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The Regulatory Relationships Between the Gonad-Inhibiting Hormone and Insulin-Like Androgenic Gland Hormone-Binding Protein Genes in the Eyestalk-Androgenic Gland-Testis Axis of *Macrobrachium rosenbergii*

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Gonad-inhibiting hormone (GIH) belongs to a family of neuropeptides that are released from the eyestalks of male crustaceans and plays key roles in gonadal maturity, reproduction, and molting. However, the detailed mechanisms underlying the effects of GIH on sexual regulation have yet to be elucidated. In the present study, we aimed to demonstrate how GIH mediate the activity of the androgenic gland (AG) to affect sexual regulation. To do this, we cloned and characterized a GIH sequence from *Macrobrachium rosenbergii* (*Mr*GIH). The open reading frame (ORF) of *Mr*GIH was 360 bp and codes for a polypeptide of 119 amino acids and a putative protein of 13.56 KDa. Tissue analysis showed that *Mr*GIH is widely expressed in a range of tissues but particularly, the eyestalk, intestine, and nerve cord. Following the *ds*RNA silencing of *Mr*GIH for 24 h, the expression levels of *Mr*GIH were down-regulated in both the eyestalk and AG when compared with the negative control, but significantly increased the expression of *Macrobrachium rosenbergii* insulin-like androgenic gland hormone-binding protein (*Mr*IAGBP) in AG, thus suggesting that *Mr*GIH is an inhibitory factor for *Mr*IAGBP. In addition, we found that eyestalk removal on certain days led to increased expression levels of *Mr*IAGBP expression. The expression levels of *Mr*IAGBP peaked at 2 d in the AG after unilateral and bilateral eyestalk ablation, exhibiting a 7.27- and 6.03-fold increase, respectively. Afterward, the expression of GIH protein levels were down-regulated and IAGBP protein levels were up-regulated after GIH silencing using immunohistochemistry method, combined with the increase of IAGBP protein levels after eyestalk ablation, confirming that *Mr*GIH is an inhibitory factor that can moderately

regulate AG development and IAGBP expression. Collectively, our findings enriched the mechanisms that control the sexual regulation pathway of male *M. rosenbergii*, and provided significant information for further explorations of the mechanism underlying sex regulation in other decapod crustaceans.

Keywords: *Macrobrachium rosenbergii*, GIH, IAGBP, eyestalk-AG-testis, sex-biased regulation

INTRODUCTION

The mechanisms of sex determination and differentiation in *Macrobrachium rosenbergii*, a commercially important species of giant freshwater prawn, have been the subject of significant research interest in terms of evolutionary biology and endocrinology. Previous work, based on the pattern of sexual dimorphism in many species of crustaceans, led to the use of monosex culture (all-male or all-female) in the commercial sector as this was the best strategy to achieve the highest yield (Ventura and Sagi, 2012; Levy et al., 2016). In addition, following sexual maturation, growth rate decline and more energy is partitioned toward reproductive events (Wang and Xu, 2019). Recent research has shown that environmental factors have led to the onset of sexual maturation in some crustaceans at a much earlier time than normal, thus resulting in slow growth and reduced production (Wilder et al., 2010). Therefore, it is of great significance to study the mechanisms associated with sex differentiation that involves gonad-related gene expression as this may be advantageous with regards to improving yield. However, the dynamic processes underlying the regulation of gonad-inhibiting factors in the giant freshwater prawn have yet to be elucidated.

In crustaceans, sexual maturation is an elaborate molecular process that is regulated by an endocrine network (Huberman, 2000). Gonad-related hormones are closely involved with sexual regulation and have the activity to inhibit spermatogenesis (Fingerman, 1997a) in the androgenic gland (AG) of male crustaceans. In mammals, spermatogenesis relies upon a number of hormones that act within the hypothalamic-pituitary-gonadal axis (Godwin, 2010; Kanda, 2019). As with the mammalian hypothalamic nervous system, decapodal crustaceans also use an endocrine axis to regulate male sex differentiation; this is referred to as the eyestalk-androgenic gland (AG)-testis (Khalaila et al., 2002). The AG is an endocrine organ that is unique to male crustaceans and exerts functionality by secreting a sex-controlling switch element known as insulin-like androgenic gland (IAG) hormone (Levy and Sagi, 2020). A series of experiments have proven that AG is able to control the sexual differentiation of the male reproductive system. For example, the transplantation of the AG into females can lead to the masculinization of females while the removal of the AG from males can result in the feminization of males (Suzuki and Yamasaki, 1991; Sagi et al., 1999; Hoang et al., 2006). Furthermore, the eyestalk is also an endocrine organ and contains an endocrine regulatory center-X-organ-Sinus gland (XO-SG) complex (Hanström, 1931). The XO-SG mainly secretes neurohormones from the crustacean hyperglycemic hormone (CHH)-superfamily; some of these are involved in a range of complex biological activities (Webster et al., 2012).

Gonad-inhibiting hormone (GIH) is a key member of the CHH-superfamily and is produced by the XO-SG complex in the eyestalk (Hopkins, 2012). GIH is also called vitellogenesis-inhibiting hormone (VIH) in females for its main function to inhibit the vitellogensis of female shrimps and crabs (Medina et al., 1996; Yano et al., 1996). GIH is indispensable because it can inhibit gonad-relate hormones in male crustaceans. Previously, GIH has been studied with regards to reproductive function (Warrier et al., 2001; Treerattrakool et al., 2008); such studies have revealed that GIH plays roles in ovarian maturation and testicular development. *In vivo* research involving *Scylla serrata* (Haihui et al., 2006), *Procambarus clarkii* (Kulkarni et al., 1991), and *Parapenaeus longirostris* (Tom et al., 1987) demonstrated that the GIH gene can exert direct influence on the ovary by inhibiting the expression of vitellogenin. A previous study also demonstrated that GIH exhibited the same activity of molt-inhibiting hormone (MIH) which also belongs to the CHH superfamily (Chang et al., 1987; de Kleijn et al., 1994; Gu et al., 2002). It has been well established that sex differentiation in crustaceans is jointly regulated by neuropeptides and hormones, and that the endocrine organs that are involved in the pathway of sexual regulation mainly include the eyestalk and glands. Surprisingly, GIH acts directly on the AG rather than the testis in male crustaceans. The GIH gene interacts with other AG genes containing insulin-like androgenic gland hormone (IAG) (Ventura et al., 2011), insulin-like androgenic gland hormone receptor (IAGR) (Sharabi et al., 2016), and insulin-like androgenic gland hormone-binding protein (IAGBP) (Yang et al., 2020) to regulate sexual reproduction and the maturation of gonads (Fingerman, 1997a,b). The GIH gene, which encodes a specific eyestalk-derived hormone, exerts action on the eyestalk-androgenic gland (AG)-testis axis to mediate the activity of the AG (Khalaila et al., 2002). However, the regulatory mechanisms underlying the role of GIH in the sexual differentiation of *M. rosenbergii* have yet to be systematically reported.

In this study, we cloned a cDNA encoding GIH from *Macrobrachium rosenbergii* (*Mr*GIH) and investigated its potential role on the eyestalk-androgenic gland (AG)-testis axis. We also used double-stranded (*ds*)RNA to create a functional knockdown of *Mr*GIH. We also applied eyestalk ablation to demonstrate the effects of GIH on IAG, IAGR, and IAGBP expression in the eyestalk and AG of male *M. rosenbergii*. Our research sheds light on the mechanisms underlying the regulation of sexual reproduction and provides evidence that GIH plays a critical role in a number of key processes.

