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G-protein-coupled estrogen receptor 1 is involved in brain development during zebrafish (*Danio rerio*) embryogenesis

Yanan Shi [a], Xiaochun Liu [a], Pei Zhu [b,1], Jianzhen Li [b,2], Kathy W.Y. Sham [b], Shuk Han Cheng [c], Shuisheng Li [a], Yong Zhang [a], Christopher H.K. Cheng [b,*], Haoran Lin [a,d,*]

[a] State Key Laboratory of Biocontrol, Institute of Aquatic Economic Animals and Guangdong Provincial Key Laboratory for Aquatic Economic Animals, School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, China
[b] School of Biomedical Sciences, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China
[c] Department of Biology and Chemistry, City University of Hong Kong, Kowloon, Hong Kong, China
[d] College of Ocean, Hainan University, Haikou 570228, Hainan, China

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ABSTRACT

G-protein-coupled estrogen receptor 1 (Gper, formerly known as GPR30) is found to be a trophic and protective factor in mediating action of estrogen in adult brain, while its role in developing brain remains to be elucidated. Here we present the expression pattern of Gper and its functions during embryogenesis in zebrafish. Both the mRNA and protein of Gper were detected throughout embryogenesis. Whole mount *in situ* hybridization (WISH) revealed a wide distribution of *gper* mRNAs in various regions of the developing brain. Gper knockdown by specific morpholinos resulted in growth retardation in embryos and morphological defects in the developing brain. In addition, induced apoptosis, decreased proliferation of the brain cells and maldevelopment of sensory and motor neurons were also found in the morphants. Our results provide novel insights into Gper functions in the developing brain, revealing that Gper can maintain the survival of the brain cells, and formation and/or differentiation of the sensory and motor neurons.

1. Introduction

17β-Estradiol (E_2) is a pleiotropic hormone. Its primary action in reproduction is exerted in every reproductive stage [1]. Besides, it can also regulate metabolism, the cardiovascular and immune systems, and many other physiological processes [2].

More and more studies have proved that E_2 is an important factor in regulating brain functions. After the discovery of aromatase in certain hypothalamic areas in adult rat [3], the brain was regarded as a unique source of E_2. Classically, E_2 action on brain functions is mainly in the central control of reproduction, such as sex behaviors [4]. Additionally, E_2 acts as a neurotrophic and neuroprotective factor for fine motor control, mental state regulation and many other brain functions and behaviors [5].

Apart from functions in the adult brain, E_2 also influences neuronal development. During ontogeny, it is synthesized throughout the brain [6], and capable of promoting the proliferation of hippocampal neuron progenitor cells and neurogenesis in rat [7]. Moreover, E_2 could affect the migration of neuroblasts from the inner side of the neural tube to the settled location where they develop into mature neural cells [8]. It also regulates synapse formation through enhancing the growth and arborization of axons and dendrites [9].

It is generally accepted that E_2 elicits its effects through estrogen receptors (ERs). Classical nuclear ER (i.e., ERα and ERβ) are ligand-regulated transcription factors, mediating long-term genomic effects on the target genes. ERα is considered being crucial for neuroreproduction [10]. ERβ predominantly mediates many of the non-reproductive functions of E_2 in the adult brain [11]. However, in the developing brain of rat, ERβ is the predominant form distributing extensively in the limbic forebrain, midbrain and hypothalamus [12]. Similarly, ERβ2 is the main form during embryogenesis of zebrafish, and its knockdown affects development of sensory hair cells [13].

A novel membrane estrogen receptor Gper is found to mediate the rapid non-genomic actions of E_2 [14]. Gper-immunoreactive cells are located in many brain regions of rat [15], and positioned to mediate cognitive performance [16], sensory nociceptive transmission [17] as well as neuroprotection [18]. Despite considerable

* Corresponding authors. Address: School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, China. Fax: +86 20 84113717 (H. Lin). Address: School of Biomedical Sciences, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China. Fax: +852 2603 5123 (C.H.K. Cheng).
E-mail addresses: chkcheng@cuhk.edu.hk (C.H.K. Cheng), lsslhr@mail.sysu.edu.cn (H. Lin).
[1] Present address: Julius L. Chambers-Biomedical/Biotechnology Research Institute North Carolina Central University, USA.
[2] Present address: College of Life Sciences, Northwest Normal University, Lanzhou, China.

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advances in knowledge about Gper in adult brain, its involvement in the developing brain remains elusive. Although the non-genomic signaling of E_2 in the developing mammalian brain was described a long time ago showing that its activation of intracellular signal transduction pathways can promote neuronal growth and differentiation [19], the nature of this membrane-associated ER was still unclear at that time. Recently, Gper is found to be involved in zebrafish embryogenesis [20]. However, whether it is involved in the development of nervous system, and how it mediates action of E_2 in the developing brain is still poorly understood.

