–7.4 Gb of paired-end 151 bp reads from each sample. The sequenced reads were trimmed using Trim_galore (ver. 0.6.7) [40], followed by *de novo* assembly using Trinity (ver. 2.14.0) [41].

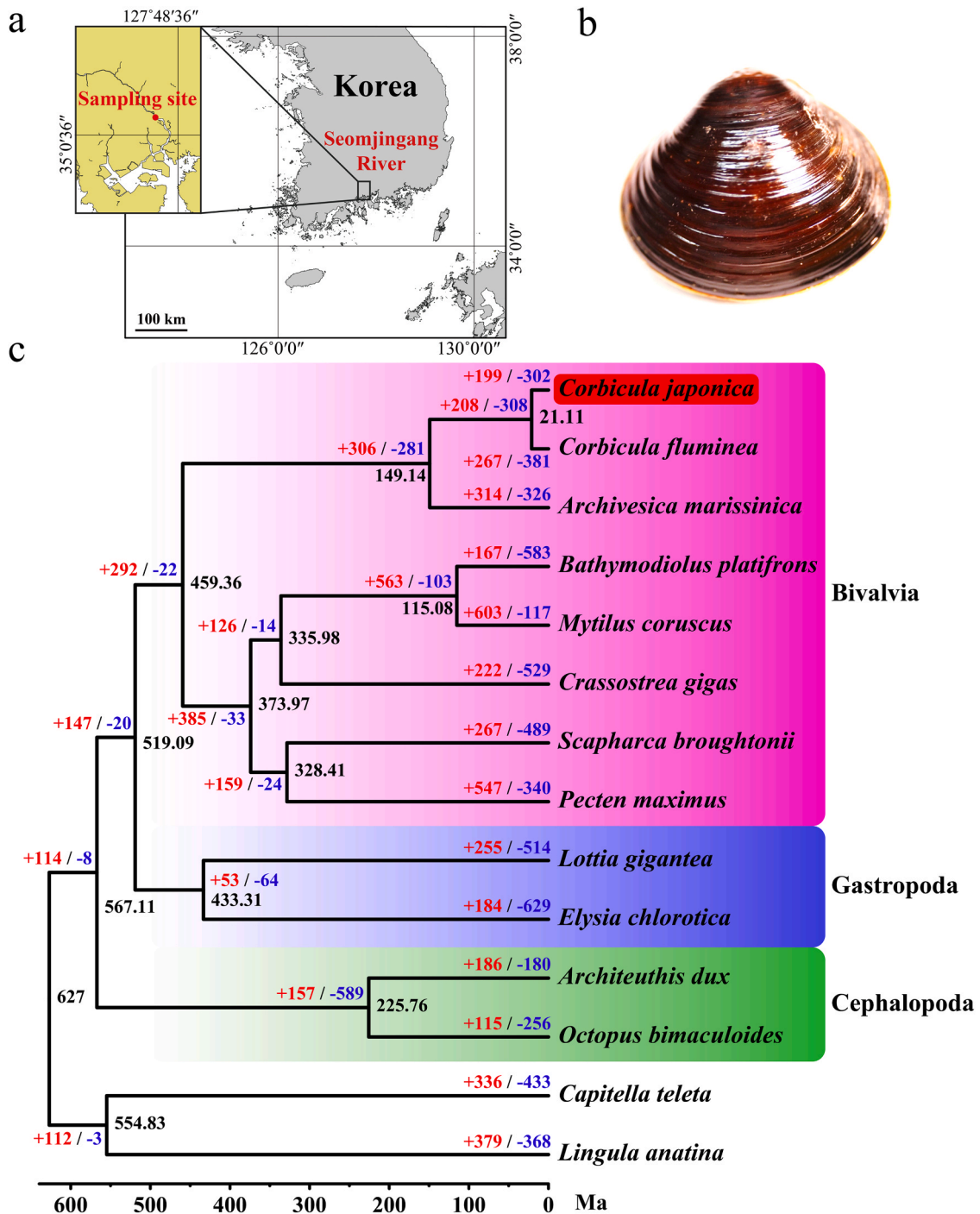


Fig. 1. Gene family expansions and contractions of the brackishwater clam, relative to 12 molluscan and two outgroup species. (a) Location of the sampling site along the Seomjingang River (red dot). The map was created on QGIS software (ver. 3.26.2, <https://www.qgis.org>) using OpenStreetMap (<https://www.openstreetmap.org/copyright>). (b) Shell of the brackishwater clam, *Corbicula japonica* (1.5 cm). (c) Phylogenetic tree constructed using single-copy orthologues from 14 lophotrochozoan species (including 12 molluscan species) showing the position of *C. japonica* (highlighted in red). The numbers in black on the right side of each node represent divergence times, while red and blue numbers represent the number of gene families undergoing significant expansion and contraction ($p < 0.05$), respectively. The time scale is indicated in million years ago (Ma) and displayed at the bottom.

2.3. Genome annotation

To screen repetitive sequences in the brackishwater clam genome, RepeatModeler (ver. 2.0.1) [42] and RepeatMasker (ver. 4.1.2) [43] pipelines were used for *de novo* prediction with the NCBI searching engine RMBlast (ver. 2.9.0). A custom repeat library for the brackishwater clam was built with RepeatModeler and repeats were identified, classified, and masked in two steps: one using the custom repeat library and the other using the well-curated library Repbase [44].

The BRAKER3 (ver. 3.0.6) [45] pipeline was employed to predict gene sets in the genome of brackishwater clam. First, repeats in the genome sequences were soft-masked using RepeatModeler and RepeatMasker. And GeneMark-ETP (ver. 1) [46,47] was used to produce hints from RNA-seq data and protein data (Metazoa orthologs from OrthoDB 11 [48] and four species (GCF_902806645.1, GCF_902652985.1, GCF_000327385.1, and CF_000002035.6)). To train gene sets, *ab initio* gene prediction was performed by AUGUSTUS (ver. 3.4.0) [47] using those hints from GeneMark-ETP. Finally, TSEBRA [49] integrates results from GeneMark-ETP and AUGUSTUS.

