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MANF regulates dopaminergic neuron development in larval zebrafish

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ABSTRACT

Mesencephalic astrocyte derived neurotrophic factor (MANF) is recognized as a dopaminergic neurotrophic factor, which can protect dopaminergic neurons from neurotoxic damage. However, little is known about the function of MANF during the vertebrate development. Here, we report that MANF expression is widespread during embryonic development and in adult organs analyzed by qPCR and *in situ* hybridization in zebrafish. Knockdown of MANF expression with antisense splice-blocking morpholino oligonucleotides resulted in no apparent abnormal phenotype. Nevertheless, the dopamine level of MANF morphants was lower than that of the wild type larvae, the expression levels of the two tyrosine hydroxylase gene transcripts were decreased and a decrease in neuron number in certain groups of *th1* and *th2* cells in the diencephalon region in MANF morphants was observed. These defects were rescued by injection of exogenous *manf* mRNA. Strikingly, *manf* mRNA could partly restore the decrease of *th1* positive cells in Nr4a2-deficient larvae. These results suggest that MANF is involved in the regulation of the development of dopaminergic system in zebrafish.

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder. The hallmark symptoms of PD, resting tremor, rigidity, and bradykinesia, result from loss of dopaminergic neurons in the substantia nigra pars compacta and the ascending innervations of the striatum, leading to depletion of dopamine (Dauer and Przedborski, 2003). L-3,4-dihydroxyphenylalanine (L-dopa) is the most widely used therapy for PD. However, after long-term treatment, it becomes less effective and causes various side effects such as dystonia and dyskinesia. Alternatively, discovering how to improve the PD symptoms by enhancing the survival of the remaining dopaminergic neurons to increase availability of dopamine in the striatum may become a potential treatment. Lately, a novel evolutionarily conserved neurotrophic factor family including cerebral dopamine neurotrophic factor (CDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF) has emerged as potentially useful target (Lindholm et al., 2008; Lindholm and Saarma, 2010; Lindholm et al., 2007; Petrova et al., 2004; Yasuda and Mochizuki, 2010).

MANF, a 20 kDa secreted protein, was primarily found to have selectively protective function on nigral dopaminergic neurons, but not GABAergic or serotonergic neurons in ventral mesencephalic cell cultures from embryonic rat brains (Petrova et al., 2003). In rodent brains, relatively high *manf* mRNA levels are detected in the cerebral cortex, hippocampus and cerebellum; in non-neuronal mouse tissues, it can be detected in the liver, kidney and testes (Lindholm et al., 2008). In the 6-hydroxydopamine (6-OHDA) rat model of PD, following an intrastriatal injection of MANF, nigrostriatal dopaminergic neurons are protected from degeneration and the dopaminergic function in the striatum can be restored (Voutilainen et al., 2009). Furthermore, MANF transcripts and protein levels are increased after ischemic and epileptic insults in the cerebral cortex, suggesting that MANF may have neuroprotective effects against neurotoxins and cerebral ischemia (Lindholm et al., 2008; Voutilainen et al., 2009; Yu et al., 2010). The crystal structure of mammalian MANF has revealed that the amino-terminal region of MANF family members contains a saposin-like domain which may interact with lipids and membranes whereas the carboxy-terminal region includes an intradomain cysteine bridge in a CXXC motif which may protect cells from endoplasmic reticulum stress induced apoptosis (Hellman et al., 2010; Hoseki et al., 2010; Parkash et al., 2009). MANF is also found in invertebrates; for instance, Drosophila MANF is expressed in glia and neurons, and knocking out *manf* expression leads to neuronal degeneration causing embryonic lethality, indicating that MANF is required for the maintenance of neural cells in the fly (Palgi et al., 2009). In vertebrates, several factors, such as pax2/5, Lmx1a, Nr4a2 and pitx3, have been identified to play crucial roles in determination of the developmental fate of midbrain dopaminergic neurons (Andressoo and Saarma, 2008; Simon et al., 2003). Nonetheless, it is currently not known if MANF is involved in development of dopaminergic neurons.

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Zebrafish is increasingly used as a vertebrate animal model, also in studies on neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease and Huntington's disease due to a short generation time, external development, and the high similarity of neuroanatomy and circuit formation between the zebrafish CNS and mammalian brain (Bandmann and Burton, 2010; Panula et al., 2010). In the vertebrate nervous system, MANF is mainly known due to its neuroprotective functions (Airavaara et al., 2009; Voutilainen et al., 2009; Yu et al., 2010). In this study, we used antisense morpholino oligonucleotides to reduce MANF protein expression and analyzed the effect on catecholaminergic, serotonergic, orexinergic and histaminergic systems to characterize the biological role of MANF in developing zebrafish brains.

Materials and methods

Zebrafish strain and maintenance

Zebrafish were obtained from our breeding line maintained in the laboratory for more than 10 years (Kaslin et al., 2004; Kaslin and Panula, 2001; Sallinen et al., 2009a; Sallinen et al., 2009b). Developing embryos were staged in hours post-fertilization (hpf) or days post-fertilization (dpf) as described previously (Kimmel et al., 1995). Embryos and larvae were raised at 28 °C. For *in situ* hybridization, embryos less than 3 dpf of age were treated with 0.03% phenylthiourea added in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.4 mM $CaCl_2$, and 0.16 mM $MgSO_4$) to inhibit pigmentation. The permits for the experiments were obtained from the Office of the Regional Government of Southern Finland in agreement with the ethical guidelines of the European convention.