To address the above issues, we set out to explore the role of Gper in the development of the nervous system during embryogenesis in zebrafish. Through Gper expression and functional studies, we discovered that Gper is essential in maintaining brain development in zebrafish, paving the way for an in-depth understanding of how Gper and E_2 within the brain exert their effects during ontogeny.

2. Materials and methods

2.1. Animals

Wild-type zebrafish were purchased locally. Adult fish were raised at 26–28 °C in a re-circulating aquaculture system on a 14 h:10 h (light:dark) cycle, and fed twice daily with newly hatched brine shrimp (Brine Shrimp Direct, USA). Embryos were generated from natural crosses and incubated in Versatile Environmental Test Chamber (MLR-350H, SANYO) at 28.5 °C in egg water [60 μg/ml sea salt (Red Sea Fish Pharm., Israel) in MILLI Q water with 0.001% methylene blue], and staged by hours post fertilization (hpf) as described [21]. Embryos after 12 hpf for all experiments except RT-PCR and Western blot were cultured in egg water with 0.003% 1-phenyl-2-thiourea (Sigma, USA) for removing zebrafish pigment.

2.2. Chemicals

AR grade chemicals were obtained from Sigma–Aldrich (USA), culture media from Gibco Life Technologies (USA), and enzymes from Promega (USA) unless otherwise stated.

2.3. RT-PCR

Total RNAs of zebrafish ovary and embryos was extracted using TRIzol Reagent (Life Technologies, USA). The quality and quantity of RNA were determined on a NanoDrop 2000C Spectrophotometer (Thermo, USA). A genomic test primer pair (5′-TGATCTACAAATGCGGTGGA-3′ and 5′-TCGAATTTCCAGAGAGCAATG-3′) was used to detect the genomic contamination during cDNA synthesis. One μg RNA was reversely transcribed into cDNA using the Reverse Transcription System (Promega, USA), and the product was amplified into *gper* using the primer pair [22]: 5′-CTCCATCCTGGCAACTCT-3′ and 5′-TATCCCTGAAGGTCTCCC-3′, and *elfα* using the primer pair: 5′-CTTCTCAGGCTGACTGTGC-3′ and 5′-CCGCTAGCATTACCCTCC-3′.

2.4. Western Blot analysis

A polyclonal antibody of zebrafish Gper was raised in New Zealand rabbit against a synthetic peptide (CGTADPSKRTDTTLWHDY) in the first extracellular region near the C-terminus of the Gper protein. The specificity of the antibody was tested by Western blot on extracts from zebrafish brain or Gper-transfected COS-7 cells with/without pre-treatment with the zebrafish Gper blocking peptide (1:4). Gper-transfected COS-7 cell line was prepared and confirmed previously [22]. Secondary antibody was goat anti-rabbit IgG (Life Technologies, USA). About 50 embryos of each group were used for protein preparation, and Western blot analysis was performed as previously described [22].

2.5. Morpholino and mRNA microinjections

Gene-specific morpholino-modified oligonucleotides (MO) were purchased from Gene Tools, LLC (Philomath, OR, USA): zebrafish standard control MO (5′-CCTCTTACCTCAGTTACAATTTATA-3′), zebrafish *gper* ATG-form MO1 (5′-TCACATTGGTAGTCTGCTCCTCCAT-3′), zebrafish *gper* ATG-form MO2 (5′-AGGTGCTACATACTTCATCTGTGTC-3′), zebrafish *p53* MO (5′-GCGCCATTGCTTTGCAAGAATTG-3′). Stock MO solutions were diluted in 1× Danieau buffer [58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5.0 mM HEPES pH 7.6]. Microinjection was employed as described [23].

For mRNA synthesis, cDNA encoding the full-length *gper* was amplified by RT-PCR using *pfu* DNA Polymerase (Promega, USA) and the primer pair: 5′-TAGGGATCCATGGAGGAGCAGACTACCAATGTG-3′ and 5′-AGTCGGCCGCTACACCTCAGACTCACTCCTGACAG-3′. The product was purified by PCR Purification Kit (Qiagen, USA) and digested with *Bam*HI and *Eag*I endonuclease (NEB, UK). The fragment was cloned into *Bam*HI and *Eag*I digested $pCS2^+$ vector, and transcribed *in vitro* into mRNA by mMESSAGE mMACHINE Kit (Ambion, USA) and purified by RNeasy Mini Kit (Qiagen, USA). For rescue experiments, 200 pg mRNA was co-injected with *gper* MO in each embryo as described [24]. *p53* MO was co-injected at equal dose as *gper* MO to suppress off-target effects caused by morpholino knockdown [25].

