Distinctive Alteration of Neuropeptide Y Expression Responsible for Neuro-Proliferation Following Zebrafish Spinal Cord Injury

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Abstract

In strong contrast to the limited repair within the mammalian central nervous system, the spinal cord of adult zebrafish is capable of regeneration following injury. Understanding the mechanism underlying neural regeneration and functional recovery in spinal cord-injured zebrafish may lead to effective therapies for human spinal cord injury (SCI). Since neuropeptide Y (NPY) plays a protective role in the pathogenesis of several neurological diseases, in the present study, the effects of NPY on neuronal repair and subsequent recovery of motor function in adult zebrafish post-SCI were evaluated. Real-time quantitative PCR (qRT-PCR), *in situ* hybridization (ISH) and immunostaining of NPY revealed decreased NPY expression at 12 hours (h), 6 days (d) and 21 d after SCI. Double-immunostaining for NPY and Islet-1, a motoneuron marker, showed that NPY was expressed in spinal cord motoneurons. NPY morpholino (MO) treatment resulted in suppressed locomotor recovery and axon regrowth. PCNA and Islet-1 double-staining showed suppressed motoneuron proliferation in NPY-MO zebrafish. Similar to NPY, the mRNA level for NPY1R was also expressed within motoneurons and downregulated at 12 h and 21 d after SCI. Collectively, these data suggest that NPY expression in motoneurons promotes locomotor recovery and axon regrowth in adult zebrafish, possibly by regulating motoneuron proliferation through the activation of NPY1R.

Introduction

In mammals, spinal cord injury (SCI) is characterized by a lack of neuron regeneration throughout the lesion site, and results in permanent functional impairment caudal to the affected region.(Bareyre 2008; Cao and Dong 2013) However, different from mammals, motor neurons in adult SCI zebrafish demonstrated BrdU positive (Reimer et al. 2008), and adult zebrafish have a prominent regenerative capacity that enables the spinal cord to regenerate damaged nerves and recover locomotor function in a remarkably short period following SCI (Peng et al. 2017b; Liu et al. 2016; Fang et al. 2014). Since several molecular pathways are shared between zebrafish and mammals, zebrafish are considered to be an ideal SCI regeneration model for discovering key neural regeneration molecules for human SCI therapy (Becker and Becker 2008).

Neuropeptide Y (NPY) is a 36-amino acid peptide that is highly conserved in vertebrates and widely expressed in both the central nervous system (CNS) and peripheral nervous system (Blomqvist et al. 1992; Gray et al. 1986; Tatemoto et al. 1982). NPY plays roles in several processes, including stress, food intake, anxiety, circadian rhythm, epilepsy, and chronic pain (Silva et al. 2002; Yoon and Kim 2019). Importantly, NPY has been implicated in neuroprotection and neurogenesis (Malva et al. 2012; Duarte-Neves et al. 2016; Decressac and Barker 2012; Peng et al. 2017a; Chen et al. 2018). NPY plays a neuroprotective role in a mouse model of Alzheimer's disease, by inhibiting endoplasmic reticulum stress-induced cell death or intracellular oxidative stress through inhibiting caspase-3 and caspase-4 activities, and by increasing the level of BDNF and NGF (Chen et al. 2018). NPY is also able to exert neuroprotection in Parkinson's disease by inhibiting excitotoxicity (Decressac and Barker 2012), as well as regulate the

survival and proliferation of different types of stem cells, including neural stem or precursor cells (Peng et al. 2017a).

In mammalian neural injury models, such as peripheral nerve injury or SCI, upregulation of NPY expression, either at the RNA or protein level, could be observed in the spinal cord (Fodor and Palkovits 1991; Zhang et al. 1993; Song et al. 2001; Coronel et al. 2017; Hwang et al. 2019). All of these studies have focused on NPY-related algesthesia research, because the dorsal horn of the spinal cord is functionally responsible for receiving sensory information. However, the function of NPY in neuro-repair and its alteration in zebrafish SCI remains unclear.