RNA isolation and cDNA synthesis

Total RNA was extracted using RNeasy mini Kit (Qiagen Inc., Valencia, CA) according to the instructions of the manufacturer. For the developmental expression analysis and morpholino oligonucleotide efficacy assay, 30-pooled embryos were collected at certain stages. For tissue-specific expression study, brain, eye, liver and kidney tissues were collected from five individual one-year-old male zebrafish. Two microgram of total RNA was reverse-transcribed using SuperScriptTM III reverse transcriptase (Invitrogen, USA) according to the manufacturer's instruction.

Quantitative real-time PCR (qPCR)

qPCR was performed on the SmartCycler II instrument (Cepheid,-Sunnyvale, CA) using the SYBR®Premix Ex Taq™ (TAKARA BIO, Tokyo, Japan). Primers for amplification were designed by Primer-BLAST (NCBI) and sequences are shown in Table 1. The primer sequence of *pax2a* and *pax5* were according to Lin et al., 2009. Two housekeeping genes, β-*actin* and ribosomal protein L13a (*rpl13a*), were used as reference controls. β-*actin* primers were obtained from the Real-time PCR Primer Databank (http://medgen.ugent.be/rtprimerdb/). Cycling parameters were as follows: 95 °C for 30 s and 45 cycles of the following, 95 °C for 10 s and 62 °C for 45 s. Fluorescence changes were monitored with SYBR Green after every cycle. Dissociation curve analysis was performed (0.2 °C per s increase from 60 °C to 95 °C with continuous fluorescence readings) at the end of cycles to ensure that only single amplicon was obtained. All reactions were performed in duplicates. Results were evaluated with the SmartCycler II software. The data were calculated by the comparative method using Ct values of β-*actin* and *rpl13a,* respectively, as the reference control (Livak and Schmittgen, 2001). Since the gene expression changes showed the same trend when normalized to different housekeeping genes (data not shown), the results referred to β-*actin* are shown in this study.

Morpholino oligonucleotide (MO) design, use and mRNA injections

Three antisense MOs (Gene Tools LLC, Philomath, OR, USA) were designed to target the 5′ untranslated region of *MANF* (MOt, 5′-ATCTGAACGACCACTAATGATACCG3′), splicing-donor sites of exon 2 (MOsp1, 5′-GACGGGTACTTACAAATCGGTTTTC-3′) and splicing-donor sites of exon 3 of *MANF* (MOsp2,5′-TGCAAACAACT-CACCGTATTTGAGT-3′). Nr4a2 MO (5′-CATACTGAGCCTGGACGCAG-GGCAT-3′) that targets both 5′ untranslated region of Nr4a2a and Nr4a2b was based on Blin et al., 2008. The working concentration was determined by injecting serial dilutions of MO. Therefore, the dose of 8 ng was found to produce the most effective inhibition without causing any unexpected gross phenotype for the translation-blocking MO (MOt) and two splice-blocking MOs (MOsps) morphants. A standard control MO (ctrl MO, 5′-CCT CTT ACC TCA GTT ACA ATT TAT A 3′) purchased directly from Gene-Tools Inc. (Philomath, OR, USA) was injected 8 ng per embryo. To assess the efficacy of the splice-blocking MOsp, RT-PCR was performed using primers F1 and F2. In this study, the combination of two MOsps was co-injected into one-cell stage embryos at the dose of 4 ng for each MOsp. The MOt injection at the dose of 8 ng also caused the same effects as MOsps did. The *manf* full-length open-reading frame cDNA construct was prepared by RT-PCR using primers F1 and F2 and Phusion High-Fidelity PCR Master mix (Finnzymes, Espoo, Finland). The PCR amplicon was cloned into the pGEM-T Easy vector (Promega, Madison, WT) and verified by sequencing. The clone with no mutations was digested with *Eco*RI and the insert was cloned into the pMC expression vector kindly given by Dr. Thomas Czerny (Fink et al., 2006) and linearized with *Not*I. Capped sense transcripts from the cDNA expression clone were generated by the mMESSAGE mMACHINE kit (Ambion, Austin, TX) using T7 RNA polymerase. For the mRNA rescue experiment, 500 pg of *manf* mRNA with 8 ng of splice-blocking MOsps were co-injected into embryos at one-cell stage.

Table 1
List of primers for the qPCR analysis.

Gene	Forward primer	Reverse primer	Accession no.	Note
dat	CGTCACCAACGGTGGAATCTA	TGCCGATGGCCTCAATTAGTA	AF318177.1	qPCR
hcrt	TCTACGAGATGCTGTGCCGAG	CGTTTGCCAAGAGTGAGAATC	BX005093	qPCR
hdc	TTCATGCGTCCTCTCCTGC	CCCCAGGCATGATGATGTTC	EF150846.1	qPCR
MANF	AGATGGAGAGTGTGAAGTCTGTGTG	CAATTGAGTCGCTGTCAAACTTG	NM_001076629	qPCR
F1/F2	TAGCGCTTTTCACATCGTATTTAACT	CCCCGCTGTTAGGTGCTCA		RT-PCR
Nr4a2b	GAAGACGGCGAAATCGATGC	CTGGCGGTTCTGACAACTTCC	NM_001002406	qPCR
Nr4a2b	TTCTAACACTGCAGCCATGC	CCTGCTTCAGTTCAGACGAG		RT-PCR
th1	GACGGAAGATGATCGGAGACA	CCGCCATGTTCCGATTTCT	XM_682702.1	qPCR
th2	CTCCAGAAGAGAATGCCACATG	ACGTTCACTCTCCAGCTGAGTG	NM_001001829	qPCR
β-actin	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC	RTPrimerDB ID:705	qPCR

Whole mount in situ hybridization (WISH)

Whole-mount *in situ* hybridization (WISH) was performed on 4% paraformaldehyde (PFA) fixed zebrafish embryos at various stages less than 5 dpf as described earlier (Chen et al., 2009). Antisense and sense digoxigenin (DIG)-labeled RNA probes were generated using the DIG RNA labeling kit (Roche Diagnostics, Germany), following the instruction of the manufacturer. The WISH procedure followed Thisse's protocol (Thisse and Thisse, 2008). The prehybridization and hybridization were conducted at 65 °C for all riboprobes. *In situ* hybridization signals were detected with sheep anti-digoxigenin-AP Fab fragments (dilution 1:10,000; Roche Mannheim, Germany). The color staining was carried out with chromogen substrates (NBT and BCIP) as described earlier (Chen et al., 2009).