2.6. Whole mount in situ hybridization

WISH was performed as described with modifications [26]. Full-length *gper* was prepared by RT-PCR from zebrafish ovary RNA using *pfu* DNA Polymerase and the primer pair: 5′-ATGGAGGAGCAGACTACCAATGTG-3′ and 5′-CTACACCTCAGACTCACTCCTGACAG-3′. The product was purified and cloned into pGEM-T Easy Vector (Promega, USA), and then linearized by the restriction enzymes *Nco*I and *Nde*I (NEB, UK) respectively, followed by *in vitro* transcription with either T7 or SP6 RNA polymerase to generate the antisense or sense RNA riboprobes using the DIG RNA Labeling Kit (Roche, USA) and subsequent purification of the riboprobes. The staining reaction was monitored by color examination on a stereomicroscope and pictured on a Leica camera DFC420. The embryos were finally stored in PBT at 4 °C. The *dlx2*, *emx1*, *pax2.1*, *otx2*, *krox20* probes were prepared as reported previously [27].

2.7. Whole mount immunofluorescence histochemistry

Embryos were prepared as described above in WISH and rehydrated before treating with 10% goat serum in PBS for 4 h at room temperature to block the non-specific binding sites, and incubated in primary antibodies overnight at 4 °C. Primary antibodies used were: acetylated-tubulin (AcTub) (1:1000 St. Louis, MO, USA), Zn-5 and Zn-12 (1:100; DSHB), Znp-1 (1:10; Zebrafish International Resource Center). After thorough washing, embryos were incubated in secondary antibody (goat anti-mouse Alexa 488, Molecular Probes Inc., USA) to develop the fluorescent signals. Fluorescence images were visualized and photographed on a spinning disk confocal microscope (Olympus BX61 DSU) connected to an EM-CCD camera.

2.8. AO staining

Embryos at 30 hpf were manually dechorionated and incubated in AO solution (5 μg/ml in egg water) in egg chamber for 30 min.

Followed by 5 times PBS washes, embryoswere visualized under a Leica DMIL Inverted Microscope equipped with fluorescence optics. Photographs were then taken using a Leica DFC420 5 M Color CCD camera.

2.9. TUNEL assay

Cell apoptosis was detected by TUNEL using the *In Situ* Cell Death Detection Kit (Roche,USA). Pictures were taken on a Leica DFC420 5 M Color CCD camera.

2.10. Annexing V and PI staining

About 100 dechorionated embryos were deyolked, and embryo cells were pelleted and filtered to obtain single-cell suspensions as described [28,29]. After PBS wash, cells were fixed in 70% ethanol for 1 h, incubated in PI solution (50 μg/ml PI, 100 μg/ml RNase Type I, 0.1% sodium citrate, 0.0002% Triton X-100) at 4 °C for 1 h in dark before analysis on a FACScan Flow Cytometer (BD Biosciences, USA).

2.11. Edu Incorporation

Cell proliferation was detected by the Click-iT Edu Imaging Kit (Life Technologies, USA), and then examined on a Leica DFC420 5 M Color CCD camera.

3. Results and discussion

3.1. Gper is expressed during early embryogenesis of zebrafish

RT-PCR was firstly employed to investigate *gper* expression during zebrafish embryogenesis. Genomic test PCR showed no genomic DNA contamination during cDNA synthesis (data not shown). As shown in Fig. 1A, *gper* mRNA can be detected throughout early embryonic development from 0 hpf to 72 hpf, with relatively higher levels after 24 hpf.

Gper specific polyclonal antibody was developed to detect its protein expression. The specificity of the antibody was validated by Western blot analysis on protein preparations of zebrafish brain or Gper-transfect COS-7 cells in which a band around 40 kDa was detected as predicted (Fig. 1B). While another band around 30 kDa disappeared when the antibody was pre-incubated with Gper blocking peptide (Fig. 1C), indicating the authenticity of the 40 kDa signal. Gper protein was detected throughout embryogenesis with a gradually increasing pattern (Fig. 1D), being consistent with its mRNA expression pattern. The non-specific 30 kDa band was detected only at 24 hpf and 36 hpf, suggesting that the 30 kDa protein may be more important for embryogenesis at these developmental stages.