NPY performs its wide range of functions mainly via Y1, Y2, Y4, Y5 and Y6 receptors (Silva et al. 2002; Yi et al. 2018). Among these receptors, Y1 (NPY1R) is the most prevalent NPY receptor in the CNS and shows the highest affinity for NPY (Alexander et al. 2013). NPY also exhibits neuro-proliferative properties mainly via activation of NPY1R (Yi et al. 2018; Peng et al. 2017a). In addition, the zebrafish NPY1R corresponds structurally to its mammalian counterpart (Larsson et al. 2008; Salaneck et al. 2008; Fallmar et al. 2011; Matsuda et al. 2012), which suggests that NPY might function in mammals and zebrafish in a similar manner. Accordingly, these studies have led us to investigate the neuroprotective effect of NPY and NPY1R in the context of SCI in the zebrafish.

Materials And Methods

Animals

All experiments were approved by the Animal Care and Use Committees of Jiangnan University in China and carried out in accordance with the Guide for the Care and Use of Laboratory Animals (the eighth edition) from the National Institutes of Health. Wild-type adult zebrafish (*Danio rerio*, 6 months old) were bought from the Jiayu Aquatic Animals Company (Shanghai, China). All zebrafish (both sexes) used in the experiments were maintained on a 14 h light and 10 h dark cycle at 28°C, and were fed twice daily.

SCI surgery of adult zebrafish

Spinal cords of zebrafish were lesioned as described previously (SCI group) (Liu et al. 2016; Peng et al. 2017b; Fang et al. 2012). Briefly, fish were anesthetized in 0.033% tricaine methane sulfonate (MS-222, Sigma, St. Louis, MO, USA) in phosphate-buffered saline (PBS), pH 7.4, until respiratory movement of the opercula stopped (around 3-5 mins). At the center between the dorsal fin and the operculum, corresponding to the eighth vertebra (4-5 mm caudal to the operculum of the spinal cord), a longitudinal incision was made through the muscle layer, and the vertebral column was exposed. The spinal cord was cut completely between the two vertebrae with micro-scissors, then the wound was sealed with Histoacryl (B. Braun, Melsungen, Germany). Then, fish were kept individually in 28°C water, Fish resumed breathing within a few seconds. The sham-injured control (Sham) underwent the identical surgical procedure except that the spinal cord was not severed. All surgical procedures were performed on ice under a microscope.

The schematic diagram for SCI and tissue preparation for other experiments is shown in Supplemental Figure 1.

qRT-PCR for NPY and NPY1R

Total RNA was extracted from the 4 mm segment of spinal cord, caudally extending from the lesion site, with an EZgeneTM Tissue RNA Miniprep Kit (Biomiga, San Diego, CA, USA) according to the manufacturer's instructions. First-strand cDNA was generated using random primers and a PrimeScriptTM RT Master Mix (Perfect Real Time) (TAKARA BIO, Otsu, Japan). qRT-PCR was carried out with SYBR[®] Premix Ex TaqTM II (Tli RNase H Plus) (TAKARA BIO, Otsu, Japan), and the comparative cycle threshold Ct method ($\Delta\Delta$ Ct method) was applied for data analysis. Primers for qRT-PCR were designed using Primer Express 5.0 software (Applied Biosystems, Foster City, CA, USA). All experiments were performed in duplicate, and PCR products were validated by melting curves to confirm the presence of single PCR products. GAPDH served as the internal control. Primers used were as follows: zebrafish NPY forward: GACTCTCACAGAAGGGTATCC, reverse: GGTTGATGTAGTGTCTTAGTGCTG. NPY1R forward: GTAGCAGTGAGTCTAAACGGATAA, reverse: GAAGTTGCGGTTTCAGAAAGC. GAPDH forward: GTGTAGGCGTGGACTGTGGT, reverse: TGGGAGTCAACCAGGACAAATA.(Pei et al. 2007) A no-template control group was performed in each experiment using the same reaction conditions at different time points. The number of fish for this assay was 10 for each group.