MATERIALS AND METHODS

Animals, Reagents, and Experimental Design

Male giant freshwater prawns (*M. rosenbergii*), weighing 15–40 g in weight and 10–20 cm in length, were obtained from Jin Yang Aquaculture Co., Ltd. (Guangzhou, China) and then cultured in a recirculating tank system at 28 ± 2°C with uninterrupted aeration in our laboratory. Young males, weighing 35 ± 5 g, were separated and had their eyestalks surgically removed. A group of smaller individuals, weighing 20 ± 5 g, were separated and used for *ds*GIH gene knockdown. These animals were grown in a feed-free culture system after manipulation. Total RNAs (1 μg) were extracted using RNAiso plus (TaKaRa) and first−strand cDNA was synthesized using HIScript® Q Select RT SuperMix for qPCR (Vazyme, Dalian, China) in accordance with the manufacturer's instructions. The specific primers used in this study are shown in **Table 1**.

RNAiso Plus was purchased from TaKaRa Bio (TaKaRa, Japan). HIScript® Q Select RT SuperMix and ChamQ SYBR qPCR Master Mix were both obtained from Vazyme (Nanjing, China). Rabbit polyclonal antibodies against *Mr*GIH and *Mr*IAGBP (Yang et al., 2020) were produced and purified by Frdbio Bioscience and Technology Company (Wuhan, China) and stored at −80°C prior to use.

Nucleotide Sequences and Bioinformatic Analysis of *Mr*GIH

Once identified, we used the full-length of *Mr*GIH nucleotide sequence as a basis to predict the open reading frame (OFR) of *Mr*GIH and its amino acid (AA) sequence by Emboss.[1] The signal peptide prediction of *Mr*GIH was used the SignalP-5.0[2] (Chen et al., 2014). SMART servicer[3] was used to identify putative protein domains from the *Mr*GIH sequence and SWISS-MODEL[4] was used to model protein structure homology (Lu et al., 2020). Clustal X 2.0 program and DNAMAN software package were then used to perform multiple sequence alignments. We also generated a phylogenetic tree, based on the ORF AA sequences of *Mr*GIH proteins; this was carried out by the neighbor-joining (NJ) method using the molecular evolution genetics analysis tool (MEGA 6.0).

Tissue Distribution Profile of *Mr*GIH in *M. rosenbergii*

Next, we investigated the tissue distribution of *Mr*GIH in *M. rosenbergii* by collecting a range of tissues from healthy prawns, including brain (B), heart (Ht), stomach (S), gill (G), testis (Te), androgenic gland (AG), eyestalk (Es), intestine (In), and ventral nerve cord (VNC). Once harvested, these tissues were stored at −80°C for RNA extraction with the RNAiso Plus system (TaKaRa, Dalian, China). First-strand cDNA was synthesized by reverse transcription, in accordance with the manufacturer's instructions, using the HiScript® Q Select RT SuperMix for qPCR (+gDNA wiper) kit. In brief, the expression profile of *Mr*GIH was tested in each of the tissue types by quantitative polymerase chain reaction (qPCR). The β−actin gene (Yang et al., 2020) was used as an internal reference and the $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression levels of *Mr*GIH.

The Preparation of *ds*RNA and *Mr*GIH Silencing

To silence the function of *Mr*GIH *in vivo*, we used specific primer sequences (**Table 1**) that were linked to the T7 promoter by using a commercial transcription T7 kit (Fermentas, United States) and followed previously described methods (Qin et al., 2019a).

[1] https://www.bioinformatics.nl/emboss-explorer/
[2] http://www.cbs.dtu.dk/services/SignalP/
[3] http://smart.embl.de/
[4] https://swissmodel.expasy.org/

TABLE 1 | A list of primers used in the study.

Primers	Sequences (5′–3′)	Purpose
*ds*GIH-1-F	GCGTAATACGACTCACTATAGGGCATCCTGTTCGCATCTTGCC	*ds*GIH
*ds*GIH-1-R	GCGTAATACGACTCACTATAGGGGATGTGTTTTTTCATTTTCT	
*ds*GIH-2-F	GCGTAATACGACTCACTATAGGGCCAGGTACCTAGACGACGAA	*ds*GIH
*ds*GIH-2-R	GCGTAATACGACTCACTATAGGGGTGGAAACAAGTCAGGACAG	
*Mr*GIH-qF	CAACAGGGATCTCTACGAGAAGG	qRT-PCR
*Mr*GIH-qR	CACAGGAAGTCCACGTTGTAGAA	
*Mr*IAG-qF	CCGAGATCAAGTGTGTGTTGTTC	qRT-PCR
*Mr*IAG-qR	TATAGATGTCAGCAGATCGTCGC	
*Mr*IAGR-qF	TACATCATAGACCCCAAGGACCT	qRT-PCR
*Mr*IAGR-qR	GTTTATTGAGGTCCTTGATCGCG	
*Mr*IAGBP-qF	GGAACTCTTCTGGGAACTGAACA	qRT-PCR
*Mr*IAGBP-qR	CGGACGTTGATGTTCATGATCTG	
β-actin-F	GTCGTGACTTGACCGATTACCT	qRT-PCR
β-actin-R	ATCTCCTGCTCGAAGTCCAATG	
pEGFP-GIH-F	CCGCTCGAGATGTCTACGCAAAAGGGCCTGCG	plasmid construction
pEGFP-GIH-R	AAACTGCAGTTTTCTACCGGCTCGTAGGATGC	

In order to determine the gene silencing efficiency, a group of small individuals (20 ± 5 g) were divided into three groups (n = 4); animals from two groups were injected with *ds*GIH-1 or *ds*GIH-2 (5 μg/g body weight), respectively (Sharabi et al., 2016), while individuals from the other group were injected with *ds*GFP to serve as a control group. We evaluated the silencing efficacy of *Mr*GIH and its effect on three gonad-related genes (*Mr*IAG, *Mr*IAGR, *Mr*IAGBP) by performing a single injection into the abdominal muscles between the prawn's 4th and 5th pleopods (Levy et al., 2016) and then collecting a range of target tissues 24 h post-injection for gene expression analysis by quantitative real-time (qRT)–polymerase chain reaction (PCR) as described in our previous report (Lu et al., 2021).

Eyestalk Ablation Assays

Young males were equally divided into three groups (n = 44 per group): (1) a unilateral eyestalk ablation group; (2) a