To determine the function of the integrated gene models, blastp was performed against the SWISS-PROT protein database [50] and the NCBI non-redundant database (<https://www.ncbi.nlm.nih.gov/refseq>) with

the following parameter: -evalue 1e-5 -dbsize 3400000000 -max_hsps 1 -max_target_seqs 1. The genes were also functionally classified with Gene Ontology (GO) and euKaryotic Orthologous Groups (KOG) using eggNOG-mapper (ver. 2.1.12) [51] with the default options. BUSCO analysis using the metazoan_odb10 database indicated that the final gene models contained 92 % complete BUSCOs (Table S6).

2.4. Gene-expression profiling

We determined gene expression patterns based on the coding sequences deduced from BRAKER3. The abundance of transcripts was expressed in Fragments Per Kilobase of transcript per Million mapped reads (FPKM) calculated using RSEM [52]. The filtered reads were mapped to the transcriptomes using bowtie2 (ver. 2.4.5) [53]. To investigate differential expression, normalization was performed using the Trimmed Mean of M-values (TMM) method [54] across samples. The DESeq2 package was used to identify differentially expressed genes (DEGs), and DEGs with $|\log_2(\text{fold change})| \geq 2$ and FDR-adjusted p -value ≤ 0.05 were considered significantly differentially expressed [55]. The Benjamini and Hochberg FDR procedures were applied to control the false discovery rate by adjusting p -values. To compare expression patterns, hierarchical clustering was performed. Each DEG

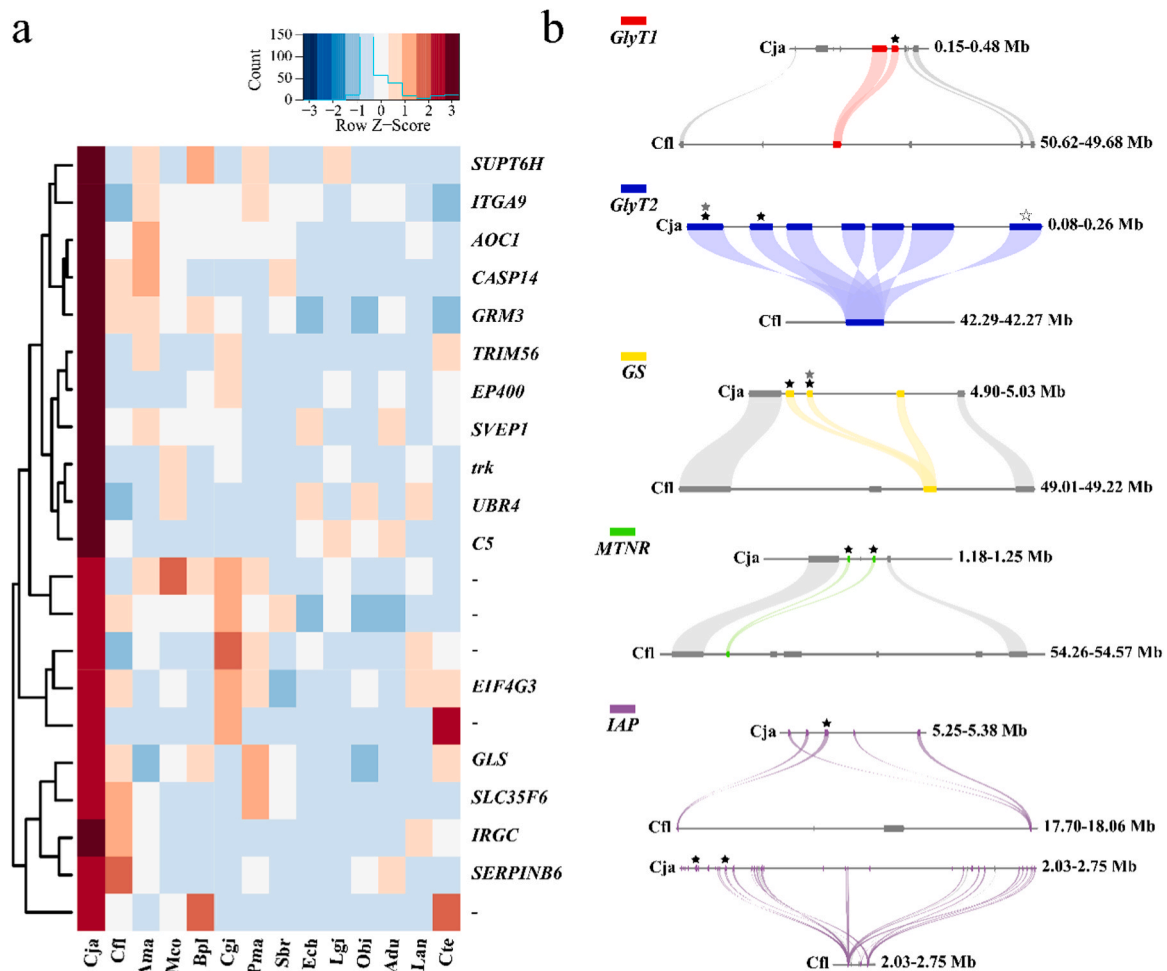


Fig. 2. Gene family expansion and tandem repeats in the brackishwater clam genome. (a) Heat map of gene families that are significantly expanded ($p < 0.05$) and enriched (Z-score > 1.96) in *Corbicula japonica* compared with 13 lophotrochozoans. (b) Microsynteny analysis of the brackishwater and freshwater clam genomes showing *GlyT1* (red), *GlyT2* (blue), *GS* (yellow), *MTNR* (green), and *IAP* (purple) genes, which are arrayed in tandem repeats in the genome of brackishwater clam. Genes upregulated in high and low salinity groups were denoted by black and dashed stars, respectively. The gray star on each corresponding gene indicated stained genes. Abbreviation: Cja, *Corbicula japonica*; Cfl, *Corbicula fluminea*; Ama, *Archivesica marissinica*; Mco, *Mytilus coruscus*; Bpl, *Bathymodiolus platifrons*; Cgi, *Crassostrea gigas*; Pma, *Pecten maximus*; Sbr, *Scapharca broughtonii*; Ech, *Elysia chlorotica*; Lgi, *Lottia gigantea*; Obi, *Octopus bimaculoides*; Adu, *Architeuthis dux*; Lan, *Lingula anatina*; Cte, *Capitella teleta*.

was subject to eggNOG-mapper analysis for functional annotation using the GO, KOG, and KEGG databases. Custom scripts were used to calculate person Chi-Square test *p*-value of every input data and visualize.