ISH for NPY and NPY1R

In situ hybridization (ISH) probes (sense and anti-sense) for NPY and NPY1R mRNA were transcribed *in vitro*. Purified PCR fragments were cloned into the pGM-T vector (Tiagen, Beijing, China), and the sequences were verified by sequencing. Digoxigenin (DIG)-labeled sense and anti-sense RNA probes were generated using the MEGAscript system (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Non-radioactive detection of mRNA in sections of the adult zebrafish was performed as described with slight modifications (Liu et al. 2016; Fang et al. 2014). At different time after SCI, wild-type zebrafish were deeply anaesthetized with 0.033% MS-222, then a 4 mm spinal cord segment, extending caudally from the lesion, was rapidly removed and fixed with 4% paraformaldehyde in PBS at 4°C for 12 h, then incubated in 15% sucrose in PBS overnight at 4°C. The spinal cord tissue was prepared as frozen sections at 10 mm thickness longitudinally, and the sections through the central canal were used for ISH. DIG-labeled sense and anti-sense RNA probes for NPY (NM_131074.2) and the NPY1R (NM_001102391.1) were generated by *in vitro* transcription using T7 and SP6 RNA polymerases. The frozen sections were initially pre-hybridized for 2 h at 55°C, and then hybridized with the DIG-labeled probes for 18 h at 55°C. After hybridization, the sections were washed and blocked, then alkaline phosphatase-coupled anti-DIG fragment antibodies (Roche, Indianapolis, IN, USA) were applied for 1 h at room temperature and developed with NBT/BCIP (nitro-blue-tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate) (Roche, Indianapolis, IN, USA). Following staining, sections were imaged using a microscope (Eclipse 80i, Nikon, Tokyo, Japan). Five fish were analyzed for each group.

Immunofluorescent co-staining of NPY with Islet-1 or PCNA

All spinal cords were processed for immunofluorescence after fixation in 4% formaldehyde in PBS at 4°C overnight. Sections were prepared as described above and processed for immunostaining (Guo et al. 2011). The spinal cords (10 µm-thick, sagittal, from the 4 mm segment of spinal cord caudal to and including the lesion site) were blocked with 5% goat serum in PBS containing 0.3% Triton-X-100 for 20 mins at room temperature, followed by primary antibody incubation. Primary antibodies used were: rabbit anti-zebrafish NPY (1:500, ImmunoStar, Hudson, WI, USA), mouse anti-zebrafish Islet-1 (motoneuron marker) (1:200, Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City, IA, USA), and rabbit anti-zebrafish PCNA (proliferating cell nuclear antigen) (1:1000, Abcam, UK). Secondary antibodies were CY3-conjugated goat anti-rabbit IgG (1:800, Beyotime, Shanghai, China) and FITC-conjugated goat anti-mouse IgG (1:800, Beyotime, Shanghai, China). DAPI (4',6-diamidino-2-phenylindole, nuclear staining reagent) (1:1000, Beyotime, Shanghai, China) was applied to stain the cell nucleus. Fluorescence images were collected using a fluorescence microscope (Eclipse 80i, Nikon, Tokyo, Japan). Five fish were used for each group.

Inhibition of NPY expression by morpholino treatment

Morpholinos (MOs) have high mRNA binding affinity and exquisite specificity, and can modify pre-mRNA splicing in the nucleus by targeting splice junctions or splice regulatory sites. In this study, a splice-blocking MO was used to knock down NPY expression. The NPY anti-sense MO (NPY MO group) (5'-TACAAACGAGGAGCTCACCTCTGCC-3', Vivo-Porter-coupled) and standard control MO (CT MO group) (5'-CCTCTTACCTCAGTTACAATTTATA-3', Vivo-Porter-coupled) (Gene Tools, Philomath, OR, USA) were dissolved in Danieau solution and absorbed by small pieces of gelfoam (Upjohn, Kalamazoo, MI, USA). The gelfoam was allowed to dry and then was divided into smaller pieces, each yielding 800 ng of MO, which were then inserted into the transection site of the spinal cord immediately after SCI. Each animal was allowed to survive the surgery for 6 weeks.

Swim-tracking of zebrafish

A swim-tracking test was used to assess the locomotor recovery of zebrafish at 1, 2, 3, 4 and 6 weeks after SCI, as described previously (Fang et al. 2014). In each trial, a zebrafish was placed in a brightly illuminated tank (22.5×11×11 cm) filled with aquarium water (5 cm deep) at 25°C. The total swimming distance of the zebrafish within a 5-min period was recorded with video tracking and EthoVision XT software (Noldus, Wageningen, Netherlands). The number of fish analyzed for this assay was twelve for each group.