Protein extraction and Western blotting

The protocol was modified based on the zebrafish book (Westerfield, 2000). The 3-dpf dechorionated embryos were deyolked in cold Ringer's solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl2 and 5.0 mM HEPES, pH 7.2) by triturating with a glass pipette with a tip diameter approximately the size of the yolk. The dechorionated, deyolked embryos were transferred and homogenized in the lysis buffer 100 mM Tris–HCl, pH 8.0, 1 M NaCl, 2% Trition X-100, 2% bovine serum albumin, 4 mM EDTA, 1 mM Na-orthovanadate and Mini EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany). The homogenized lysate was incubated on ice for 30 min, and centrifuged at the speed of 13,000 rpm for 15 min at 4 °C. The protein concentration of the supernatants was measured and 15 μg was used for immunoblotting with rabbit polyclonal anti-MANF antibody (4347, 1:2000; ProSci Inc, Poway, CA) and the same blot was stripped and incubated with mouse anti-actin antibody (AC-40, 1:1000; Sigma, St. Louis, MO). To determine the specificity of the MANF antibody, an immunizing peptide blocking experiment was performed. The MANF antibody was incubated with 100 μg per ml of the blocking MANF peptide (4347P, ProSci Inc, Poway, CA) that corresponds to the immunogen peptide used for immunization for 2 h at 37 °C. The neutralized antibody was used side-by-side with the antibody alone.

Immunocytochemistry

Whole-mount immunostaining was performed on 2% PFA or 4% 1-ethyl-3(3-dimethylaminoproply)-carbodiimide (for GABA staining) fixed zebrafish embryos at the stage less than 3 dpf. For 5 dpf fixed larvae, brains were dissected to enhance antigen presentation. Antibody incubations were carried out with 4% normal goat serum and 1% DMSO in 0.3% Triton X-100/ PBS for 16 h at 4 °C with gentle agitation. Primary antibodies were anti-acetylated tubulin mouse antibody (T6793, 1:1000; Sigma, St. Louis, MO), anti-HuC/D mouse antibody (A21271, 1:1000; Invitrogen, Eugene, USA), anti-MANF rabbit antibody (4347, 1:3000; ProSci Inc, Poway, CA), anti-serotonin rabbit antibody (S5545, 1:1000; Sigma, St. Louis, MO) anti-tyrosine hydroxylase monoclonal mouse antibody (MAB318, 1:1000; Chemicon), and anti-zebrafish GFAP (zrf 1, 1:5000; Zebrafish International Resource Center, Oregon, USA), and anti-GABA rabbit antibody (GABA 1H, 1:1000, (Karhunen et al., 1993)) The specificity of the TH and serotonin antibodies has been verified earlier (Kaslin and Panula, 2001). The following secondary antibodies were applied: Alexa Fluor® 488 or 568 goat anti-mouse or anti-rabbit IgG (1:1000; Invitrogen, Eugene, OR).

BrdU saturation labeling

The protocol was based on Mueller and Wullimann, 2002. 5 dpf larvae were incubated in 10 mM bromodeoxyuridine (BrdU, Sigma) in E3 medium at 28.5 °C for 14 h. Larvae were sacrificed immediately and fixed in 4% PFA. Fixed samples were washed with PBDT (1xPBS, 1% DMSO and 0.1% Tween-20) and placed in 100% of methanol for 1 h at −20 °C and rehydrated in a graded methanol in PBDT, following rehydration, all larvae were digested with proteinase K in PBDT (10 μg/ml) for 20 min, refixed in 4% PFA for 20 min, washed three times in PBDT (3 × 10 min). To denature DNA, samples were incubated in 1 N hydrochloric acid in PBDT for 1 h at room temperature and washed three times (3 × 20 min) in PBDT. Thereafter, samples were incubated in 4% blocking solution for 1 h at room temperature and in rat anti-BrdU (ab6326, 1:300; Abcam, Cambridge, MA) 16 h at 4 °C. The secondary antibodies were applied as described in the immunocytochemistry section.

Imaging

Brightfield images were taken with a Leica DM IRB inverted microscope with a DFC 480 charge-coupled device camera and z-stacks were processed with Leica Application Suite software and Corel DRAW X3 software using the multifocus algorithm. Immunofluorescence samples were examined using a Leica TCS SP2 AOBS confocal microscope. For excitation, an Argon laser (488 nm) and green diode laser (561 nm) were used. Emission was detected at 500–550 nm and 560–620 nm, respectively. Cross-talk between the channels, and background noise were eliminated with sequential scanning and frame averaging as described earlier (Sallinen et al., 2009b). Stacks of images taken at 0.2–1.2 μm intervals were compiled, and the maximum intensity projection algorithm was used to produce final images with Leica Confocal Software.

Quantification of dopaminergic neurons

After WISH staining, specimens were mounted in 80% glycerol and cell numbers were counted using a 20× objective in the multifocus mode. All experiments were independently repeated three times.

High performance liquid chromatography (HPLC)

At the stage of 5 dpf, 30-pooled larvae were collected and sonicated in 150 μl of 2% perchloric acid. After centrifugation, 10 μl of supernatant was assessed for dopamine concentration by HPLC. The detection details are described in Sallinen et al., 2009a, 2009b.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Dunnett's test. Student's *t*-test was performed to determine significant differences in relative expression levels between the control and morphant group in stage-relative and tissue-specific experiments. Data analysis was performed by GraphPad Prism v.4.1 software (San Diego, CA).