WISH was further employed to determine the spatial expression of *gper*. As shown in Fig. 1E, *gper* can be detected in blastomeres at 4 hpf. At 18 hpf, its expression was obviously concentrated in the central nervous system. At 24 hpf, the signal aggregated towards the head including the anterior diencephalon, midbrain and hindbrain. The presence of *gper* at 36 hpf was similar to that at 24 hpf, being more remarkable in the eye area, diencephalon, midbrain and mid-hindbrain boundary (MHB). At 48 hpf and 72 hpf, *gper* was detected in the trigeminal ganglia as well as in the heart, pancreas and intestinal bulb [30]. The synchronous WISH using the *gper* sense probe can hardly detect any signal in embryos at these stages (data not shown). Trigeminal ganglia contains sensory neurons mediating the perception of pain and temperature [31]. Localization of Gper in a population of neurons of the sensory ganglia (dorsal root, nodose and trigeminal ganglia) in adult rat implies its involvement in the processing of sensory information [32]. Therefore, involvement of zebrafish Gper in the development

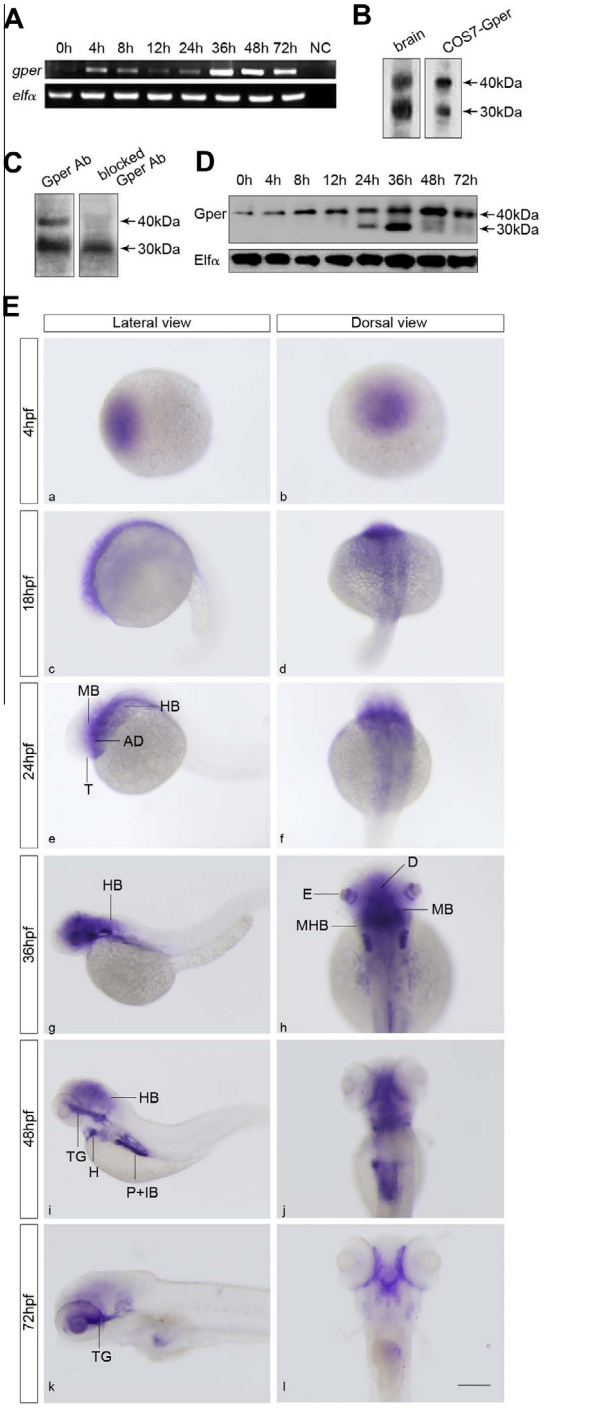

Fig. 1. Gper expression in zebrafish embryogenesis. (A) Expression of *gper* in embryogenesis. Double distilled water was added instead of cDNA template in the negative control. Amplification of *elfα* was used as an internal control. (B) Validation of antibody of zebrafish Gper in zebrafish brain or Gper transfected COS-7 cells. (C) Validation of antibody of zebrafish Gper with blocked Gper antibody. (D) Western blot analysis of Gper in embryogenesis. Rabbit polyclonal antibody against Elfα (Abcam, UK) was used as the internal reference. (E) Localization of *gper* by WISH in embryogenesis (lateral view from left: a, c, e, g, i, k; dorsal view: b, d, f, h, j, l). h: hours post fertilization; NC: negative control; cos7-Gper: Gper transfected COS-7; Gper Ab: antibody of zebrafish Gper; blocked Gper Ab: Gper Ab pre-incubated with Gper blocking peptide; T: telencephalon; MB: midbrain; AD: anterior diencephalon; HB: hindbrain; D: diencephalon; H: heart; TG: trigeminal ganglia; P + IB: pancreas and intestinal bulb; E: eye. Scale bar = 350 μm.

of sensory neurons stands to reason. Our results on Gper expression profile are consistent with recent findings [20]. The present study supplies additional information on protein expression of Gper in further substantiation of its physiological function in zebrafish embryogenesis.