Anterograde tracing of zebrafish motoneurons at 6 weeks following MO treatment

To study whether regenerated axons had crossed the lesion site, biocytin (Sigma, St. Louis, MO, USA) was applied via a second surgery at the site of the brain stem/spinal cord junction at 6 weeks after SCI (Becker et al. 1997) (Fig 3. A). Biocytin was dissolved to approximately 1 mg/10 µl PBS. A small piece of

gelfoam saturated with either vehicle or biocytin was put into the secondary injury site. Then the injury site was sealed by Histoacryl (B. Braun, Melsungen, Germany). After 24 h, 4 mm of the spinal cord segment just beneath the first injury site was collected (Fig 3. A) and fixed in 4 % paraformaldehyde in PBS at 4°C overnight, embedded in 15 % sucrose, and sectioned coronally (in 25 µm-thick sections) or longitudinally (in 16 µm-thick sections) on a cryostat. Biocytin labeling was detected with streptavidin-Cy3 (1:400, Bioss, Beijing, China) and imaged with a fluorescence microscope. The intensity of biocytin-positive axons reflects the axonal regrowth. Five random coronal sections through the central canal in each fish were measured to obtain mean density values for immunofluorescence with Image J software (Wayne Rasband, National Institutes of Health, USA). Five fish were in each group (sham, control MO, NPY MO group) (Fang et al. 2012).

Co-localization of NPY1R mRNA with NPY or motoneurons by immunofluorescence and ISH

To co-localize NPY1R with NPY and motor neurons, cells expressing NPY1R mRNA were identified by ISH (we were unable to obtain antibodies capable of specifically recognizing zebrafish NPY1R, thus immunohistochemistry was not performed), and NPY and motoneurons were identified by immunohistochemistry. ISH was performed as described above. Following ISH, the sections were processed using standard immunohistochemical procedures. In brief, spinal cord sections prepared as above were incubated with primary antibody against rabbit NPY and mouse Islet-1 at 4°C overnight, followed by incubation with FITC-conjugated goat anti-rabbit/mouse secondary antibody at room temperature for 2 h. Fluorescence was observed with a fluorescence microscope. Five fish were used for each group.

Statistical Analysis

SPSS 19.0 software was used for data analysis (two-way ANOVA or one-way ANOVA). All data are presented as means ± SEM. The level of significance was set at $p < 0.05$ for the threshold for significance ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$). All experiments were performed in triplicate.

Results

SCI induces decreased NPY expression in the adult zebrafish spinal cord

To examine the possible involvement of NPY in spinal cord regeneration of adult zebrafish, the expression of NPY in the 4 mm segment of spinal cord immediately caudal to the lesion site was detected by qRT-PCR at 12 h, 6 d, 21 d after SCI. We found that compared to the sham group, expression of NPY mRNA in the SCI group was significantly decreased to 26.9% at 12 h ($***p < 0.001$), 80.3% at 6 d ($*p < 0.05$) and 42.1% at 21 d ($**p < 0.01$), with NPY expression 6 d post-SCI showing a transient peak (Fig. 1A). The qRT-PCR results were confirmed by ISH where, compared to the sham group, NPY mRNA-positive signals in SCI group were less at 12 h, 6 d and 21 d, especially in the large cells along the central canal, which were inferred to be motoneurons based on the morphology (Fig. 1B). Immunofluorescence analysis also showed that the NPY protein level in the SCI group was less than the sham control at 12 h,

6 d and 21 d. NPY-positive cells were located predominantly in the area surrounding the central canal (Fig. 1C).

NPY knock-down retards locomotor recovery following SCI in zebrafish

To determine the locomotor recovery of NPY MO-treated adult zebrafish (NPY MO group) following SCI, we measured weekly the distance that the fish could swim in a period of 5 min. Standard control MO was used as the control (CT MO group). By qRT-PCR analysis, NPY mRNA expression was found to be reduced at 3 d and persisted for 1 w after SCI in the NPY MO group (Fig. 2A), confirming knock-down of NPY. At 1 w after SCI, no difference in locomotion was observed between the NPY MO and control MO groups. However, NPY MO zebrafish swam a shorter distance in 5 min than CT MO zebrafish at 2, 3 and 6 w (Fig 2B) after SCI. At 2 w after SCI, the total distance moved by the NPY MO group (200.73 ± 17.36 cm) was 68% of that in the CT MO group (295.34 ± 35.84 cm, $*p < 0.05$). At 3 w, the total swimming distance of the NPY MO group (216.96 ± 35.12 cm) was 57.8% of that in CT MO group (375.08 ± 38.08 cm, $***p < 0.001$), and at 4 w after SCI, the total distance moved by the NPY MO group (272.69 ± 67.11 cm) was 48.5 % of that in the CT MO group (561.86 ± 91.96 cm, $*p < 0.05$). Similarly, at 6 w after SCI, the total distance moved by the NPY MO group (267.33 ± 86.62 cm) was further reduced to 36.6 % of the control group (730.07 ± 119.42 cm, $*p < 0.05$) (Fig. 2C). These results indicate that the recovery of locomotor function of SCI zebrafish is inhibited by NPY MO treatment, suggesting that NPY may promote locomotor recovery.