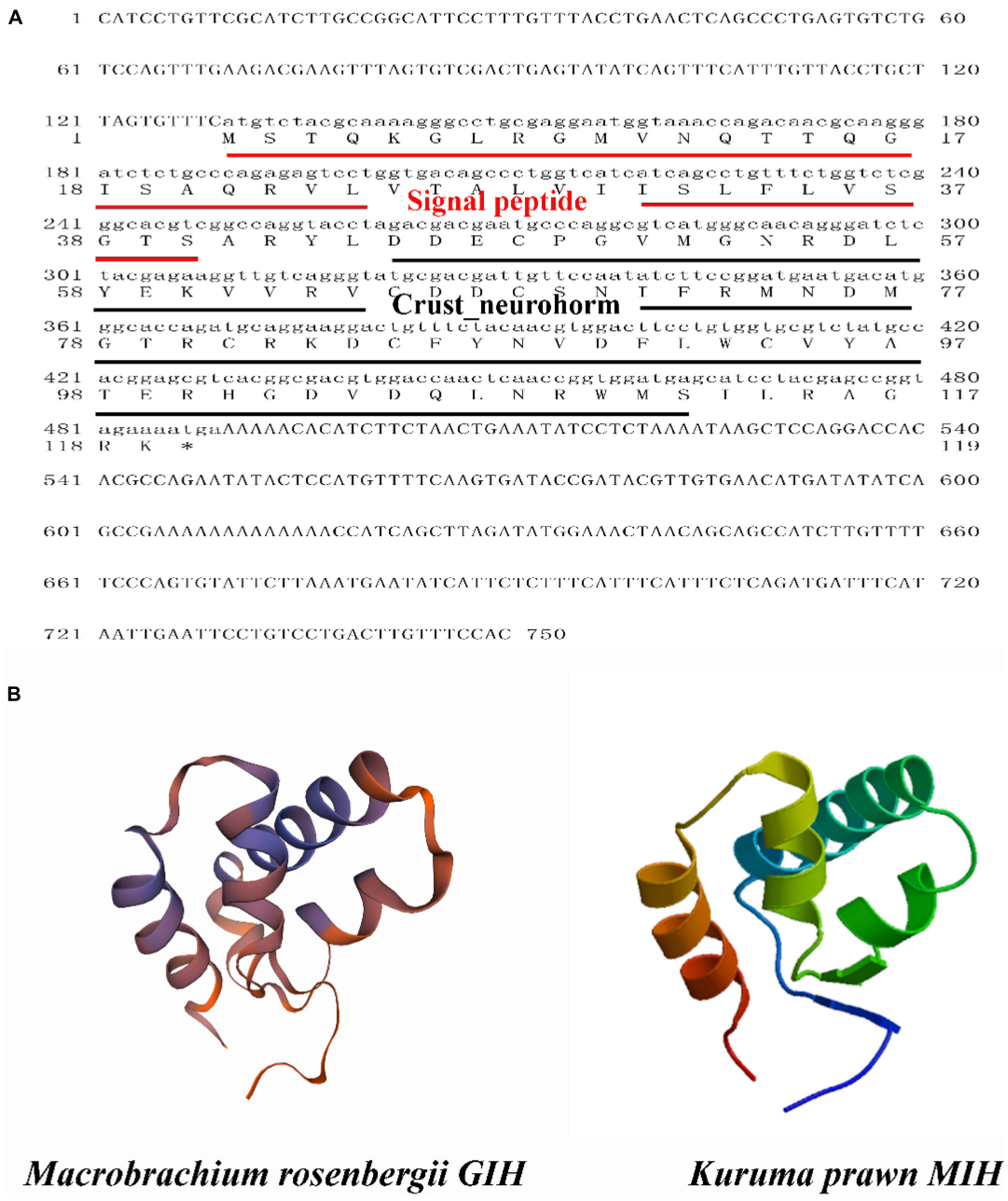

FIGURE 1 | The sequence analysis of *Mr*GIH. **(A)** The full-length ORF nucleotide sequence and deduced amino acid sequence of *Mr*GIH. Signal peptide was underlined in red lines. Crustacean neurohormone was underlined in black lines. **(B)** Protein structural homology of *Mr*GIH was modeled by SWISS-MODEL service, which built a high quality of 3D model with *kuruma prawn* MIH protein.

bilateral eyestalk ablation group; and (3) a control group (intact individuals). We performed treatments on different days (2, 4, 6, and 8 days). Following eyestalk removal, we acquired AG tissues from both the experimental group and the control group, respectively. In total, we cultured *M. rosenbergii* which had undergone eyestalk ablation for 8 days; four individuals during were dissected and sampled every 2 days. Then, relative gene expression levels were determined by qRT-PCR.

Immunofluorescence Assays

To monitor the release of *Mr*GIH and *Mr*IAGBP protein from target sections following *ds*GIH knockdown and eyestalk ablation, we conducted immunofluorescence signal assessment,

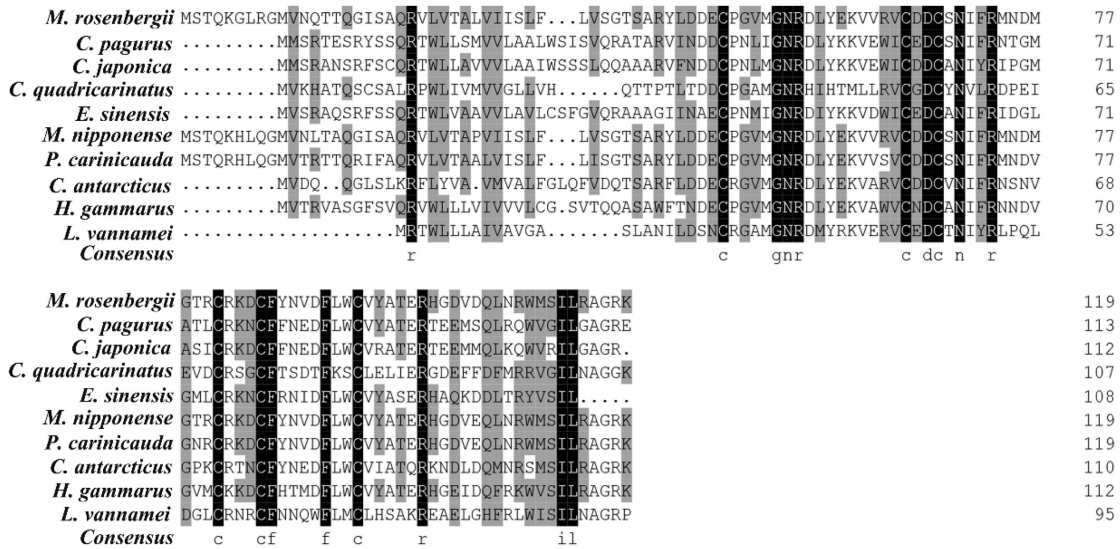

FIGURE 2 | Multiple-sequence alignments of *Mr*GIH with other crustaceans. Species of *Cancer pagurus* (CAB61424.1), *Charybdis japonica* (ACD11361.1), *Cherax quadricarinatus* (AWK57516.1), *Eriocheir sinensis* (AAQ81640.1), *Macrobrachium nipponense* (AEJ54622.1), *Palaemon carinicauda* (AIJ49750.1), *Chorismus antarcticus* (ANQ38670.1), *Homarus gammarus* (ABA42181.1), *Litopenaeus vannamei* (KF879913.1) were aligned. Highly conserved amino acids were represented by black background.