3.2. Gper knockdown caused growth retardation and developmental deformity

Two independent MOs of ATG-form were employed to further elucidate the role of Gper during zebrafish embryogenesis. As demonstrated by Western blot analysis, 8 ng MO1 or 8 ng MO2 per embryo could efficiently block translation of the endogenous *gper* at 24 hpf. The non-specific 30 kDa band still remained, indicating the specificity of gene knockdown by morpholinos (Fig. 2A and B). Thus, 8 ng MOs were injected in the subsequent experiments. *gper* has only two exons and intron insertion in its transcript may not result in gene knockdown. Therefore, two distinct MOs of ATG-form were employed instead of splice-form to confirm MO knockdown effects.

The Gper-knockdown embryos were examined at 24 hpf under a microscope. It was found that MO1 and MO2 morphants displayed similar malformed morphology. The degrees of deformities were classified into mild and severe defects. As shown in Fig. 2C, control embryos were morphologically indistinguishable from their wild-type siblings, but *gper* morphants with mild defect possessed slightly smaller hindbrain. The morphants with severe defects were more abnormal with underdeveloped heads and shrunken body size whereas the rescued embryos recovered to some extents. However, Gper antagonist G15 was previously used to block Gper in embryos, but no effects on gross morphology was resulted [20]. It is reasonable because it is still controversial whether G15 effectively blocks Gper [33]. Nevertheless, *gper* morpholino knockdown in our study is more specific in defining the function of this receptor in normal embryogenesis.

Approximately 66.2% and 61.4% of MO1 and MO2 morphants displayed abnormal phenotypes, and 11.8% and14.5% of them were normal respectively. However, about 37.9% and 46.1% of rescued MO1 and MO2 morphants could restore normal morphology, and 43.1% and 28.9% remained abnormal respectively (Table 1). These percentages are the average of the results from three independent experiments. Partial success in rescue experiment of MO1 may be due to the fact that *gper* mRNA contains MO1 target sequences which sequester *gper* mRNA, resulting in low successful rate of rescue. While *gper* mRNA is free of MO2 target sequence, and the rescued morphology is more obvious, verifying the specificity of *gper* MO in causing developmental deformity.

3.3. Gper knockdown resulted in apoptosis of brain cells

WISH analysis of several brain markers failed to show any notable changes in *gper* morphants, except for a slight reduction in the expression of midbrain marker *otx2* (Supplemental Fig. 1). Since *otx2* is required for the embryonic development of sensory organs and maintenance of brain functions [34], it indicates that Gper knockdown may influence the development of sensory organs and specification of the brain.

In pursuit of the mechanism leading to growth retardation in the *gper* morphants, we have conducted AO staining and TUNEL in embryos at 30 hpf. It was found that unlike the control group, *gper* morphants were trended to be stained by AO which indicated a greater number ofapoptotic cells in the brain. The observed level