Inhibition of NPY impairs axonal regrowth after SCI in zebrafish

To further elucidate the requirement of zebrafish for NPY in recovery from SCI, we examined axonal regrowth beyond the lesion site by anterograde tracing at 6 w post-SCI (Fig. 3A). An uninjured group was used as the normal control. Biocytin-positive signals in both coronal and sagittal sections from the NPY MO group were considerably less than that of uninjured- and the CT MO-group (Fig. 3B). In coronal sections, compared with uninjured group, the CT MO and NPY MO groups showed an 8.5% ($**p < 0.01$) and 32.2% ($***p < 0.001$) decrease of immunofluorescence intensity, respectively. Compared with the CT MO group, the NPY MO group showed a 25.3% decrease in axonal regrowth ($***p < 0.001$) (Fig. 3C). These observations support the view that NPY promotes axonal regrowth, and enhances locomotor recovery during regeneration after SCI in zebrafish.

NPY MO treatment reduces proliferation of motor neurons in the spinal cord following SCI

To characterize the predominant NPY-positive cell types, we performed double-immunofluorescence labeling for NPY and Islet-1 (a marker for motoneurons) at 12 h and 21 d after SCI. The results showed that NPY was expressed in motor neurons. Moreover, two subtypes of motoneurons, of small and large size, were observed (Fig. 4A). To reveal the effect of NPY on motoneurons, double immunostaining of PCNA and Islet-1 was used after NPY MO treatment. We found that CT MO-treated zebrafish showed more proliferating motor neurons than the sham group (Fig. 4B), indicating that neurogenesis occurred following SCI in zebrafish. NPY MO-treated zebrafish showed reduced numbers of proliferating motor neurons than CT MO-treated zebrafish (Fig. 4B), indicating the reduced neurogenesis was induced by NPY

knock-down. These findings prompted us to speculate that NPY could work as a modulator of motoneuron proliferation.

SCI induces decreased NPY1R expression in the zebrafish spinal cord

Given that NPY possesses the capacity to modulate neurogenesis and has previously been implicated in proliferation through activating the NPY1R subtype (Hansel et al. 2001), we sought to determine whether NPY could exert its effects on motor neuron regeneration through NPY1R. ISH and qRT-PCR was used to detect the expression of NPY1R in the 4 mm segment of spinal cord including and caudal to the lesion site at 12 h, 6 d, 21 d after complete spinal cord lesion. We found the expression of NPY1R mRNA in the spinal cord of the SCI group decreased to 47.4% at 12 h ($*p < 0.05$), 37.2% at 6 d ($***p < 0.001$) and 51.6% at 21 d ($*p < 0.05$) compared to the sham control (Fig. 5A). The qRT-PCR results were confirmed by ISH. Compared to the sham control, NPY1R mRNA expression was lower at 12 h, 6 d, and 21 d after SCI (Fig. 5B). The change in expression of NPY1R was consistent with that of NPY.

NPY1R co-localizes with NPY and motor neurons

Co-localization of NPY1R mRNA (due to the absence of zebrafish NPYR1 antibody for zebrafish) and NPY protein were determined by ISH and immunofluorescence, respectively, showed that NPY1R co-localizes with most NPY-positive cells at 12 h and 21 d after SCI. SCI zebrafish showed fewer co-labeled cells than the sham group (Fig. 6A). In addition, ISH for NPY1R mRNA and Islet-1 immunostaining for motoneurons, in the same longitudinal sections of the spinal cord, displayed co-localization at 12 h and 21 d after SCI. Cells showing co-localization were fewer in SCI zebrafish than in sham-treated zebrafish (Fig. 6B). NPY and NPY1R showed the same expression trend. These results indicate that NPY1R mRNA is expressed in spinal cord motoneurons, suggesting that NPY is likely to exert its effects on motoneuron proliferation through the NPY1R.