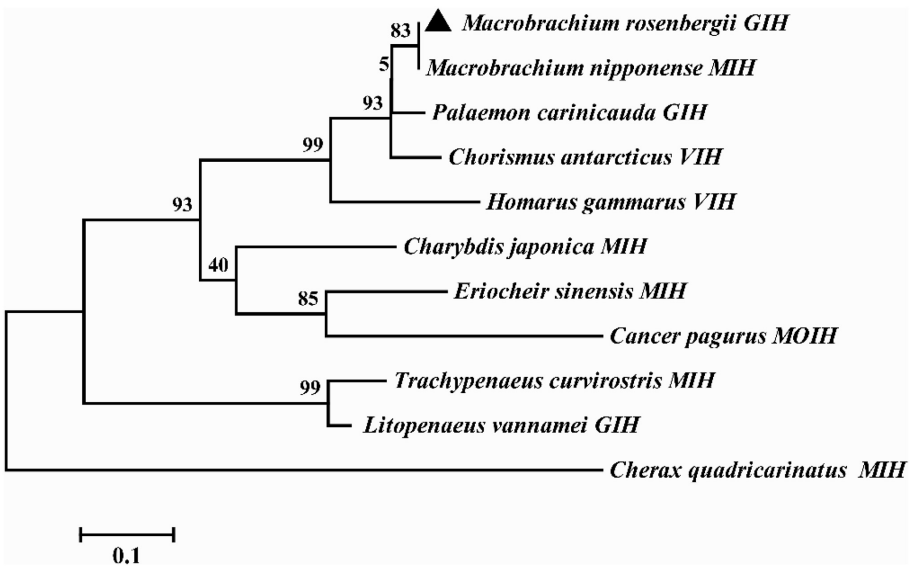

FIGURE 3 | Phylogenetic tree of *Mr*GIH with other homologous species. The amino acid sequences of *Mr*GIH from *M. rosenbergii* (marked with a triangle) and other species are listed below: *Macrobrachium nipponense* (AEJ54622.1), *Palaemon carinicauda* (AIJ49750.1), *Chorismus antarcticus* (ANQ38670.1), *Homarus gammarus* (ABA42181.1), *Charybdis japonica* (ACD11361.1), *Eriocheir sinensis* (AAQ81640.1), *Cancer pagurus* (CAB61424.1), *Trachypenaeus curvirostris* (AAL55259.1), *Cherax quadricarinatus* (AWK57516.1), *Litopenaeus vannamei* (KF879913.1).

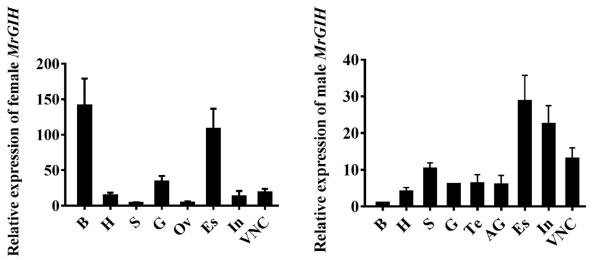

FIGURE 4 | Tissue distribution analysis of *Mr*GIH. Different tissues from healthy female and male *M. rosenbergii* were as followed: Brain (B), Heart (Ht), Stomach (S), Gills (G), Testis (Te), Ovary (Ov), Androgenic gland (AG), Eyestalk (Es), Intestine (In), and Ventral Nerve Cord (VNC). β-actin gene is as an internal reference to normalize *Mr*GIH mRNA.

as described previously (Qin et al., 2019b) but with minor modifications. Following *ds*RNA silencing and surgical removal experiments, we harvested eyestalk and AG tissues and fixed these at 4°C for 24 h in 4% paraformaldehyde solution. These tissues were then rehydrated in ethyl alcohol, dewaxed in xylene, and then cut into small sections. Later, the samples were incubated with anti-*Mr*GIH, anti-*Mr*IAGBP and dye-linked goat Cy3 conjugated anti-rabbit IgG (Guge Biotech, China), successively. DAPI was used to stain nuclei. Immunofluorescence signals were monitored by fluorescence microscopy (Leica DMI8, Germany).

Statistical Analyses

All data are represented as mean ± standard deviation (SD; $n = 3$). Statistical analyses were performed using SSPS version 23.0. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. Figures were created by GraphPad Prism 7 and statistical significance was set at $p < 0.05$.

RESULTS

Sequence Analysis and Molecular Characteristics of the *Mr*GIH Gene

Sequence analysis showed that the ORF of *Mr*GIH was 360 bp in length and encoded a *Mr*GIH protein that was 13.56 kDa in size and 119 amino acids (AAs) in length, while has a 40-aa signal peptide (**Figure 1A**). Moreover, SMART showed that *Mr*GIH was a crustacean neurohormone. Protein modeling revealed that the three-dimensional (3D) structure of the *Mr*GIH gene featured alpha helices in the N-terminal region and shared 51.28% sequence identity with MIH from the Kuruma prawn (**Figure 1B**).

Multiple Alignment and Phylogenetic Analysis of *Mr*GIH

Multiple alignment showed that the amino acids of *Mr*GIH shared 98.18% identity with *Macrobrachium nipponense* (AEJ54622.1), 89.09% identity with *Palaemon carinicauda* (AIJ49750.1), 83.64% identity with *Chorismus antarcticus* (ANQ38670.1), 58.93% identity with *Homarus gammarus* (ABA42181.1), 48.21% identity with *Charybdis japonica* (ACD11361.1), 47.22% identity with *Eriocheir sinensis* (AAQ81640.1), 48.05% identity with *Cancer pagurus* (CAB61424.1), 44.93% identity with *Trachypenaeus curvirostris* (AAL55259.1), 34.62% identity with *Cherax quadricarinatus* (AWK57516.1), and 31.67% identity with *Litopenaeus vannamei* (KF879913.1) (**Figure 2**). Phylogenetic analysis further showed that in the GIH of *M. rosenbergii* was most similar to MIH from *M. nipponense* (with a sequence similarity of 98.18%), followed by GIH from *P. carinicauda* (with a sequence similarity of 89.09%) (**Figure 3**).

Tissue Expression Profile of *Mr*GIH

Target tissues were harvested and qRT-PCR was used to determine the spatial distribution profile of *Mr*GIH. We found that *Mr*GIH mRNA was expressed at very high levels in the eyestalk both of females and males; Interestingly in the brain, female GIH is highly expressed, while male GIH is low expressed; *Mr*GIH was also expressed in a range of other tissues, but with lower levels; for example, in the androgenic gland, ovary, testis, stomach, gill, and heart (**Figure 4**).

The Effects of dsRNA-Induced *Mr*GIH Silencing on Sexual Regulation

Next, we performed experiments to investigate the effects of *Mr*GIH silencing on sexual regulation in the eyestalk

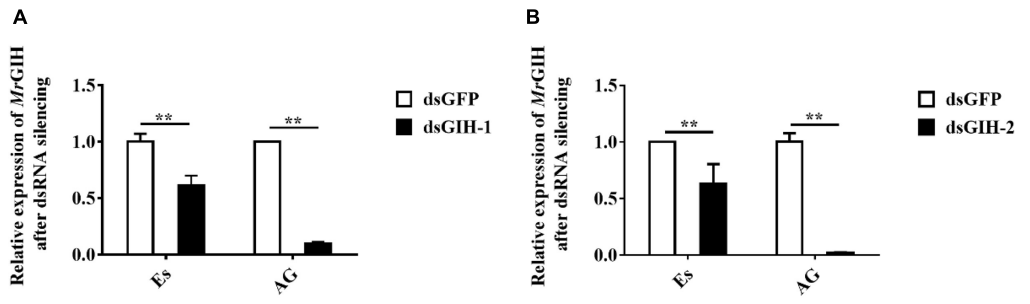

FIGURE 5 | The mRNA expression levels of *Mr*GIH after gene silencing. **(A)** The silencing efficiency of *Mr*GIH injected with *dsMr*GIH-1. **(B)** The silencing efficiency of *Mr*GIH injected with *dsMr*GIH-2, $^{**}P < 0.001$.

and AG. First, we investigated the efficiency of the two *ds*RNAs (*ds*GIH-1, *ds*GIH-2) to influence the expression of *Mr*GIH in the eyestalk and AG. Analysis showed that *ds*GIH-1 resulted in a knockdown efficiency of 39% in the eyestalk and 90% in the AG; the *ds*GIH-2 approach resulted in a knockdown efficiency of *Mr*GIH expression by 37% in the eyestalk and 98% in the AG (**Figure 5**), thus suggesting that the *ds*RNAs created by our special primers led to a