2.5. Signal transduction-related gene identification and phylogenetic analysis

We examined the putative neuron-related genes used in this study to identify that they are homologous to vertebrates. The occurrence of gene duplications in certain transporters, such as the solute carrier family 6 (SLC6; *GAT1*, *GAT2*, and *GAT3*), necessitated a detailed phylogenetic analysis. Subsequently, their identification as orthologous genes was affirmed using amino acids sequence.

To identify and cluster orthologue groups between different species, OrthoFinder2 (ver. 2.5.4) [56] was used with protein sequences from 14 species: *C. japonica* (in this study), *Mytilus coruscus*, *Pecten maximus*, *Lottia gigantea*, *Architeuthis dux*, *Lingula anatina*, *Capitella teleta* (obtained from the National Center for Biotechnology Information), *Archivesica marissinica*, *Bathymodiolus platifrons*, *Crassostrea gigas*, *Scapharca broughtonii*, *Elysia chlorotica*, *Octopus bimaculoides* (downloaded from MolluscDB) [57], and *Corbicula fluminea* (downloaded from <https://doi.org/10.1038/s41598-021-94545-2>, after performing gene prediction) [58]. DIAMOND (ver. 2.0.15) [59], a high-performance sequence search program, was used, and a total of 319 single-copy orthologues were identified.

Multiple sequence alignment was conducted using MAFFT (ver. 7.475) [60] with the default parameters for each dataset. The best models for each tree were determined with ModelFinder [61] and maximum-likelihood (ML) and Bayesian trees were constructed using RAXML-NG (ver. 1.2.0) [62] and MrBayes (ver. 3.2.4) [63], respectively. We used the 'make_ultrametric.py' python script provided by OrthoFinder2 to construct an ultrametric tree for divergent time estimation. The root age was calibrated to 627 million years ago (MYA) based on the TimeTree database [64]. The gene trees were visualized using FigTree (ver. 1.4.4, <http://tree.bio.ed.ac.uk/software/figtree>).

2.6. Gene family analysis

We employed Computational Analysis of gene Family Evolution (CAFE, ver. 5.0.0) [65] to identify significantly expanded and contracted gene families using the ultrametric tree and orthologue gene families file from previous steps as input. We removed gene families for which at least one species had more than 100 gene members while at least seven other species had 0 gene members. Gene families with Viterbi *p*-values less than 0.05 were considered to have undergone significant expansion or contraction. To identify expansions in the brackishwater clam, gene families that showed an increase compared to their parent clade were extracted from the file "model_change.txt". Next, to identify gene families which were enriched in brackishwater clam, a Z-score above 1.96 and gene families containing at least five gene members were selected. The function of each gene family was annotated using eggNOG-mapper. We determined the representative functions for each gene family by selecting the gene name with the highest number of overlaps within the gene family. The enrichment of Gene ID was analyzed by KOBAS [66] using the KEGG database.

2.7. Comparative analysis between two *Corbicula* genomes

To identify macrosynteny pairs between the brackishwater clam and freshwater clam, MCScan (<https://github.com/tanghaibao/jcvi/wiki/MCscan>, Python-version) [67] was used with default parameters. An all-against-all alignment was performed using LAST to obtain micro-collinearity blocks containing at least ten gene pairs [68]. Visualizations of micro-synteny were generated in python using scripts from MCScan.

2.8. Detection of positively selected genes in the genomes of ten molluscs

Since distantly related taxa are included in Fig. 1c (e.g., Mollusca, Brachiopoda, and Annelida), we created a new species tree exclusively containing Mollusca for the PAML analysis (please refer to Table S11 for species list). Positively selected genes (PSGs) were identified by analyzing single-copy orthologous genes from ten Molluscs using OrthoFinder2. The ML tree obtained from OrthoFinder2 was subsequently unrooted and had its branch lengths removed. Amino acid sequences were subjected to multiple sequence alignment using MAFFT. Multiple codon alignment was performed using pal2nal (ver. 14) [69]. To identify rapidly evolving genes under evolutionary pressure, we employed a branch model implemented in codeml within the PAML package (ver. 4.10.5) [70]. A likelihood ratio test was conducted to compare two models: (i) a null model (model = 0 NSsites = 0), where all branches have one d_N/d_S ratio; and (ii) an alternative model (model = 2 NSsites = 0), where the target lineage (*C. japonica* was used as a foreground) has a different d_N/d_S ratio compared to the background branches. We used the chi-square (χ^2) distribution to assess the statistical significance of the *p*-value. Genes were considered PSGs when $p < 0.05$ and the d_N/d_S ratio on the foreground branch was higher than that on the background branch.

2.9. Gene cloning and probe synthesis

The PCR reactions were performed under the following cycling conditions: pre-denaturation at 98 °C for 5 min, followed by 36 cycles of denaturation at 98 °C for 30 s, elongation at 72 °C for a sequence length-specific duration, and a final elongation step at 72 °C for 5 min. The primers used were as follows (gene name, forward primer, reverse primer): *GS*, 5'-GTT-GCT-GGA-TAC-CGA-TGG-CT-3', 5'-ACC-ACA-TCC-ATT-TAC-CGC-CA-3'; *GAT2*, 5'-CTA-TTC-CAA-GGT-GTG-GCG-G-3', 5'-ACT-CCA-CCA-AGC-AGG-CAA-T-3'; *GlyT2*, 5'-TAG-CGT-CAT-AGC-GGG-TTT-CG-3', 5'-TGT-TCA-CTG-CCC-CTG-GAT-3'. The amplified gene fragments were subcloned into pGEM T-easy vectors (Promega, Madison, WI, USA) and sequenced to confirm their identities. Then, the subcloned inserts were amplified using universal SP6 and T7 promoter primers (SP6 primer, 5'-TAT-TTA-GGT-GAC-ACT-ATA-G-3'; T7 primer, 5'-TAA-TAC-GAC-TCA-CTA-TAG-G-3'). The PCR-amplified linear templates were used to synthesize an *in vitro*-transcribed anti-sense riboprobe using a MEGAscript Transcription kit (Ambion, Austin, TX, USA).