Discussion

Molecules that promote regeneration and functional recovery following zebrafish SCI could provide effective therapeutic clues for human SCI. NPY is widely distributed throughout all levels of the spinal cord in a variety of mammals, including the rat, guinea pig, cat, marmoset, and horse (Gibson et al. 1984), and also functions as a neuroprotective peptide against excitotoxicity and apoptosis (Decressac and Barker 2012; Malva et al. 2012; Duarte-Neves et al. 2016). In the zebrafish CNS, NPY has been reported to be expressed in the hypothalamus and medulla (Mathieu et al. 2002; Jeong et al. 2018), but its expression in zebrafish spinal cord has not been described. Here, we explore the changes of NPY and the NPY1R, and their function in zebrafish SCI neuro-repair.

We found that in both normal control zebrafish and SCI zebrafish, NPY and NPY1R are present in spinal cord motoneurons. Morphologically, in normal control zebrafish, both small and large size motoneurons were present, with the large motoneurons being in the majority. Following SCI, in the early post-SCI stage (12 h post-SCI), NPY-positive motoneurons mainly displayed a small-sized appearance along the central

canal, whereas in the late post-SCI stage (21 d post-SCI), NPY-positive motoneurons were mainly large-sized, which is in line with that in rats following axotomy (Zhang et al. 1993). Both large and small motoneurons were NPY-positive. The two types of motoneurons might correspond to early-developing primary (small-size, immature) and late-developing secondary (large-size, mature) motoneurons after spinal lesion (Grunwald et al. 1988) and persist through adulthood in the zebrafish (Stil and Drapeau 2016). It has been reported that NPY is expressed in both immature and mature neurons, and might function in modulation of proliferation in immature neurons and neuroprotection in mature neurons (Decressac and Barker 2012). Numerically, both NPY and NPY1R are down-regulated at the injury site following SCI. NPY shows down-regulation in spinal cord motoneurons at 12 h, 6 d and 21 d post-SCI, which is contrary to findings in mammalian neural injury. In patients or mammalian models suffering from peripheral nerve injury (such as transection, loose ligation, a crushed sciatic nerve or its branches, or chronic constriction injury of the infraorbital nerve), upregulation of NPY in the dorsal root ganglia (DRG) and spinal cord dorsal horn (SCDH, downstream of the DRG in the nociceptive afferent pathway) following injury has been reported (Brumovsky et al. 2007; Diaz-delCastillo et al. 2018). Since NPY is closely related with nociceptive afferent neurons, the underlying mechanism might be due to peripheral nerve injury causing NPY up-regulation in DRG neurons (hub of the nociceptive afferent pathway), which further leads to increased NPY transport from the DRG to the SCDH, manifested as the upregulation of NPY in the SCDH (Brumovsky et al. 2007; Diaz-delCastillo et al. 2018). In mammalian SCI models, several studies have described the upregulation of NPY, NPY1R and NPY2R in the spinal cord (Song et al. 2001) (Coronel et al. 2017; Hwang et al. 2019). Hwang and colleagues described the upregulation of NPY in the rat DRG following SCI. SCI rats, that had received transplantation of GDNF-hNSPCs (human neural stem progenitor cells overexpressing glial cell line-derived neurotrophic factor), show a reduction of glial scar formation, and enhanced neurite outgrowth and axonal extension. Interestingly, the upregulation of NPY in the DRG is also reversed (Hwang et al. 2019). All previous studies of NPY, NPY1R and NPY2R in mammalian neural injury models have only focused on their alteration and roles in nociceptive afference, but their function in neuro-repair was not involved (Song et al. 2001; Coronel et al. 2017; Hwang et al. 2019). Using a zebrafish SCI model, we explore the function of NPY in neuro-repair. When NPY is knocked-down by MO treatment, locomotor recovery, axon regrowth and neuro-proliferation are inhibited. Hence, our observations suggest that NPY is a beneficial regulator in the progression of zebrafish spinal cord regeneration, probably by promoting neuro-proliferation via NPY1R. The contrary expression of NPY and NPY1R at the injury site in mammalian and zebrafish neural injury models suggests a special role for NPY in zebrafish SCI recovery. A comparative study concerning different gene regulation mechanisms between species should also be explored.