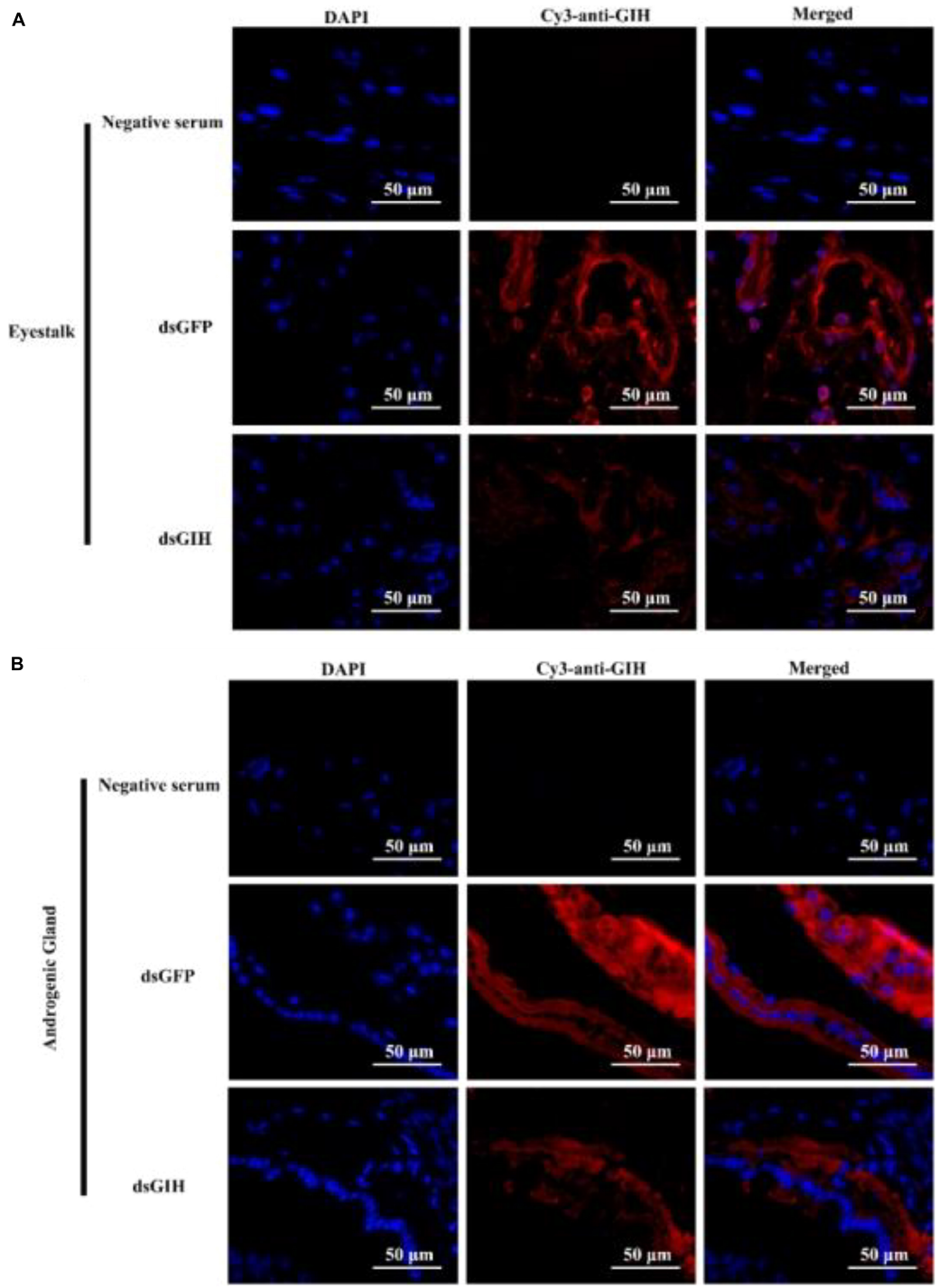

FIGURE 6 | The protein expression levels of *Mr*GIH after gene silencing using immunohistochemistry. *ds*GFP, injected with *ds*GFP RNA; *ds*GIH, injected with *ds*GIH RNA. *Mr*GIH was in red fluorescence signals. The protein expression levels of *Mr*GIH in the eyestalk **(A)** and androgenic gland **(B)** after injected with *ds*MrGIH at 24 h. Negative serum served was as the control. Scale bar was indicated 50 μm.

significant reduction in the expression of *Mr*GIH. It is suggested that *ds*GIH-2 should be used to detect *Mr*GIH expression in the subsequent immunofluorescence assays. As shown in **Figure 6**, the expression of *Mr*GIH was down-regulated by *ds*GIH-2 in the eyestalk and AG when compared to the *ds*GFP group.

Twenty-four hours after the knockdown of *Mr*GIH, qRT-PCR showed that the mRNA expression of *Mr*IAG and