Results

manf Orthologue gene in zebrafish is highly conserved

MANF is reported as an evolutionarily conserved neurotrophic factor according to the amino acid identity and phylogenetic analysis among invertebrate and vertebrate species (Lindholm et al., 2008). A BLAST search using human MANF amino acid sequence (NP_006001) only identified a single zebrafish *manf* homologous gene. RT-PCR was performed to obtain the full-open reading frame of zebrafish *manf* cDNA and the predicted amino acid sequence matched exactly the annotated MANF in the Genebank database. The zebrafish *manf* gene consists of four exons on chromosome 22 (NM_001076629) and the exon–intron boundaries were highly conserved with human *MANF* gene. The open reading frame includes 180 amino acids, sharing approximated 80%, 66% and 60% of amino acid identity with human, fly and worm MANF, respectively, and eight structurally conserved cysteine residues were found in zebrafish MANF. Using the protein homology/analogy recognition engine Phyre (Kelley and Sternberg, 2009), the predicted secondary structure showed seven α-helices and the 3D model had the highest similarity to the human MANF (Hellman et al., 2010). Moreover, the structure of N-terminus domain was highly similar to the human saposin D whereas the C-terminus was similar to SAP domain (Hellman et al., 2011).

manf has widespread expression during embryonic development and in adult organs.

The spatiotemporal expression of *manf* was analyzed by whole mount *in situ* hybridization (WISH), immunohistochemistry and qPCR. The *manf* transcript was detectable at 2 hpf (Fig. 1A). It appeared in the polster (Fig.1B) and notochord (Fig. 1C) at 11 hpf. At 1 dpf, *manf* was expressed in the head and otic vesicles (Fig. 1D); at 2 dpf, the expression was mainly detected in pharyngeal arches but very little in the head (Fig. 1E). At 3 dpf, it was primarily expressed in peripheral organs such as cranial cavity, intestine, liver and posterior neuromasts (Fig. 1F and F'). On the other hand, positive signals were completely abolished when the sense riboprobe and omission of the antisense probe were performed as negative control (data not shown). By qPCR analysis, the *manf* transcript had the highest expression level in whole embryos at 2 hpf (Fig. 1G), and then gradually declined at later stages, indicating that *manf* mRNA had maternal contribution during early development. In male adult tissues, the liver had the highest expression level compared to the brain, eyes, and kidney (Fig. 1H).

manf Expression is observed in several adult brain regions

In order to characterize the expression pattern of *manf* in adult brain, *in situ* hybridization and immunohistochemistry were