NPY has been reported to stimulate neural cell proliferation, as well as enhance growth and survival (Decressac et al. 2009; Palkovits 1995). It exhibits neuro-proliferative properties and regulates inflammation mainly by activating the NPY1R (Hansel et al. 2001; Howell et al. 2005; Milenkovic et al. 2004; Decressac et al. 2009; Peng et al. 2017a; Yi et al. 2018; Wheway et al. 2007; Malva et al. 2012; Farzi et al. 2015). In our study, NPY1R expression was also detected. Consistent with NPY expression, NPY1R is also downregulated at 12 h, 6 d, and 21 d following SCI. By immunostaining, we showed that NPY1R

staining overlaps with most NPY-positive motoneurons in the spinal cord. Thus, our data favor the possibility that NPY may promote motoneuron proliferation mainly by activating NPY1R in zebrafish.

As a widely expressed peptide in both the CNS and PNS, NPY was also found to regulate the release and function of dopamine and serotonin (Myers et al. 1992; Quarta and Smolders 2014), which are also reported to be involved in neural repair (Barreiro-Iglesias et al. 2015; Chapela et al. 2019; Cigliola et al. 2020). The further study on the mechanism and application of NPY in neural repair is needed.

In conclusion, our results suggest that NPY may play a beneficial role in neuro-recovery from SCI by promoting axonal regrowth and regulating motoneuron proliferation via the NPY1R in adult zebrafish. The different expression patterns of NPY and NPY1R between mammalian and zebrafish SCI models might contribute to the different capacities for neuro-regeneration. The profound mechanism and detection of NPY and NPY receptors over a longer period needs to be further explored. In addition, NPY is of particular interest as it has the ability to cross the blood-brain barrier (Kastin and Akerstrom 1999), in contrast to most of the neurotrophic and neuro-proliferative factors identified so far. Thus, adjunctive therapies with appropriate molecules, such as NPY, NPY1R agonists or pharmacological derivatives, may be of significant potential to manage SCI therapy.

Declarations

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Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: This research was approved by the Animal Ethics Committee of Jiangnan University. The zebrafish work was performed according to Regulations for the Administration of Affairs Concerning Experimental Animals (approved by the State Council on October 31, 1998 and promulgated by decree No.2 of the State Science and Technology Commission on November 14, 1988).

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Figures

Fig1

Figure 1

Time course of NPY expression in spinal cord tissue of SCI-lesioned zebrafish. (A) qPCR detection of NPY mRNA in the spinal cord caudal to the lesion site at 12 h, 6 d and 21 d after SCI. Significant downregulation was observed at 12 h, 6 d and 21 d after SCI compared to the sham-injured group. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-way ANOVA with Tukey's post hoc test; $n = 10$ fish/group). Values represent means $\pm$ SEM. (B) ISH for NPY mRNA in longitudinal sections of the 4 mm segment of spinal

cord, extending caudally from and including the lesion site, at 12 h, 6 d and 21 d after SCI (n = 5 fish/group). Numbers of positive cells were lower at 12 h, 6 d, and 21 d after SCI compared to the sham-injured group. Asterisks indicate the central canal. Arrows indicate rostral-caudal direction. (C) Immunofluorescence staining of NPY in longitudinal sections of the 4 mm caudal segment of spinal cord. NPY-positive cells were reduced along the central canal at 12 h, 6 d and 21 d. NPY protein expressed in the cytoplasm. Asterisks indicate the central canal. Arrows indicate rostral-caudal direction. NPY, red; DAPI, blue. No signal was detected in sections incubated with the sense probe in ISH and without primary antibody in immunohistology, respectively.

Fig 2

Figure 2

Locomotor recovery was impaired in spinal cord-injured zebrafish by treatment with NPY MO. (A) The NPY morpholino significantly reduces NPY mRNA expression at 3 d and 1 w after SCI compared to the control (CT) MO group (** $p < 0.01$, *** $p < 0.001$, two-way ANOVA with Tukey's post hoc test; $n = 12$ fish/group). Values represent means $\pm$ SEM. (B) Swimming tracks of the control MO and NPY MO groups at 6 weeks. (C) Quantification of the total swimming distance of undisturbed zebrafish in 5 mins in

control MO and NPY MO groups. The total swimming distance of zebrafish in 5 mins was reduced to 70.4%, 42.3%, 48.9% and 42.3% at 2 w, 3 w, 4 w and 6 w respectively, after SCI in the NPY MO groups vs. control MO groups (*p < 0.05, ***p < 0.001, two-way ANOVA with Tukey's post hoc test; n = 12 fish/group). Values represent means ± SEM.