2.10. In situ hybridization

The gills were fixed using 4 % paraformaldehyde (PFA; Sigma-Aldrich, Saint Louis, MO, USA) overnight and subsequently washed three times, each time for 10 min, in 0.1 % PBT (phosphate-buffered saline and 0.1 % Tween 20). The samples were permeabilized by treatment with 0.5 mg/mL proteinase K (Biofact, Daejeon, Korea) in PBT for 10 min at 50 °C. Following the proteinase K treatment, the samples were rinsed three times, each time for 10 min, in a solution containing 2 mg/mL glycine in PBT; washed twice, each time for 10 min, in PBT at room temperature; washed for 10 min in 0.5 mL of 0.1 M triethanolamine buffer (TEA, pH 8.0, Sigma-Aldrich, Saint Louis, MO, USA); rinsed three times, each time for 10 min, in PBT; post-fixed for 20 min in 4 % PFA; and washed four times, each time for 10 min, in PBT at room temperature. Details of the protocol were described previously [71]. Two rounds of whole-mount *in situ* hybridization were conducted for each gene, one at 4 ppt and another at 19 ppt.

3. Results and discussion

3.1. De novo assembly and features of the *Corbicula japonica* genome

The genome of *Corbicula japonica*, a brackishwater clam collected from the Seomjingang River, South Korea (Fig. 1a and Fig. 1b), was

sequenced using HiFi (59X coverage), paired-end reads from Illumina (87X coverage), and Omni-C reads from Illumina (76X coverage) platforms (Tables S2 and S3).

The assembled genome of the brackishwater clam *C. japonica* consisted of 1630 scaffolds with an N50 of 3.94 Mb and a total scaffold length of 1.07 Gb (Table S4). In this study, the assembled genome size of the brackishwater clam was similar to the estimated genome size of 0.98 Gb (Fig. S1). BUSCO analysis revealed that the genome completeness of the brackishwater clam was 91.3 %, which is comparable to that of seven other bivalve genomes (Table S4). Also, 91 % and 97 % of the Illumina reads (DNA and RNA) were aligned to the genome, indicating the completeness of its genome assembly. Furthermore, 558 Mb of the genomic sequences were identified as repeats, comprising for 52.2 % of the entire genomic sequence (Table S5). And we annotated 21,663 non-redundant protein-coding genes. The GC content of the brackishwater clam's genome exhibited a low value of 34.8 %, resembling the low value of 35.4 % of the closely related freshwater clam *C. fluminea* (Table S4).

To confirm the phylogenetic placement and genetic divergences within Order Venerida, 13 mitochondrial protein-coding genes were used for phylogenetic reconstruction (Fig. S2). Based on this phylogeny, within the family Cyrenidae, the closest relative of the brackishwater clam *C. japonica* was the freshwater clam *C. fluminea*.

3.2. Patterns of gene family expansions across the molluscan phylogeny and in *C. japonica*

To explore the evolutionary history and patterns of gene family expansions and contractions of the brackishwater clam *C. japonica*, we compared its genome with those of 14 lophotrochozoan species (12 mollusks, one brachiopod, and one annelid) (Table S6). A total of 34,303 orthogroups containing 444,382 genes were identified (Table S7). The species tree was reconstructed using 319 single-copy orthologs and revealed that *C. fluminea* is closely related to *C. japonica* among the bivalves (Fig. 1c). We estimated the timing of divergence between the brackishwater *C. japonica* and freshwater *C. fluminea* to be approximately 21.11 million years ago.

The gene family analysis among the 14 lophotrochozoans included in our study revealed 199 significantly expanded gene families and 302 significantly contracted gene families in the brackishwater clam relative to the other species ($p < 0.05$) (Fig. 1c). Furthermore, we investigated 21 gene families that were significantly enriched exclusively in *C. japonica*, comparing 13 lophotrochozoans (Z-score > 1.96) (Fig. 2a and Table S8). Among the enriched gene families, those associated with functions such as "GABAergic synapse" (KEGG PATHWAY:hsa04727), "Glutamatergic synapse" (KEGG PATHWAY:hsa04724), and "Neuroactive ligand-receptor interaction" (KEGG PATHWAY:myi04080) might be associated with the ability of the brackishwater clam to acclimate and thrive in salinity-fluctuating environments (Table S9). Among the 21 enriched gene families in *C. japonica*, we found two gene families that appear to play a significant role in modulating neuronal excitability within the gill tissues. Our findings include the identification of the metabotropic glutamate receptor type 3 (mGlu3, *GRM3*), which has shown a notable enrichment in brackishwater clam (Z-score = 2.79) (Table S8). This receptor has been observed on the glial membranes of mice [72] and its involvement in synaptic plasticity [73,74]. Additionally, glutaminase (*GLS*), an enzyme that catalyzes glutamine to produce glutamate, was identified. Considering that glutamate is the predominant excitatory neurotransmitter in the nervous system, the enrichment of this enzyme (Z-score = 2.65) is pivotal for maintaining brain homeostasis [75,76] (Table S8).

Intriguingly, we identified two and seven paralogs of Sodium- and chloride-dependent glycine transporters (*GlyT1*, *SLC6A9* and *GlyT2*, *SLC6A5*) in the brackishwater clam genome (Fig. 2b and Fig. S3). Among the paralogs, one *GlyT1* and two *GlyT2* genes showed significantly increased expression in the stressful high salinity group (HSG, 19 ppt),

relative to the control group (LSG, 4 ppt) (Table S10). Unexpectedly, one *GlyT2* paralog showed the opposite expression pattern of greater expression at the control 4 ppt conditions (Table S10). Given that glycine transporters are important regulators for maintaining homeostasis in the central nervous system (CNS) by facilitating the active reuptake of glycine into presynaptic neurons [77], this opposite expression pattern of this paralog gene suggests differentiation in its function. Our data suggest the interesting possibility that the neofunctionalization of *GlyT2* paralogs may contribute to neural plasticity in *C. japonica*. Therefore, the gene family expansion of *GlyT2* may potentially contribute to regulating the excitatory and inhibitory states, enabling the brackishwater clams to respond to salt stress effectively.