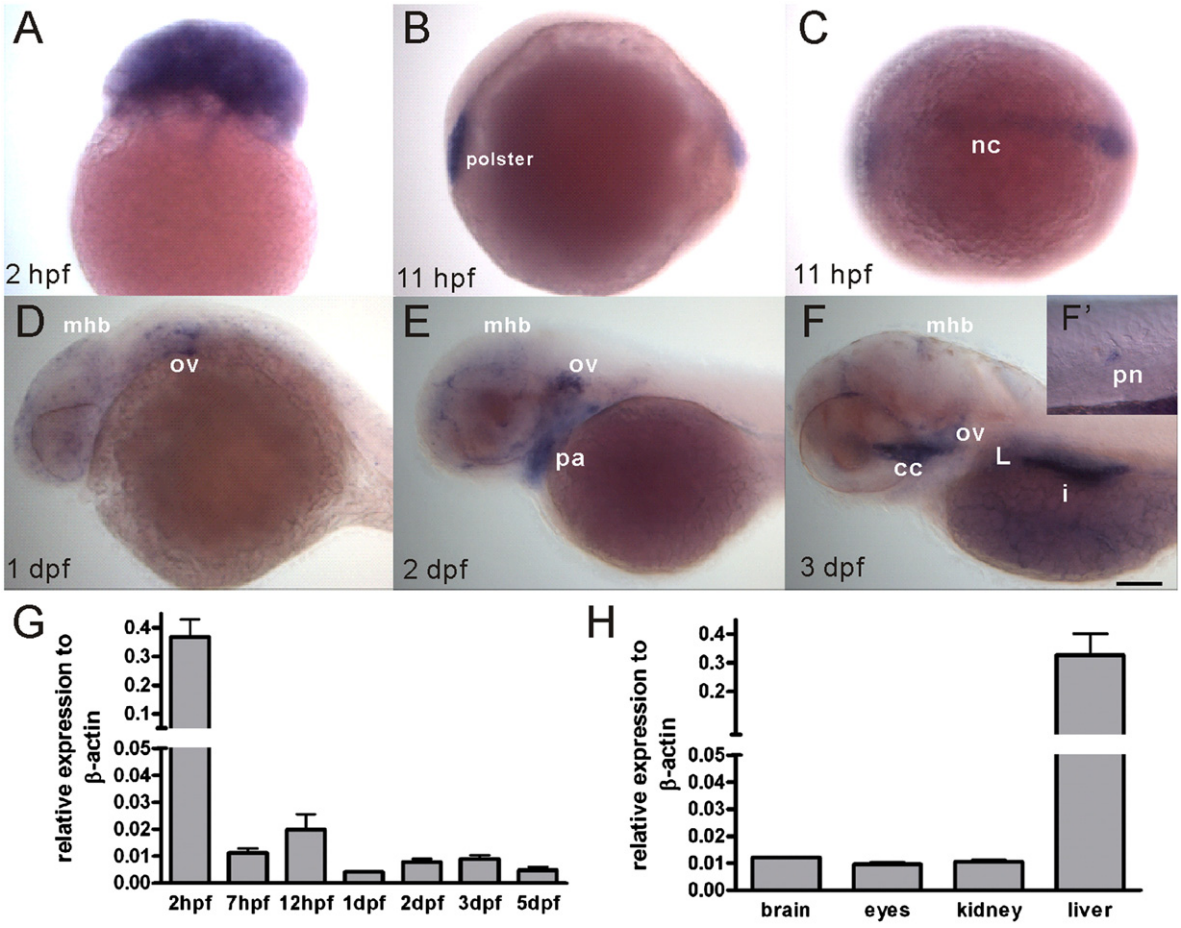

Fig. 1. Spatiotemporal expression of the *manf* transcript during zebrafish embryogenesis and in adult organs. (A–F) *manf* expression pattern was revealed by whole-mount *in situ* hybridization, anterior to the left. (A) lateral view of a 2 hpf embryo, (B) lateral view of a 11 hpf embryo, (C) dorsal view of a 11 hpf embryo, (D–F) lateral view of a one dpf, two dpf and three dpf embryo, (F') high magnification view of a posterior neuromast. (G) *manf* mRNA levels were analyzed by quantitative PCR. Thirty embryos/group were collected at defined stages. Each experiment was repeated three times using independent RNA isolations. (H) Adult tissues were collected from five individual one-year old male fish and analyzed by qPCR. cc, cranial cavity; i, intestine; L, liver; mhb, midbrain–hindbrain boundary; nc, notochord; ov, otic vesicles; pa, pharyngeal arches; pn, posterior neuromast. Scale bar=100 μm.

performed on sagittal sections of nine-month-old male fish brain. *manf* transcript was most prominently detected in ventral telencephalic, preoptic, ventral thalamic, pretectal, dorsal thalamic, and hypothalamic regions (Fig. 2A). MANF protein was mainly expressed in the dorsal zone of the dorsal telencephalic area, the ventral nucleus of the ventral telencephalic area, parvocellular preoptic nucleus, habenula, anterior thalamic nucleus, periventricular gray zone of optic tectum, telencephalic ventricle, corpus cerebelli and medial longitudinal fascicle (Fig. 2B). The MANF protein localization mostly correlated well with the mRNA expression pattern, although MANF immunoreactive cells were additionally detected in optic tectum, cerebellum and rhombencephalic ventricular regions (Fig. 2B). A slight discordance between mRNA and protein expression patterns was observed. This may be caused by the difference of protein trafficking in various brain regions since MANF is a secretory protein or by posttranscriptional regulation and/or slow protein turnover rate. To characterize which cell type specifically expressed MANF, a general neural marker (HuC/D)(Kim et al., 1996; Mueller and Wullimann, 2002) a dopaminergic neuron marker (TH1, tyrosine