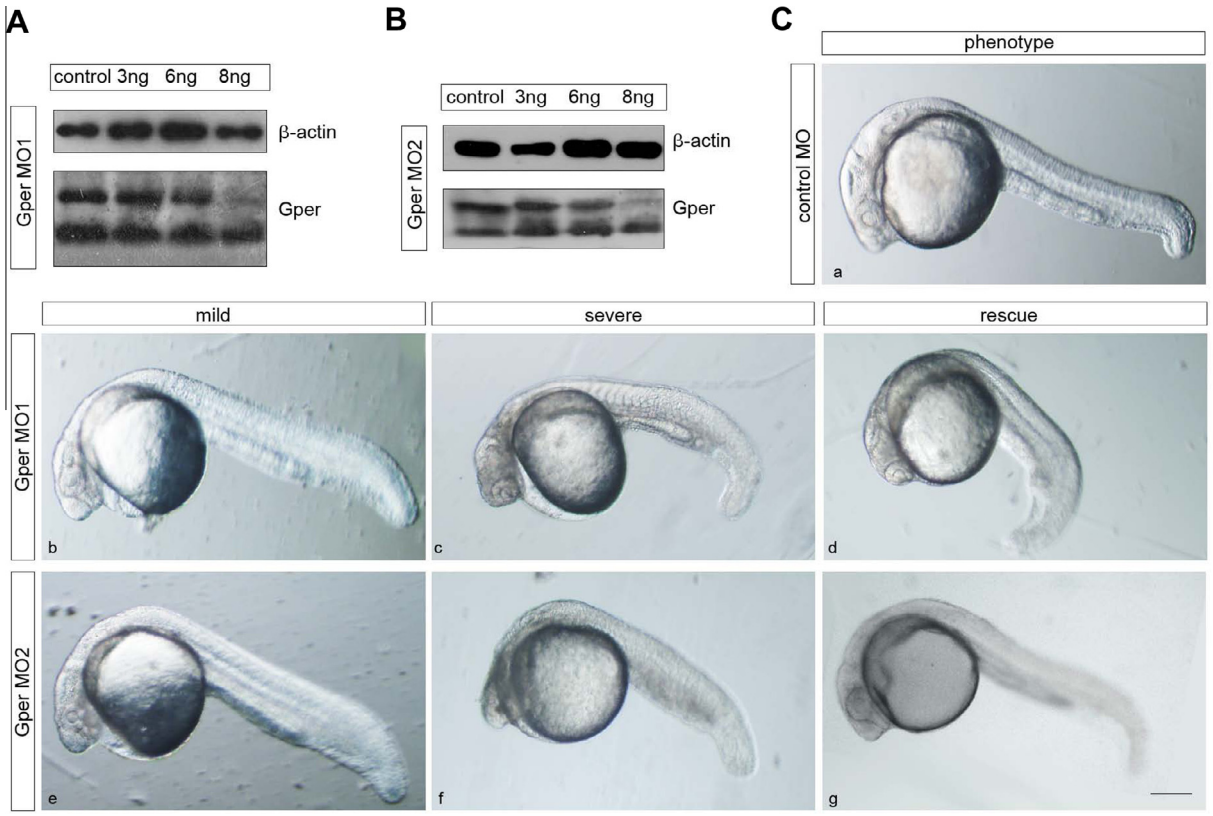

Fig. 2. Phenotypes of *gper* morphants. (A) Evaluation of *gper* knockdown by MO1 at 24 hpf by Western blot analysis. Mouse polyclonal antibody against β-actin (Santa Cruz, USA) was used as the internal reference. (B) Evaluation of *gper* knockdown by MO2 at 24 hpf by Western blot analysis. (C) Morphological phenotypes of *gper* morphants at 24 hpf. a. Standard control MO injected embryos. b, e. MO1/MO2 morphants with mild defect; c, f. MO1/MO2 morphants with severe defect; d, g. MO1/MO2 rescue with less deformity. Scale bar = 250 μm.

Table 1
Statistics of gross morphological phenotypes of *gper* morphants at 24 hpf.

	Phenotype[a]		
Group	Normal	Abnormal	Dead
Control	83.9% (52/62)	0	16.1% (10/62)
MO1	11.8% (8/68)	66.2% (45/68)	22.1% (15/68)
MO2	14.5% (12/83)	61.4% (51/83)	24.1% (20/83)
MO1 Rescue	37.9% (22/58)	43.1% (25/58)	19% (11/58)
MO2 Rescue	46.1% (35/76)	28.9% (22/76)	25% (19/76)

[a] Phenotypes were defined as shown in Fig. 2C.

of apoptosis was attenuated in the rescue group (Figs. 3A and B). Meanwhile, robust proliferating signals throughout the whole body of the control embryos were detected by Edu incorporation. As expected, proliferating signals were significantly reduced in the morphants and restored in the rescue group (Fig. 3C). When *p53* was concurrently knockdown which can specifically ameliorate the cell death induced by MO off-targeting [25], the apoptosis and proliferative signals remained essentially the same as in the morphants, thereby verifying the specific effect of *gper* MO in inducing apoptosis. Consistent with the result, flow cytometry analysis evidently detected about 20.2% of cells at the sub-G_1 phase in morphants, while only 1.2% in the control (Fig. 3D). Taken together, Gper knockdown can specifically induce apoptosis and reduce proliferation of brain cells. Whether the elevated apoptosis and decreased proliferation was responsible for the defects in embryos development awaits further investigations.

3.4. Gper knockdown can affect motor and sensory neuron development

Immunostaining of several neuron markers showed that the distribution of AcTub (axon marker) was almost abolished in *gper* morphants (Fig. 4A). During embryonic development, acetylation of tubulin can maintain the stability of the cytoskeleton as well as neurite formation, thereby maintaining the normal function of neurons [35]. Thus, reduction in tubulin acetylation levels in *gper* morphants suggests the involvement of Gper in neurite formation and normal functions. Zn-12 signal (marker of Rohon-Beard sensory neuron) in the dorsal spinal cord was lost in the morphants (Fig. 4B), suggesting that Gper may affect the development of sensory neurons. Moreover, *gper* was found to be localized in trigeminal ganglia where somatic sensory fibers lay conducting different senses, further indicating its importance in sensory nerve development. Signal of Znp-1 (marker of axon extension of primary motor neurons) and Zn-5 (marker of axon extension of secondary motor neurons) was significantly reduced in the morphants (Fig. 4C and D), indicating the importance of Gper in the development of motor neurons. In addition, the abnormal expression of these markers can be ameliorated by *gper* mRNA but not by *p53* co-injection, testifying the specific effect of Gper knockdown in neuron development. In summary, Gper is shown to be involved in embryonic development of zebrafish sensory and motor neurons in the present study (Supplemental Fig. 2).