In addition, three paralogs of *Glutamine synthetase* (*GS*) are present in the brackishwater clam genome and have undergone duplication (Fig. 2b and Fig. S4). The glutamate/GABA-glutamine cycle operates between neurons and surrounding glial cells. To sustain this continuous cycle, the amount of released glutamate or GABA from presynaptic neurons must be replenished, at least to the same extent, by glial cells in the form of glutamine. Therefore, the presence of three tandemly duplicated *GS* genes may potentially facilitate rapid replenishment of glutamine in the presynaptic neurons through synthesis. Interestingly, two *GS* genes were identified as having higher expression in the high salinity treatment (HSG) (Table S10). We further discuss the possible functions of *GlyT1*, *GlyT2*, and *GS* below (in "Hypothesized models for neural excitability").

3.3. Signatures of putative positive selection along branches of the molluscan phylogeny

We examined genes with signatures of positive selection ($d_N/d_S > 1$) along the branches of the molluscan phylogeny (Table S11). We identified 49 positively selected genes (PSGs) out of the 870 single-copy genes examined across ten mollusc species. Remarkably, more than 40 % of these genes were linked to the GO term "response to stimulus (GO:0050896)", 17.14 % were linked to "signaling (GO:0023052)", 14.29 % were linked to "signal transduction (GO:0007165)", and 2.86 % were linked to "presynapse (GO:0098793)" and "neuron to neuron synapse (GO:0098984)" (Table S12). In the same context, the most prominent category in the KOG analysis was T (signal transduction mechanisms), except for category S (function unknown) (Table S13).

Among 49 PSGs, we discovered the presence of *Cation-chloride cotransporter 6* (*CCC6*, *SLC12A9*) (Fig. S5), which plays a crucial role in neuronal signaling and ionic regulation in arthropods [78, 79, 80]. Despite the important role of CCC6 in maintaining electrochemical Cl⁻ gradients and determining the polarity of GABA effects in the brain of mice [81], to our best knowledge, the functions of CCC6 homologs in bivalves have not yet been explored. Thus, further research is needed to examine the effect of CCC6 in brackishwater clams and determine what functions it might serve in the context of adaptation to the estuarine environment.

3.4. Genome-wide gene expression patterns and potential nervous system plasticity across salinity treatments

We obtained 919 differentially expressed genes (DEGs) between salinity treatments. Of these genes, 335 were expressed more highly in the control low salinity treatment, while 584 genes displayed increased expression in the stressful high salinity treatment (Table S10).

A euKaryotic Orthologous Groups (KOG) [82] analysis was performed to investigate the dynamics of gene expression in the HSG and LSG treatments. A total of 429 (73.46 %) DEGs from the HSG (19 ppt) were annotated. We analyzed the functional categories in the HSG that showed an increased number relative to the control conditions (4 ppt) (Fig. 3a). In category P (inorganic ion transport and metabolism), genes related to neurotransmission and signal transmission, such as *GlyT2* and *Potassium voltage-gated channel subfamily KQT member 1* (*KCNQ1*), were