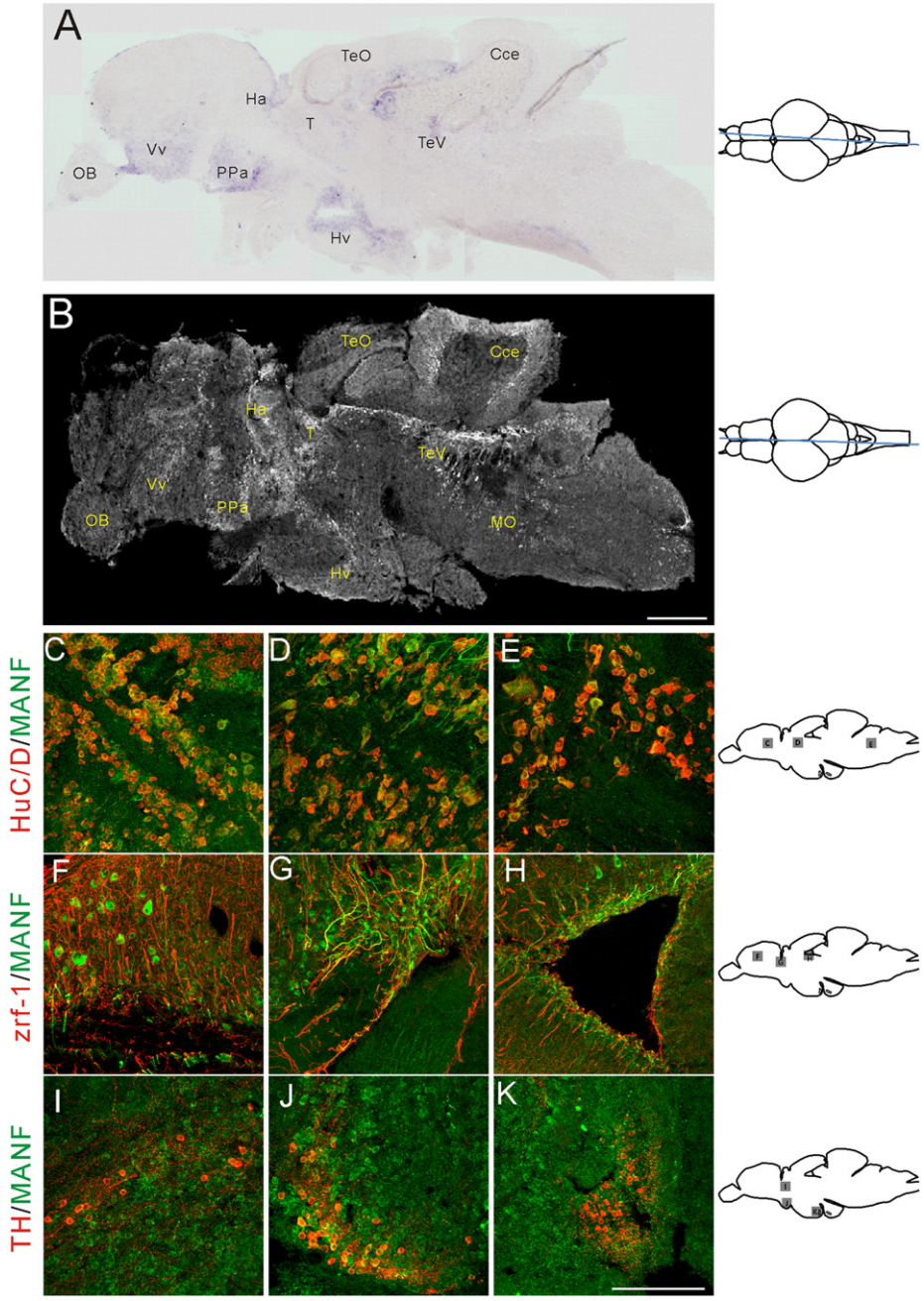

Fig. 2. *In situ* hybridization mRNA analysis and immunocytochemistry of MANF expression in adult zebrafish brain. (A) A sagittal view shows *manf* mRNA in an adult brain section. Expression can be seen in ventral telencephalic, preoptic, ventral thalamic, pretectal, dorsal thalamic, and hypothalamic regions. (B) A sagittal view of the brain shows immuoreactivity for MANF protein. Expression is shown in telencephalic, preoptic, thalamic, optic tectal, cerebellar and rhombencephalic ventricular regions. MANF antibody co-immunostaining with HuC/D antibody in ventral telencephalic (C), dorsal thalamic (D), and rhombencephalic ventricular regions (E). Co-immunostaining with zrf-1 in dorsal telencephalon (F), ventral thalamic region (G), and telencephalic ventricle (H). Co-immunostaining with TH1 in ventral thalamic area (I), preoptic complex (J), and in caudal hypothalamus (K). MANF staining is shown in green; HuC/D, zrf-1 and TH staining are shown in red. Cce: cerebellar corpus. Ha: habenula. Hv: ventral zone of periventricular hypothalamus. OB: olfactory bulb. PPa: parvocellular preoptic nucleus, anterior part. T: thalamus. TeO: tectum opticum. TeV: tectal ventricle. Vv: ventral nucleus of ventral telencephalic area. Scale bar = 300 μm in (B), 100 μm in (K).

hydroxylase 1), and a glial marker zrf-1 (GFAP, glial fibrillary acidic protein) antibodies were applied in double-staining experiments with the MANF antibody. The majority of MANF-positive cells were HuC/D positive cells in the telencephalon, optic tectum, diencephalon and rhombencephalon (Fig. 2C–E). However, a few MANF positive processes showed GFAP positive signals in ventral thalamic region and around telencephalic ventricle (Fig. 2F–H). The MANF-positive cells were located close to TH1 positive cells in preoptic, ventral and dorsal thalamic regions, and only few MANF-containing cells were found to co-express TH1 (Fig. 2I–K). MANF double-labeling with zrf-1, HuC/D and TH was also performed on 3-dpf and 5-dpf sagittal sections (Suppl Fig. 1). The result was in agreement with the adult one. The majority of MANF-containing cells were HuC/D positive cells rather than glial cells or TH positive mature neurons from early development to adulthood in zebrafish brains, indicating that zebrafish MANF appears to be mainly expressed in the mature neuronal cells.

Splice-blocking MOs perturb MANF expression

To understand the function of MANF during zebrafish development, a combination of two antisense splice-blocking MO (MOsp) were mainly used in this study. The targeting sites of the two MOs, MOsp1 and MOsp2, are depicted in Fig. 3A. The efficacy of MOsp was determined by qPCR using 3 dpf fish and immunoreactivity was studied in 5 dpf fish. The qPCR result showed that the combination of MOsps injection perturbed approxiamately 75% of normal *manf* pre-mRNA splicing at 3 dpf (Fig. 3A). Moreover, the western blot depicted that the MANF protein was reduced by maximum of 85% following *manf* MOsps injection at 3 dpf (Fig. 3B). The immunohistochemistry showed that MOsp successfully knocked down MANF protein expression in the brain, skin cells, anterior and posterior neuromasts and liver at 5 dpf (Fig. 3D). An antigen absorption control was performed to confirm the specificity of MANF antibody in zebrafish. The blocking peptide successfully wiped out the immunoblotting signal and immunoreactivity (Fig. 3B and D blocking peptide).

To confirm the specificity of *manf* MOsp, qPCR was further carried out to quantify the mRNA level of *p53* and *delta 113p53* using 1 dpf embryos (Robu et al., 2007). *p53* and *delta 113p53*, recognized as off-targeting markers, are induced usually by the MO knockdown technology resulting in non-specific morphological phenotypes such as a small size of head, neural death and curved body in MO-injected embryos at 1 dpf. The qPCR result showed that the mRNA level of *p53* was not altered although that of *delta 113p53* was four times higher than in the ctrl MO group (data not shown). Nonetheless, MANF-deficient morphants developed with a normal size of head, eyes, somites and notochord without any apparent defects in the head at 24 hpf, or any distinguishable gross phenotype at 3 dpf (Fig. 3C). Importantly, the phenotypic changes induced by *manf* MOsps were rescued by co-injection of