Selective neuronal death, a process that determines the number of neurons to reside in specific neuronal populations in the adult brain, is known to be affected by neonatal estrogen [5]. A study

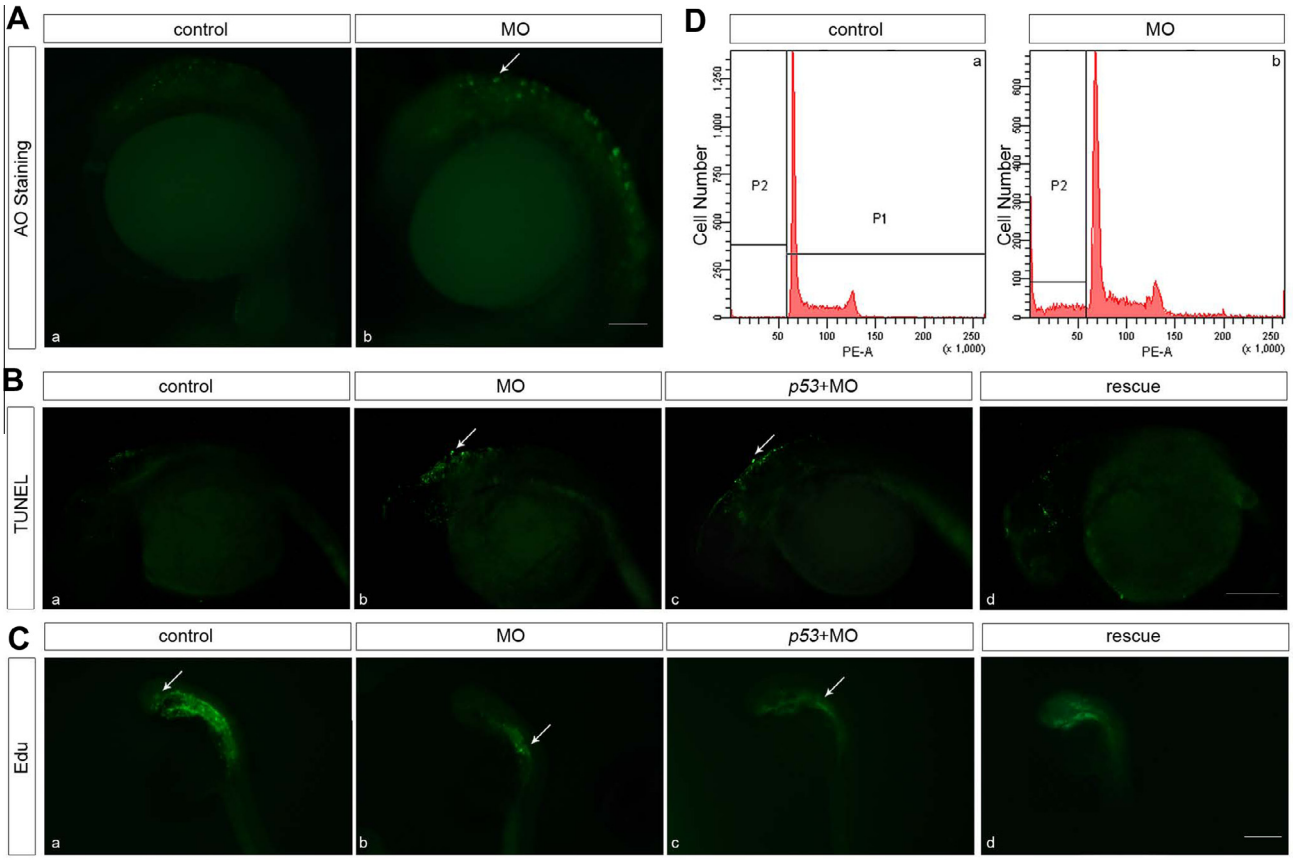

Fig. 3. Apoptosis and proliferation examination. (A) AO staining of embryos at 30 hpf. Scale bar = 200 μm. (B) TUNEL assay of embryos at 30 hpf. Scale bar = 400 μm. (C) Edu assay of embryos at 30 hpf. Scale bar = 450 μm. Similar patterns were observed in all embryos examined in each group ($N = 8–10$). Arrows: positive staining. (D) Cell cycle histogram of embryos at 30 hpf. a. control group; b. MO1 morphants; c. *p53*, *gper* MO1 co-injected embryos; d. rescued embryos.