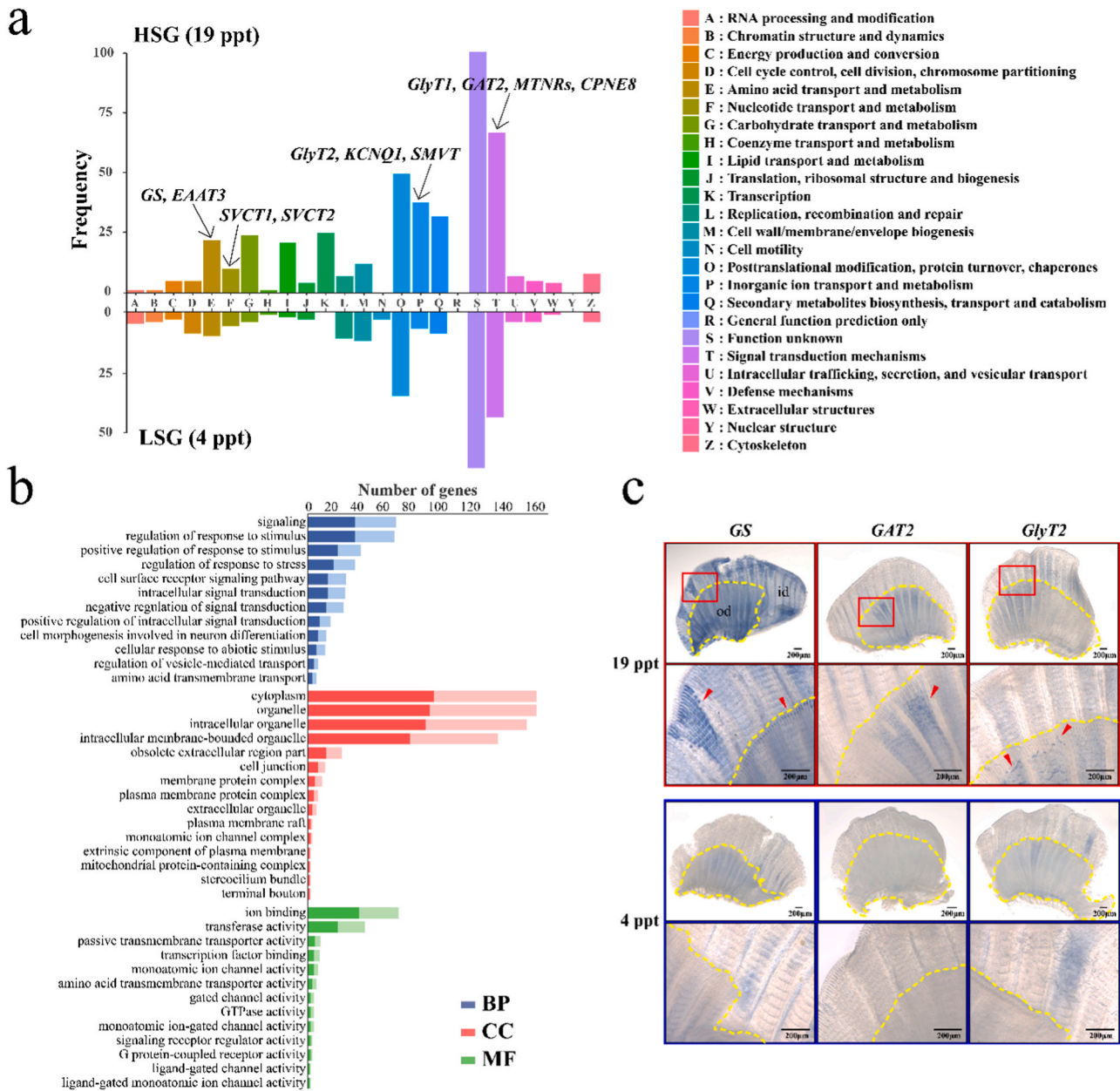


Fig. 3. Gene expression dynamics under high salinity and low salinity groups. (a) KOG analysis shows distinct expression profiles in the high salinity group (HSG, 19 ppt) and the optimal salinity group (LSG, 4 ppt). (b) GO term analysis of HSG and LSG. Significant GO terms related to the nervous system in HSG are shown ($p < 0.05$). Genes found in 19 ppt are indicated in a lighter shade, while those in 4 ppt are shown in a darker color. Abbreviations: BP, biological process; CC, cellular component. (c) Whole-mount *in situ* hybridization of brackishwater clam gills, visualizing gills after hybridization with anti-sense RNA probes of *GS*, *GAT2*, and *GlyT2*. Blue NBT/BCIP is indicative of mRNA expression. Images were taken using a Zeiss microscope Axio Zoom V16. Abbreviations: od, outer demibranch; id, inner demibranch. Red arrows and yellow dotted lines represent the mRNA expression regions and outer demibranch regions, respectively. Scale bar, 200 μ m. The expression of three genes in HSG was analyzed in two independent experiments.

identified (Table S14). These genes mediate neuronal excitability throughout the nervous system and play a crucial role in synaptic plasticity in mammals [83, 84, 85, 86] and zebrafish [87]. We then explored if there were more neuron-related genes among the increased categories in HSG. In category E (amino acid transport and metabolism), genes related to the glutamate/GABA-glutamine cycle [88], such as *GS* and *Excitatory amino acid transporter 3* (*EAAT3*, *SLC1A1*), were identified. Also, in category T (signal transduction mechanisms), we identified *Sodium- and chloride-dependent glycine transporter 1* (*GlyT1*, *SLC6A9*), *GABA transporter 2* (*GAT2*, *SLC6A13*), *Melatonin receptors* (*MTNRs*), and *Calcium-dependent membrane-binding protein* (*CPNE8*) (Table S14). Consistent with previous results, 238 (40.75 %) gene ontology (GO)

terms significantly enriched in the high salinity treatment ($p < 0.05$) were related to the signaling processes, such as “signaling” (GO:0023052), “amino acid transmembrane transporter activity” (GO:0015171), “gated channel activity” (GO:0022836), and “signaling receptor regulator activity” (GO:0030545), (Fig. 3b and Table S15). Though gills are not central nervous system organs, we identified neuron-related genes in the DEG analysis.

3.5. Genome-wide expression of genes putatively related to osmotic and ionic regulation across salinity conditions in gill tissue of *C. japonica*

In response to salinity change, osmotic regulation would be required

for cell volume regulation in the face of increases in ambient osmotic pressure, whereas ionic regulatory mechanisms would be required to maintain ion and water homeostasis [80,89,90].

With respect to genes potentially contributing to osmotic regulation, in the gill tissue of *C. japonica*, several genes related to amino acid synthesis, transport, and degradation showed significant increases in expression in the high salinity treatment (HSG, 19 ppt), relative to the control (LSG, 4 ppt). For instance, *Alanine-glyoxylate aminotransferase* (AGXT), which catalyzes the chemical reaction, L-alanine + glyoxylate = pyruvate + glycine (Fig. S6 and Table S10). This enzyme has been found to affect the accumulation of alanine in response to hyperosmotic stress in mussels [91,92].

In terms of ionic and/or osmotic regulation, two gene copies of the ion transporter Na^+/K^+ -ATPase subunit α (NKA- α) showed increased expression (4.29 and 2.55 log₂-fold increase) in *C. japonica* gill tissue in the high salinity treatment (HSG, 19 ppt) (Fig. S7 and Table S10). In ionocytes, cells specialized for ion transport, NKA is basolaterally localized and pumps 3 Na^+ out of the cell, and 2 K^+ into the cell for each ATP consumed [80,93]. Under high salinity stress, increased NKA expression might enable greater rates of ion secretion through the excretory organs (nephridia). On the other hand, in the gill tissue, the role of NKA expression under hyperosmotic stress might be related to facilitating Na^+ -dependent amino acid co-transport by creating a Na^+ deficit in the cell and thus driving Na^+ and amino acid co-transport into the cell through the apical membrane [94]. Of course, NKA participates in a multitude of physiological processes, including neural regulation, such that its increased expression at high salinity could serve multiple purposes. NKA expression is also increased under high salinity conditions in the gill tissue of the abalone *Haliotis discus hannai* [95].

Intriguingly, additional ion transporters showed increased expression in the HSG treatment, such as *Sodium-driven chloride/bicarbonate exchanger* (NDCBE, SLC4A10), *Ammonia transporter* (AMT), and a cation/ H^+ antiporter (VNXI) (Table S10). In addition, a *beta-Carbonic anhydrase* (β -CA) gene showed increased expression in HSG (Table S10). In contrast to the known ion regulatory functions of *alpha-Carbonic anhydrases* [96], β -CAs are far less studied in animals. While these putative ion transport-related genes might be involved in ionic regulation in *C. japonica* [97], physiological studies will be needed to determine the functions of these gene products under different salinity conditions. For all the genes mentioned above, extensive studies are needed to decipher their roles in salinity acclimation and/or adaptation.

3.6. *In situ* RNA transcription of putative neurotransmitter-related genes in clam gill tissue

Using *in situ* hybridization with RNA probes in clam gill tissue, we visualized the differentially expressed genes that are likely neurotransmitter-related, namely *GS*, *GAT2*, and *GlyT2*, to determine their changes in expression according to salinity (4 versus 19 ppt) (Fig. 3c). None of these genes were expressed in the low salinity control treatment (4 ppt), but all were expressed in the outer demibranch under the stressful high salinity conditions (19 ppt). In addition, relative to expression in LSG (TMM > 453), the gene *GS* was expressed seven-fold more strongly in the inner demibranch in HSG (TMM > 3474). These results indicate that high salinity environments affect the expression of putative neurotransmitter-related genes, mainly in the outer demibranch. The *in situ* hybridization images (Fig. 3c) were insufficient in resolution to determine whether the expression of these genes occurred within neurons in the gills rather than in other cell types. Thus, these genes could predominantly be performing other functions in other cell types. Recent studies have shown that branchial nerves are innervated in the gill of Bivalvia species such as *Crassostrea gigas* and *Crenomytilus grayanus*, suggesting that gills serve as chemo-mechanical sensing organs that acquire and deliver information from the external environment [98,99].