Figure 3

Regrowth of axons were inhibited after reduction NPY MO in spinal cord-injured zebrafish. (A) Schematic diagram of anterograde tracing. (B) Coronal and longitudinal sections showed fluorescence intensity of axons beyond the lesion site at 6 weeks after injury. Zebrafish treated with NPY MO showed significantly less axonal regeneration than fish treated with control MO. The sham injury group was used as a control. Regenerating axons (red). Asterisks indicate the central canal. Arrows indicate rostral-caudal direction. (To demonstrate the cells surrounding the central canal clearly, the square area was magnified in Supplemental figure 2) (C) The intensity of bands was quantified with Image J software. Biocytin staining analysis of axonal regeneration in MO-treated zebrafish indicated that the percentage of the labeled area was reduced to 74.7% in serial cross-sections from the 4 mm caudal segment of the spinal cord, from the lesion site, of fish treated with NPY MO vs. control MO. (** $p < 0.001$, one-way ANOVA with Tukey's post-hoc test; $n = 5$ fish/group). Values represent means $\pm$ SEM.

Figure 4

NPY expression in motor neurons. (A) Double immunofluorescence staining of NPY and Islet-1 in longitudinal sections showed NPY localization in motoneurons along the central canal. NPY was mainly expressed in the cytoplasm. There were large and small NPY positive motoneurons in along the central canal. NPY, red; Islet-1, green; merge, yellow. Asterisks indicate the central canal. Arrows indicate rostral-caudal direction. (B) Double-immunofluorescence staining of Islet-1 with PCNA in longitudinal sections (4 mm lesion-caudal segment of the spinal cord) showed that PCNA positive (red) proliferating motoneurons appeared after spinal cord injury in both CT MO and NPY MO groups. NPY MO group showed decreased PCNA positive motoneurons compared with CT MO group. The merged PCNA- and DAPI- and Islet-1-positive cells indicated proliferating motoneurons. PCNA, red; Islet-1, green; DAPI, blue. Asterisks indicate the central canal. Arrows indicate rostral-caudal direction.

Fig 5

Figure 5

Time course of NPY1R mRNA expression after SCI. (A) qPCR of NPY1R mRNA in the 4 mm lesion-caudal spinal cord segment showed significant down-regulation at 12 h, 6 d and 21 d after SCI vs. the sham-injured group. (* $p < 0.05$, *** $p < 0.001$, two-way ANOVA with Tukey's post hoc test; $n = 10$ fish/group). Values represent means $\pm$ SEM. (B) ISH detection of NPY1R mRNA in longitudinal sections of the 4 mm lesion-caudal spinal cord segment at 12 h, 6 d and 21 d after SCI ($n = 5$ fish/group). Numbers of positive

cells were lower at all time points after SCI compared to the sham-injured group. No signal was detected in sections incubated with the sense probe under the same conditions as the anti-sense probe. Asterisks indicate the central canal. Arrows indicate rostral-caudal direction.

Fig 6

Figure 6

Co-localization of NPY1R mRNA and NPY protein, motoneurons in the spinal cord of SCI zebrafish. (A) Longitudinal sections of the spinal cord along the central canal showed overlap of NPY1R mRNA by ISH and NPY immunofluorescent staining in the sham group and 12 h-, 21 d-treated groups after SCI (n = 5 fish/group). NPY1R, fuchsia; NPY, green. Asterisks indicate the central canal. Arrows indicate rostral-caudal direction. (B) Longitudinal sections of the spinal cord along the central canal showed co-existence of NPY1R mRNA by ISH and motoneurons labeled with Islet-1 immunofluorescent staining in both sham

and 12 h-, 21 d-SCI treated groups (n = 5 fish/group). NPY1R, fuchsia; Islet-1, green. Asterisks indicate the central canal. Arrows indicate rostral-caudal direction.

Supplementary Files

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