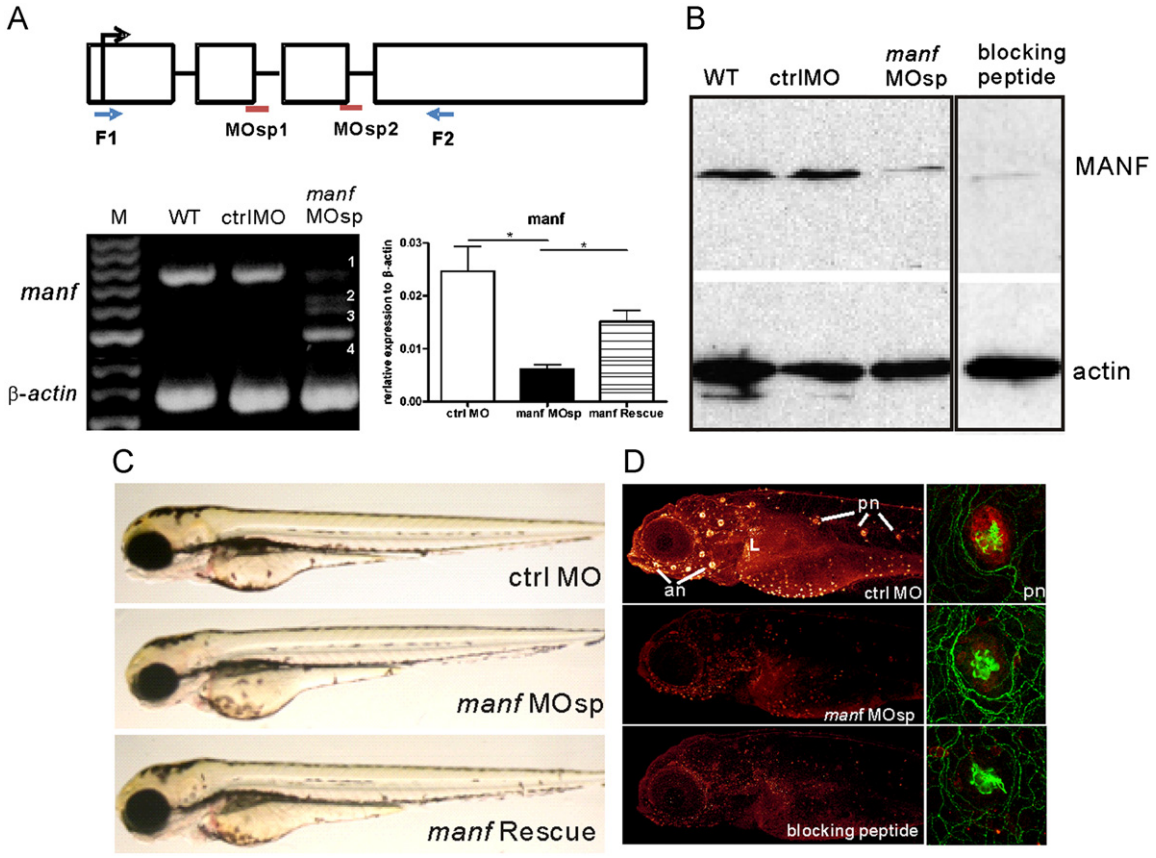

Fig. 3. Efficacy of splice-blocking morpholino oligonucletides (MO). (A) The zebrafish *manf* gene includes four exons. The translation start site is located in the exon 1. The targeting sites of two MOs are in red (MOsp1 and MOsp2). The primer pair used for RT-PCR is shown in blue (F1 and F2). Transcripts of 3-dpf uninjected, control and *manf* MOsp embryos were used to determine the MO efficiency. Band 1 in the gel electrophoresis indicates the regular *manf* transcript. Bands 2, 3 and 4 represent the aberrant splicing variants. All PCR products were verified by sequencing. qPCR was performed to quantify the mRNA expression level ($^{*}p < 0.05$, $n=3$, Student's *t*-test). (B) The western blot analysis of MANF expression in the wild type, control MO and *manf* MOsp- injected groups at 3 dpf. The blot of anti-β-actin was used as an internal control. (C) Bright-field images of control, *manf* MOsp morphants and *manf* RNA rescue larvae at 3 dpf. (D) MANF immunostaining on 5-dpf ctrl MO, *manf* MOsp injected embryos. A higher magnification of a posterior neuromast is shown by double-labeling with MANF shown in red and acetylated tubulin shown in green. The uninjected larvae stained using MANF antibody preabsorbed with MANF blocking peptide to examine the specificity of MANF antibody. MANF positive cells were labeled in bright red. an, anterior neuromast; L, liver; pn, posterior neuromast. Scale bar = 100 μm.