3.7. Hypothesized models for neuronal excitability

Earlier studies have shown that salt intake increases neuronal excitability in vertebrates [100,101]. Based on our findings above, we hypothesized that the nervous system in the gills of brackishwater clams might become more excitable when exposed to high salinity (Fig. 4). We identified putative neurotransmitter transporter-related genes *GAT2*, *GlyT1*, and *GlyT2*, including multiple gene paralogs, in the *C. japonica* genome (Fig. S3 and Fig. S8). These genes have been associated with inhibitory neurotransmitters in *Drosophila* and marine invertebrates [29, 102, 103]. For *GS*, *GAT2*, and *GlyT2*, we found increased *in situ* RNA expression in clam gill tissue at higher salinity (19 ppt) relative to lower salinity control conditions (4 ppt) (Fig. 3c). The increased expression of putative neurotransmitter transporters in the clam gill tissue potentially indicates a rapid removal of inhibitory neurotransmitters from the synaptic gap, preventing continuous receptor activation [104].

For instance, once GABA is secreted from presynaptic neurons, it is reabsorbed by *GAT2* located in glial cells. When taken up, GABA is catabolized into succinate by GABA transaminase (EC 2.6.1.19) and succinate-semialdehyde dehydrogenase (SSADH) and is further metabolized to α -ketoglutarate in the TCA cycle for glutamate synthesis [105, 106]. Glutamate is then converted to glutamine by *GS*, which was identified as an upregulated DEG in our analysis (Table S10). Glutamine synthesized in glial cells is supplied to presynaptic neurons [107] and reconverted to glutamate by a mitochondrial enzyme, phosphate-activated glutaminase (PAG, EC 3.5.1.2). In glutamatergic neurons, this glutamate is packaged into vesicles and released into the synaptic cleft, whereas, in GABAergic neurons, it is metabolized into GABA by glutamate decarboxylase (GAD, EC 4.1.1.15) and secreted [76, 108]. This recycling loop ensures the efficient utilization of inhibitory neurotransmitters.

Second, secreted glycine is absorbed by the glycine transporters *GlyT1* and *GlyT2*, which regulate glycine levels within the presynaptic vesicles [77]. There is a difference in the distribution patterns of these two transporters; *GlyT1* is found in glial cells and neurons in mouse model [109], whereas *GlyT2* is exclusively located on neuronal membranes and serves as a marker for glycinergic neurons in vertebrates [110]. Therefore, the high expression and presence of *GlyT1* and *GlyT2* in clam gills suggest that gills may reuptake glycine in the synaptic cleft, thereby reducing its inhibitory effect on the gills.

Third, we identified *KCNQ1* in the clam genome, which plays a key role in excitatory synapses (Figs. 4 and S9) [111,112]. After a nerve impulse, *KCNQ1* mediates repolarization, which is essential for modulating neuronal excitability in the nervous system [113]. This process allows continuous signal propagation as the action potential returns to the resting potential. Taken together, the increased expression of genes associated with neuronal excitability may contribute to overall network excitation and enhanced osmosensitivity, potentially aiding the survival of the brackishwater clam in estuaries.

Our intriguing finding that some of these putative neurotransmitter transporter-related genes are highly expressed in clam gill tissue (particularly at higher salinity) should be explored further to discover the specific cell types in which these genes are expressed. We predict that these genes are expressed in neural cells. It would be worthwhile to investigate the functions of the putative neurotransmitter transporter-related proteins, particularly of *GS*, *GAT2*, *GlyT1*, and *GlyT2*, to determine their roles in response to rapidly changing salinity.

3.8. Mechanisms of nerve cell damage, repair, and protection

In a previous study [15], salt stress was found to lead to the destruction of epithelial cells in *C. japonica*, causing physiological disorders. Hence, we next aimed to identify potential agents that could cause damage to the nervous system. We found that *CPNE8*, a member of the copine family, was highly expressed in HSG (Fig. S10 and Table S10). *CPNE8* encodes a Ca^{2+} -dependent phospholipid-binding

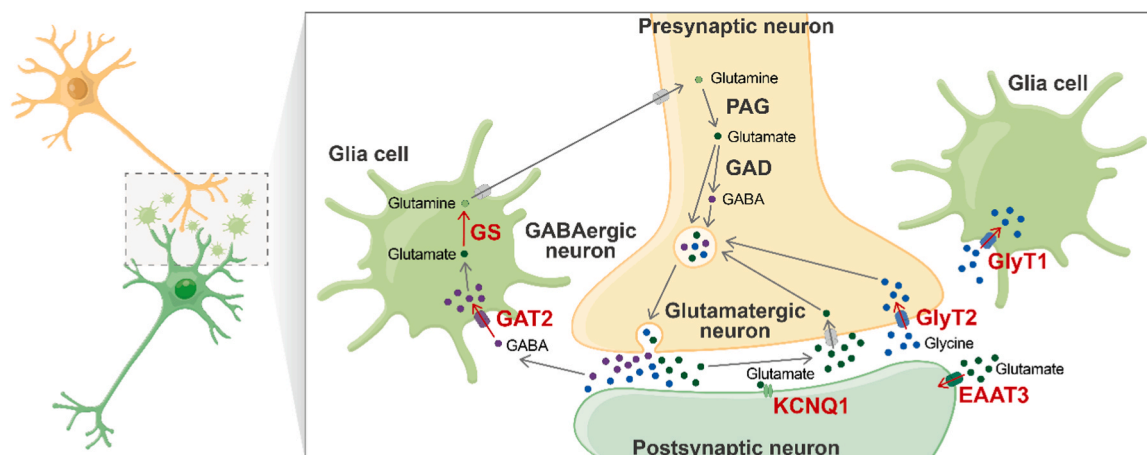


Fig. 4. Hypothesized model of the brackishwater clams under salt stress. A glutamate/GABA-glutamine and glycine cycle in the brackishwater clam is depicted. When salinity increases, neurotransmitters (GABA) involved in excitation are rapidly reabsorbed back into the presynaptic glial cell by GAT2. In the glial cell, the neurotransmitters are converted into glutamine by glutamine synthase (GS) and transferred back to the presynaptic neuron. In the presynaptic neuron, glutamine is reconverted to glutamate by phosphate-activated glutaminase (PAG). In glutamatergic neurons, glutamate is secreted directly, whereas in GABAergic neurons, it is converted to GABA by glutamate decarboxylase (GAD), which is then secreted. For continuous signal propagation, KCNQ1 mediates repolarization. GlyT1 and GlyT2 mediate the clearance of glycine from the space between presynaptic and postsynaptic neurons. Upregulated genes identified by transcriptome analysis and the direction of neurotransmitter movement are shown in red.