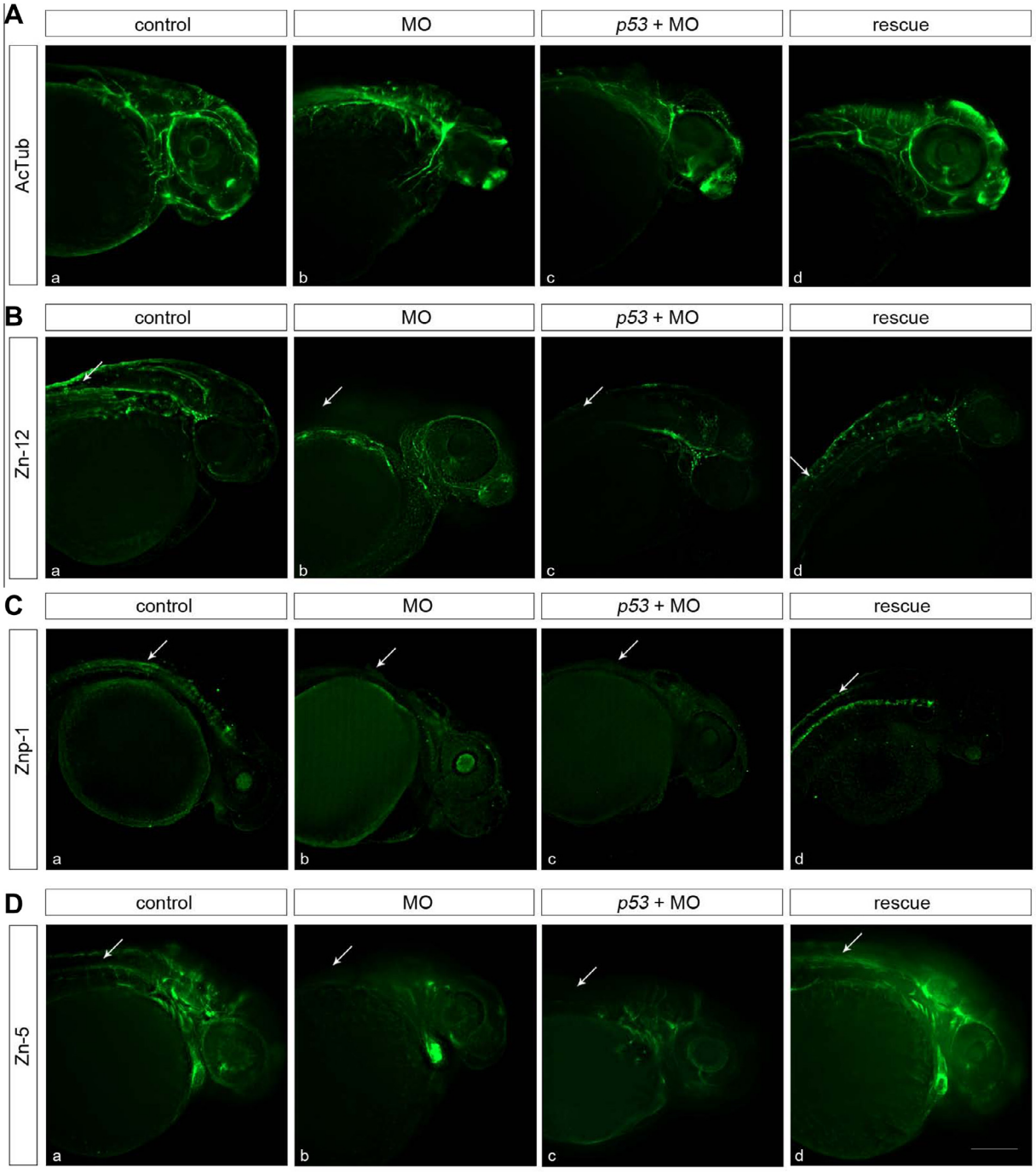

Fig. 4. Effects of Gper knockdown on neuron markers expression. Immunostaining of neuron markers in embryos at 48 hpf. a. control group; b. MO1 morphants; c. *p53*, *gper* MO1 co-injected embryos; d. rescued embryos. Similar patterns were observed in all embryos examined in each group ($N = 10–15$). Arrows in B: signal of RB sensory neuron cells; Arrows in C: signal of axon extension of primary motor neurons; Arrows in D: signal of axon extension of secondary motor neurons. Scale bar = 300 μm.

has revealed that the aromatase-inhibited zebrafish displayed abnormal sensory-motor functions such as tactile and swimming behaviors [36]. With the fact that Gper mediates estrogen effects in the brain through rapid non-genomic pathway [37], we reasonably propose that neonatal estrogen sustains brain cell survival through Gper and thus maintains brain/neural development during embryogenesis in zebrafish. However, further studies to reveal the molecular mechanisms of Gper activated downstream pathway in this developmental regulation are highly warranted.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.130.

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