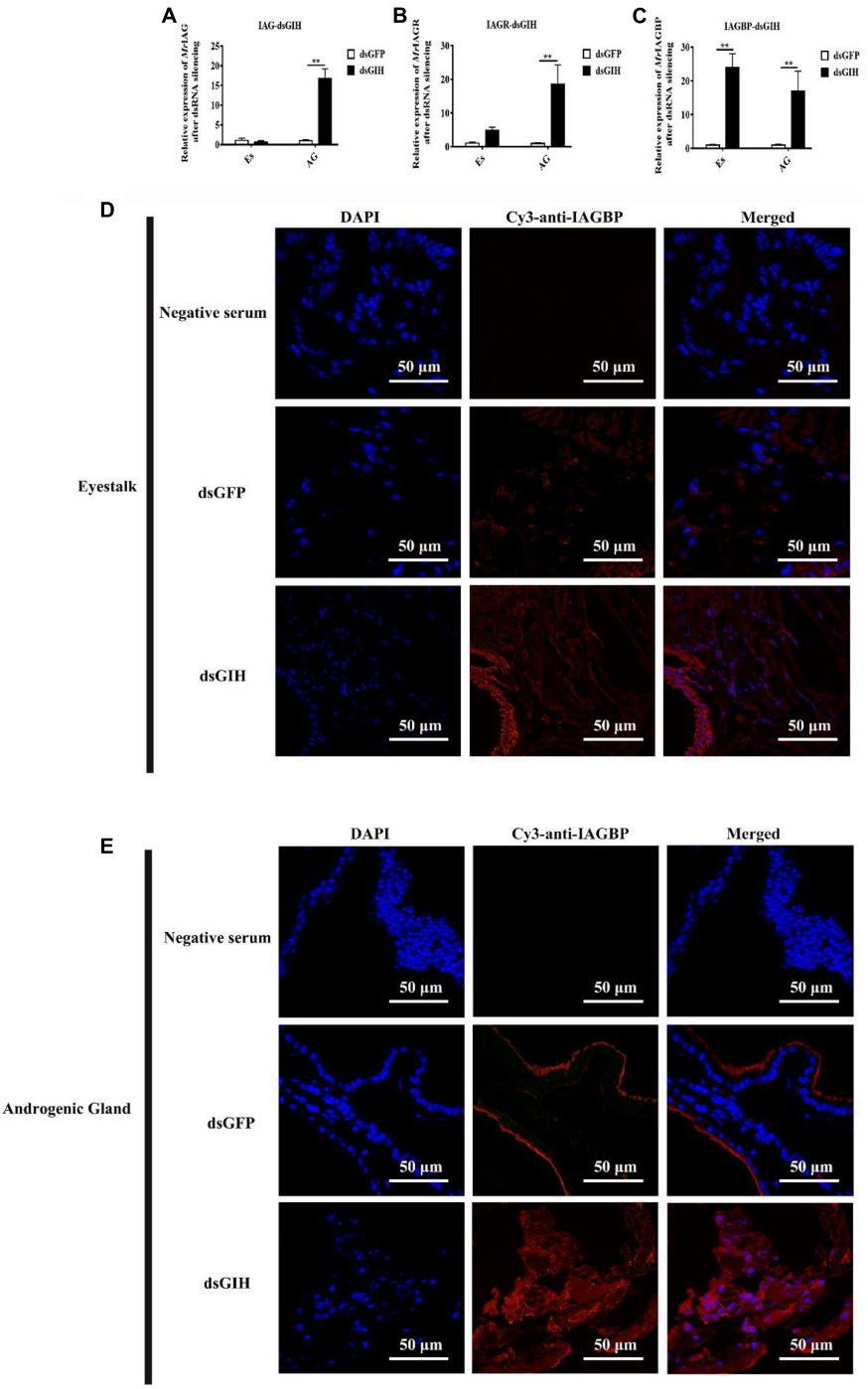

FIGURE 7 | Effects of *ds*RNA-*Mr*GIH injection. The mRNA expression levels of *Mr*IAG **(A)**, *Mr*IAGR **(B)**, and *Mr*IAGBP **(C)** in eyestalk (Es) and androgenic gland (AG) after *Mr*GIH knockdown at 24 h. *ds*GFP, injected with *ds*GFP RNA; *ds*GIH, injected with *ds*GIH RNA. The protein expression levels of *Mr*IAGBP in the eyestalk **(D)** and androgenic gland **(E)** after injected with *dsMr*GIH at 24 h. *Mr*IAGBP was in red fluorescence signals. Negative serum served was as the control. Scale bar was indicated 50 μm. $^{**}P < 0.001$.

*Mr*IAGR had increased significantly in the AG; there was no significant difference with regards to the expression levels of these two mRNAs in the eyestalk (**Figures 7A,B**). Surprisingly, the expression levels of *Mr*IAGBP mRNA were significantly upregulated in both the AG and the eyestalk (**Figure 7C**). Therefore, we carried out further immunofluorescence assays to investigate the specific relationship between the *Mr*GIH and *Mr*IAGBP proteins. Analysis showed that *Mr*IAGBP protein expression increased in the eyestalk and AG following the knockdown of *Mr*GIH (**Figures 7D,E**). Collectively, these results indicate that *Mr*GIH is an inhibitory factor that inhibits sex-related genes in the "eyestalk-AG-testis" pathway.

Surgical Removal of the Eyestalk in *M. rosenbergii*

As shown in **Figure 4**, the highest expression levels of *Mr*GIH were identified in the eyestalks of healthy male prawns. To further investigate the regulatory activity of *Mr*GIH hormone in more detail, we designed an experiment involving unilateral or bilateral eyestalk removal in male *M. rosenbergii*. Following unilateral eyestalk ablation, the expression levels of *Mr*IAG in the AG were significantly up-regulated at 4 and 8 d, exhibiting a 14.77- and 5.96-fold increase, respectively (**Figure 8A**). The expression levels of *Mr*IAGR in the AG peaked (a 15.51-fold increase) at 2 d and remained at high levels (14. 57-, 11. 86-, and 9.84-fold increases at 4, 6, and 8 days, respectively) (**Figure 8B**). In the AG, the expression levels of *Mr*IAGBP increased by 7.27, 5. 80-, 4. 04-, and 5.18-fold at 2, 4, 6, and 8 days, respectively (**Figure 8C**).

Following bilateral eyestalk ablation, the expression levels of *Mr*IAG in the AG increased significantly at 2 and 4 days, exhibiting a 19.78- and 36.27-fold increase, respectively. Levels were highest (a 74.36-fold increase) at the 6 d timepoint; by the 8 days timepoint, there was an 11.68-fold increase (**Figure 8a**). The expression levels of *Mr*IAGR exhibited a 13. 31-, 26. 50-, 40. 65-, and 7.06-fold increase at 2, 4, 6, and 8 days, respectively (**Figure 8b**). The expression levels of *Mr*IAGBP in the AG peaked at 2 d (a 6.03-fold increase) and remained high from 4 to 8 days, exhibiting a 4. 42-, 3. 03-, and 3.65-fold increase at 4, 6, and 8 days, respectively (**Figure 8c**). To further investigate the role of *Mr*GIH protein under unilateral or bilateral eyestalk ablation in male *M. rosenbergii*, we evaluated the expression levels of *Mr*IAGBP protein in the AG at 2, 4, 6, and 8 days by immunohistochemistry analyses; we used animals with intact eyestalks as controls. Our results showed strong immunostaining for *Mr*IAGBP (red staining) in animals undergoing both unilateral (**Figure 9**) and bilateral eyestalk ablation (**Figure 10**) in AG tissues at 2, 4, 6, and 8 days.