protein that plays a key role in tumorigenesis and malignancies in human [114]. Increased levels of *CPNE8* expression in humans have also been observed in Parkinsonian neurodegeneration [115]. Therefore, this gene may contribute to transmission disorders in the CNS. However, in some studies, *CPNE8* has been associated with excitatory neurons [116], as well as CNS development and mitogenesis [117], indicating the need for further research to elucidate the role of *CPNE8*.

In this study, we identified four genomic features in the brackishwater clam that might contribute to cell protection. First, to avoid excitotoxicity, excitatory amino acid transporters (EAATs in solute carrier family 1 [SLC1]) are essential for transporting extracellular glutamate within the CNS, particularly for removing glutamate in the mammalian brain [118, 119, 120]. Within the SLC1 family, EAAT3 (Fig. S11) was identified as an upregulated DEG in HSG (Table S10). Interestingly, EAAT3 exhibits an additional transport function related to neuronal stress responses. Unlike other SLC1 family members, EAAT3 can transport cysteine into neurons within mammalian cell models [121,122]. This cysteine transport activity has emerged as a significant physiological role of EAAT3, as the absence of this transporter decreases neuronal glutathione levels and increases vulnerability to oxidative stress [123, 124].

In addition, three vitamin transporter genes were highly expressed in HSG (Table S10): Sodium-dependent multivitamin transporter (SMVT, SLC5A6) and Sodium-dependent vitamin C transporters (SVCT1, SLC23A1 and SVCT2, SLC23A2) (Figs. S12 and S13). Vitamin C is known to protect mucosal tissues in human [125] and increase resistance to abiotic stress and infection in the Gastropoda [126]. Therefore, genes related to vitamins may play a crucial role in preventing gill impairment in the face of abiotic stresses such as salt stress.

Also, two paralogs of *MTNR* were found in the brackishwater clam genome (Fig. 2b). Recent studies have revealed its importance in neurogenesis impairment and neuroprotection [127,128]. Melatonin has also been reported to act as a neuroprotective substance for Parkinson's disease, which may be related to the damage caused by *CPNE8*, as mentioned earlier [129]. In plants, rapid melatonin accumulation has been observed under salt stress, resulting in enhanced resistance to abiotic stress [130,131]. Therefore, the enrichment of *MTNRs* in the brackishwater clam may prevent neuronal cell death by restoring damaged neurons.

Finally, an inhibitor of apoptosis protein (*IAP*), which regulates cell proliferation at multiple levels, was found to be enriched in HSG

(Table S10). Several studies have demonstrated that this gene controls cell death, inflammation, immune responses, and stress responses [132, 133]. Interestingly, expanded *IAP* gene families have been reported in some Bivalvia species, including *C. gigas*, *Bathymodiolus platifrons* [134], *Pinctada fucata* [135], *Saccostrea glomerata* [136], and *Mercenaria mercenaria* [133]. Among these bivalves, *M. mercenaria*, which thrives in estuaries experiencing fluctuations in salinity, has the highest number of *IAP* gene family members among all metazoans [133,137]. We identified 39 paralogs of *IAP* genes in the genome of *C. japonica* (Fig. 2b). Interestingly, among those *IAP* paralogs, two baculoviral *IAP* repeat containing 7 (*BIRC7*) and one death-associated inhibitor of apoptosis 2 (*DIAP2*) revealed increased expression in the gill tissue of brackishwater clam in HSG (Fig. 2b and Table S10). Therefore, the *IAP* gene family in the brackishwater clam suggests that the inhibition of apoptosis may potentially regulate responses to immune and abiotic stresses.

4. Conclusions

Through the comprehensive analysis of a high-quality genome and environment-specific transcriptome, our study contributes to the current understanding of mechanisms by which brackishwater clams respond to salinity stimuli and raises some intriguing hypotheses and questions. We discovered that the gill act as a sensory organ, detecting external stimuli, and triggering neuronal excitation. Previous studies on the neurological aspects of salt stress have been limited to a few model organisms. While this study offers valuable insights, further physiological experiments are needed to investigate the functions of genes under salinity stress conditions, which will deepen our understanding of the adaptive mechanisms involved. Therefore, this study provides a novel perspective on the plasticity of the nervous system in molluscs and contributes to the fields of molluscan biology, neurology, and evolution by providing novel insights and questions regarding the intricate interplay between salinity stress and nervous system adaptations.

Environmental Implication

Global warming is accelerating the rise in global temperatures, leading to a series of environmental challenges, including rising sea levels. This sea-level rise has significant implications for estuarine environments, one of the most critical impacts is saltwater intrusion, which alters the salinity of estuarine habitats. Therefore, understanding the

resilience, plastic responses, and adaptation of estuarine animals is crucial for assessing the potential impacts of sea-level rise due to global warming. However, no study has yet revealed the neural plasticity of bivalves. This study explores how bivalves respond to salt stress, potentially induced by sea level rise and resulting saltwater intrusion.

CRedit authorship contribution statement

Eun-Jeong Kim: Investigation. **Chi-une Song:** Investigation. **Chan-Jun Lee:** Formal analysis, Data curation. **Yun Keun An:** Resources, Investigation. **Hyeonwoo Choi:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Seong-il Eyun:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Sung-Jin Cho:** Writing – review & editing, Supervision, Project administration. **Carol Eunmi Lee:** Writing – review & editing, Validation, Investigation, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2024.136627](https://doi.org/10.1016/j.jhazmat.2024.136627).

Data availability

Data will be made available on request.

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