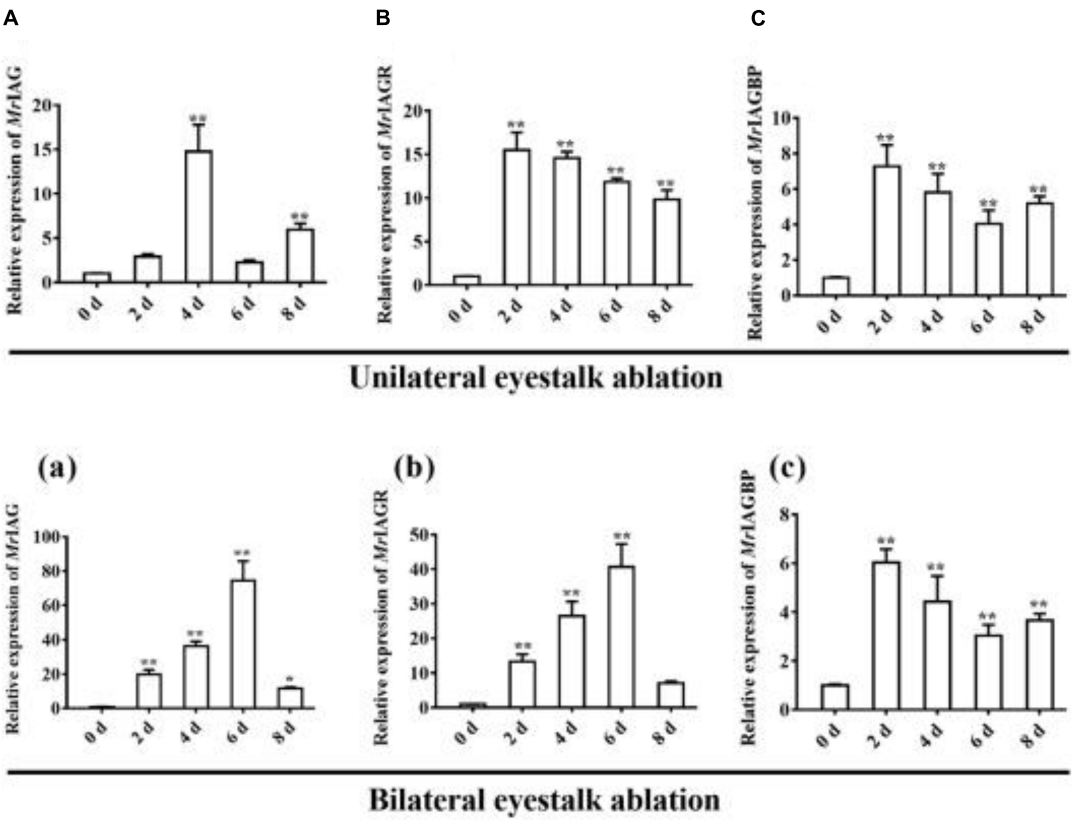

FIGURE 8 | Expression profile of IAG, IAGR, and IAGBP gene on different days in androgenic gland after eyestalk ablation. **(A–C)** unilateral eyestalk ablation, **(A)** *Mr*IAG, **(B)** *Mr*IAGR, (**C**) *Mr*IAGBP. **(a–c)**: bilateral eyestalk ablation, **(a)** *Mr*IAG, **(b)** *Mr*IAGR, **(c)** *Mr*IAGBP. *Mr*IAGBP was in red fluorescence signals. mRNA levels were analyzed by qRT-PCR and normalized to β-actin. qRT-PCR data are shown as means ± SD (standard deviation). $^{*}P < 0.05$, $^{**}P < 0.001$.

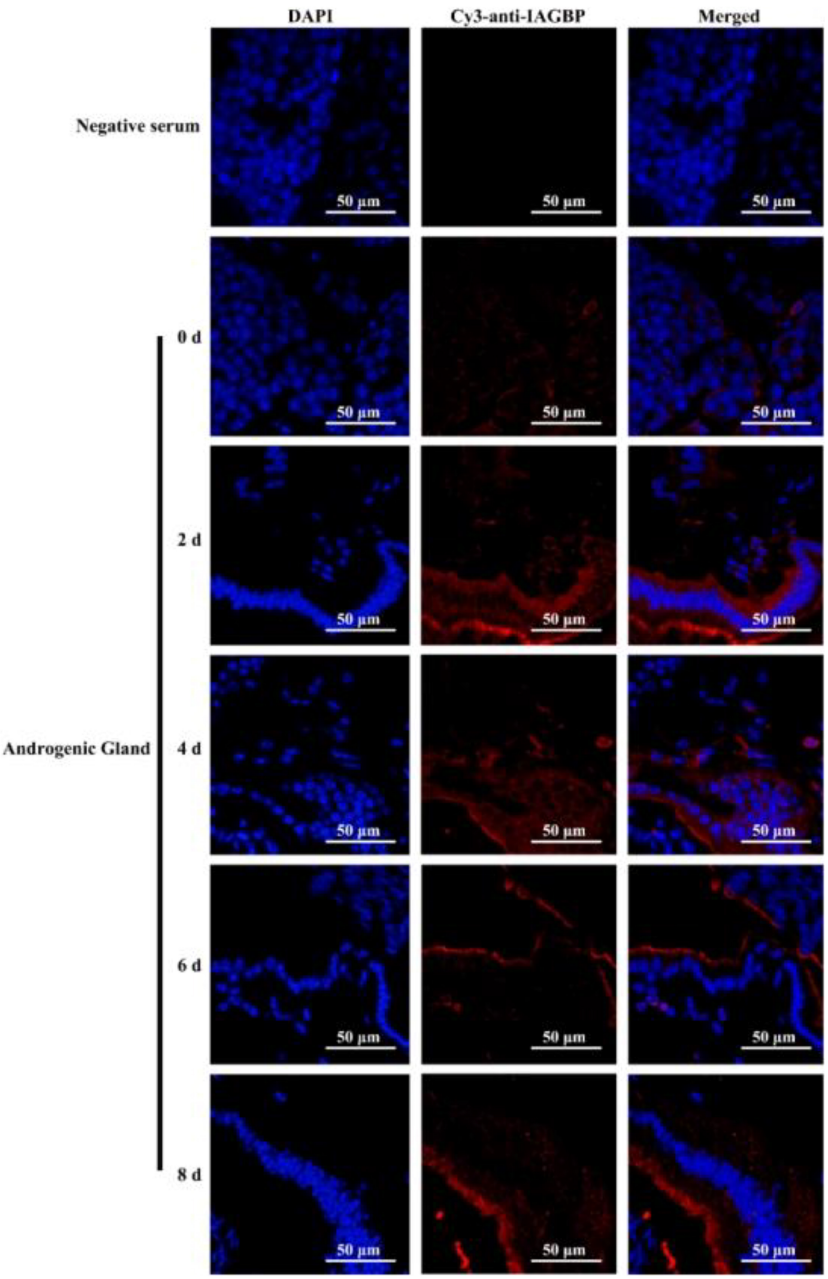

FIGURE 9 | The protein expression levels of *Mr*IAGBP on different days in androgenic gland after unilateral eyestalk ablation. *Mr*IAGBP was in red fluorescence signals. Negative serum served was as the control. Scale bar was indicated 50 μm.

These results were confirmed by qRT-PCR, which also provided evidence for high expression levels of *Mr*IAGBP in the AG following eyestalk ablation.

DISCUSSION

Secretory hormones play an important role in sex determination and sex differentiation in crustaceans, particularly AG genes and genes from the CHH superfamily. The eyestalk-AG-testis axis is also known to be a significant signaling pathway in male crustaceans and plays a key role in regulating sexual determination (Khalaila et al., 2002). The IAG signaling pathway has been investigated in different species of crustaceans (Rosen et al., 2010; Chung et al., 2011; Herran et al., 2020; Tan et al., 2020; Liu et al., 2021). However, previous research did not identify the precise relationship between GIH-mediated activity in the AG and sex differentiation in *Macrobrachium rosenbergii*. Therefore, in the present study, we isolated and characterized the GIH gene from *M. rosenbergii* (referred

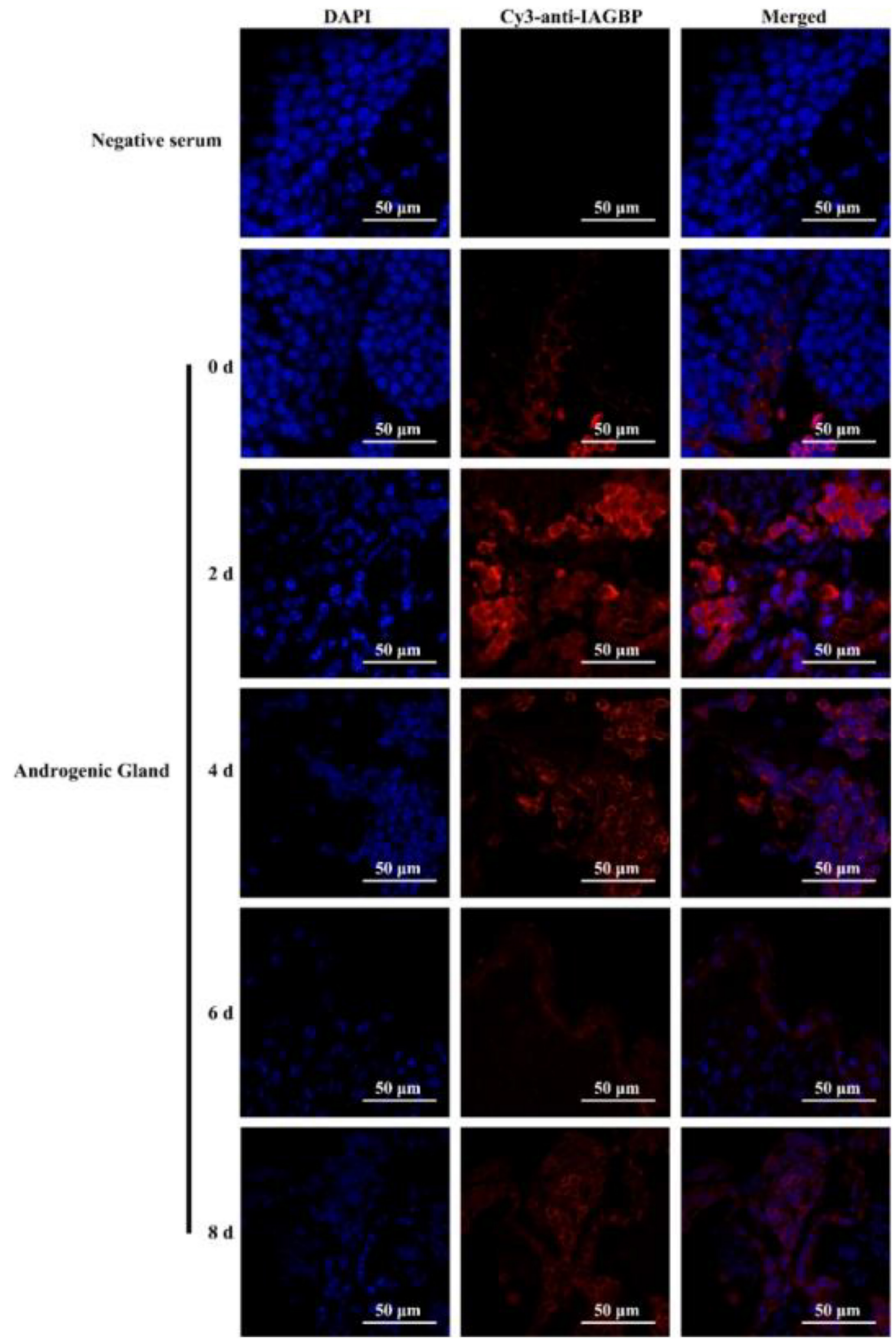

FIGURE 10 | The protein expression levels of *Mr*IAGBP on different days in androgenic gland after bilateral eyestalk ablation. *Mr*IAGBP was in red fluorescence signals. Negative serum served was as the control. Scale bar was indicated 50 μm.

to herein as *Mr*GIH) and investigated its potential role in sexual regulation. As a member of the CHH superfamily II, *Mr*GIH possess several conserved CHH domains, including the crust_neurohorm domain (Davey et al., 2000) but without a precursor-related peptide (Chung et al., 2010).

The ORF for the *Mr*GIH cDNA was 360 bp in length and encoded a protein containing 119 amino acids, as also reported in previous studies (Shi et al., 2018; Chen et al., 2020). In addition, multiple sequence alignment and the generation of a phylogenetic tree indicated that *Mr*GIH shared a high degree of similarity to MIH from *Macrobrachium nipponense* (Qiao et al., 2018). This suggests that in addition to its characteristic ability to inhibit gonads, the GIH gene can also regulate molting activity, as previously reported in *Homarus americanus* (de Kleijn et al., 1994). In the present study, we found that the *Mr*GIH gene was mainly expressed in the eyestalks of *Macrobrachium*

rosenbergii, although the transcription of GIH was also detected in the gills, mid-gut gland, hemolymph, nerve cord, thoracic ganglion, ovary, testis, muscle, and spermatophore, as previously reported for *Nephrops norvegicus* (Edomi et al., 2002), *Penaeus monodon* (Treerattrakool et al., 2008), and *Fenneropenaeus* (Shi et al., 2018).

The IAG signaling pathway is directly activated by the binding of insulin-like peptides to their receptors, including IAGR and IAGBP. For example, the knockdown of IAGBP in *M. rosenbergii* (Yang et al., 2020) and the knockdown of IAGR in *M. nipponense* (Li et al., 2015a) lead to the downregulation of IAG expression. The XO-SG, located at the base of the eyestalk, is the central-hub of the neuroendocrine system in crustaceans (Dircksen et al., 2001). Neurohormones of the CHH family are structurally related peptides that are encoded by a multigene family that is specific to arthropods (Hopkins, 2012). In decapods, these neurohormones are mainly produced by the XO-SG system, a major neuroendocrine organ situated in the eyestalk (De Kleijn and Van Herp, 1995). These hormones play important roles in metabolism, reproduction, and development in animals (Escamilla-Chimal et al., 2001). We aimed to investigate the role of GIH mediation in AG activity and establish whether eyestalk-derived genes regulate the sex-related genes in the eyestalk-AG-testis endocrine axis. In our study, we found that *Mr*GIH was a member of the CHH subfamily II, as reported previously (Soyez, 1997; Chen et al., 2020). In this research, we propose that *Mr*GIH, which serves as an upstream gene for the eyestalk-AG-testis endocrine axis and is responsible for inhibiting sexual maturation and sex differentiation in male *M. rosenbergii*. Thus, we performed *ds*RNA-*Mr*GIH knockdown. We found that the knockdown of *Mr*GIH facilitated the expression of *Mr*IAG, *Mr*IAGR, and *Mr*IAGBP hormones and promoted the expression of *Mr*IAGBP protein. Collectively, these data indicated that *Mr*GIH was associated with sexual regulation systems in the male eyestalk, and acts as an inhibitory factor that regulates sex-related genes.

Eyestalk-derived factors, acting under hormonal stimulation, can also regulate other physiological processes (Chen et al., 2020). As part of the eyestalk-AG-testis pathway, the XO-SG complex can activate the expression of multiple target genes. In the crayfish *Cherax quadricarinatus*, the addition of sinus gland extract led to the significant inhibition of amino acid incorporation into the AG (Khalaila et al., 2002). Furthermore, eyestalk ablation led to hypertrophic glands and the overexpression of IAG hormones (Chung et al., 2011; Li et al., 2015b; Guo et al., 2019). In our study, we found that the unilateral and bilateral removal of eyestalks in *M. rosenbergii* led to an increase in the expression levels of *Mr*IAG, *Mr*IAGR, and *Mr*IAGBP. This data proved that the presence of neuropeptides in the eyestalk could regulate the expression of the *Mr*IAG, *Mr*IAGR, and *Mr*IAGBP genes. Our data suggest that *Mr*GIH plays an essential role in regulating upstream components in the eyestalk-AG-testis pathway of prawns.

Collectively, we isolated and characterized GIH in *M. rosenbergii* and demonstrated its role as a key regulator of sexual differentiation and an inhibitor for the expression of sex-related genes. Furthermore, our findings have significant aquacultural implications because they highlight a pathway enabling that allows us to identify and analyze the GIH-induced mediation of AG activity and interactions with sex-regulatory mechanisms. Although previous research has described the effects of the eyestalk-AG-testis pathway on sexual activity in decapod crustaceans, the specific mechanisms underlying the effects of AG inhibitory factors produced by eyestalk and the axis itself still remain to be characterized and elucidated.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

LL and ZQ conceived and designed the experiments. MT and ZL performed the experiments and wrote the manuscript. GY, VB, and ZX analyzed the data. All authors read the manuscript and approved it in its final version.

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