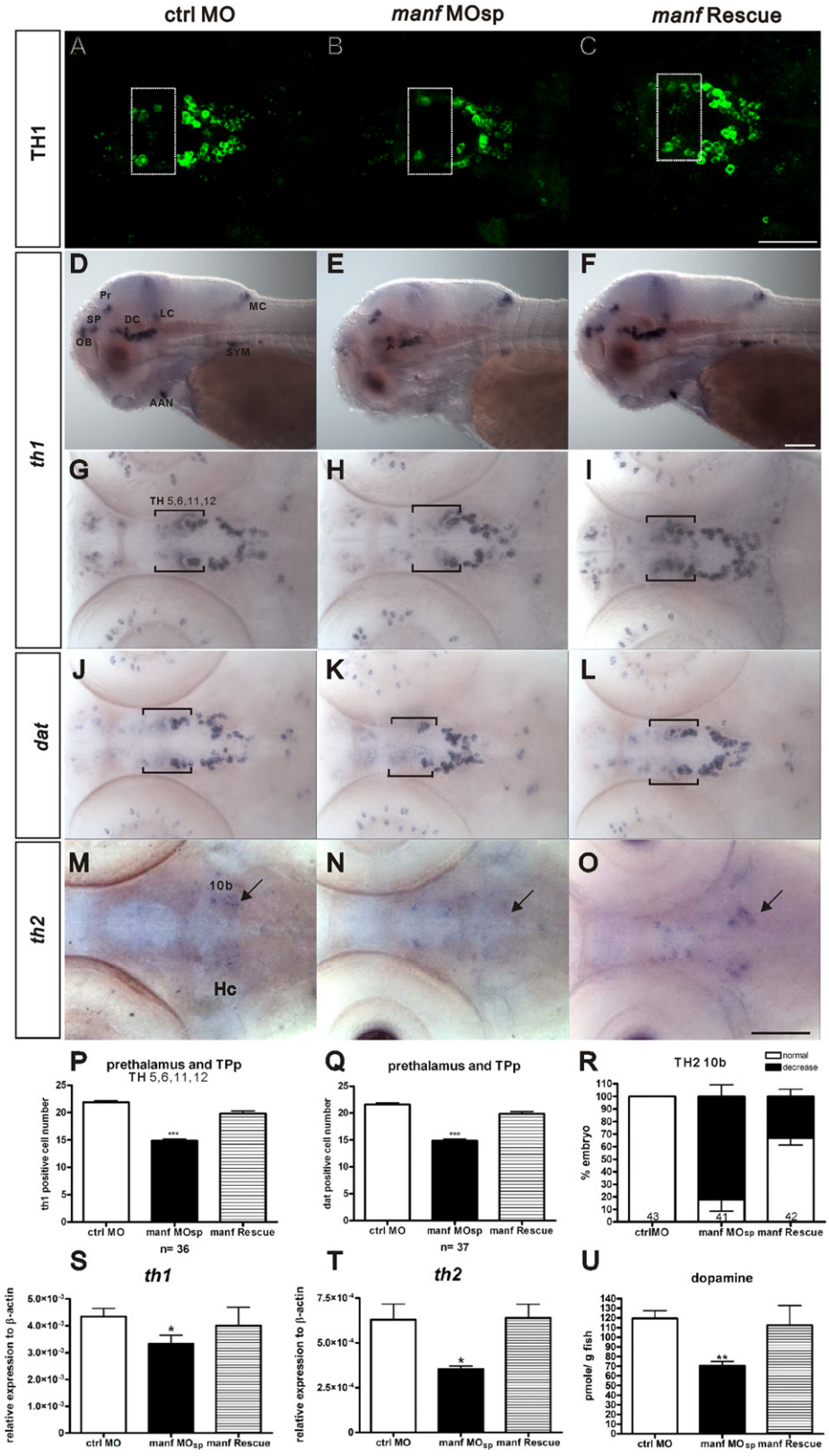

Fig. 4. Declines of gene expression of two tyrosine hydroxylases, dopamine transporter and dopamine level in *manf* MOsp morphants. Spatial distribution of *th1*, *th2* and *dat* was revealed by whole mount *in situ* hybridization at 3 dpf control MO, *manf* MOsp and *manf* RNA rescued morphants. (A–C) A dorsal view of TH 1 immunohistochemistry. (D–F) A lateral view of *th1* expression pattern. (G–I) A dorsal view of *th1* expression pattern. (J–L) A dorsal view of *dat* expression pattern. (M–O) A dorsal view of *th2* expression pattern. Quantitative analysis of the number of th1-containing cells (P) and *dat*-positive cells (Q) in prethalanus and TPp dopaminergic populations 5,6,11 and 12 defined by Sallinen et al., 2009b; DC1 and DC2 group defined by (Rink and Wullimann, 2002; Schweitzer et al., 2012). (R) The percentage of embryos with normal and decreased *th2*-positive signal (***$p < 0.001$, one-way ANOVA with Dunnett's test). (S–T) qPCR analysis of *th1* and *th2* transcript levels (*$p < 0.05$, $n=3$, Student's *t* test). (U) dopamine concentration measured by HPLC at 4 dpf (**$p < 0.01$, $n=4$, Student's *t* test). The frame shown in the G–L indicates the deficiency of *th1* cell populations (TH1 populations 5,6,11 and 12) in a *manf* MOsp morphant. The arrow in M–O indicates the site of the deficiency of *th2* cell population in caudal hypothalamus (Hc, group 10b) in a *manf* MOsp morphant. AAN: arch-associated neurons; DC: diencephalic catecholaminergic cluster; LC: locus coeruleus; MC: medulla catecholamergic cluster; OB: olfactory bulb. Pr: dorsal pretectum; Sym: sympathetic neurons; TPp: periventricular nucleus of posterior tuberculum. Scale bar = 100 μm.

manf mRNA (Fig. 4). Taken together, the MOs applied in this study enabled specific inhibition of MANF expression.

Knockdown of MANF decreases th1, th2 and dat expression

MANF is reported to have neurotrophic effects on the regulation of development of dopaminergic neurons *in vitro* (Petrova et al., 2003). Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the biosynthesis of catecholamines and commonly used as a dopaminergic neuron marker. In zebrafish, two non-allelic *th* genes, *th1* and *th2*, have been reported (Candy and Collet, 2005; Chen et al., 2009; Filippi et al., 2010; Yamamoto et al., 2010). To investigate whether knocking down MANF expression has any effects on *th1* and *th2* expression *in vivo*, whole mount *in situ* hybridization and qPCR were performed on 3 dpf ctrl MO and MANF MO injected fish. The number of *th1*-containing cells in prethalamus and periventricular nucleus of posterior tuberculum (TPp), DC1 and DC2 defined in (Rink and Wullimann, 2002; Schweitzer et al., 2012) and dopaminergic cell group 5,6,11 and 12 in Sallinen et al., 2009b, was decreased in the *manf* MOsp injected group compared to the ctrl MO group (Fig. 4D, E, G, H and P). The expression pattern of the dopamine transporter (dat) in *manf* morphants was affected: there were fewer *dat*-containing cells in the prethalamus and TPp (Fig. 4J, K, L and R). No obvious changes were observed in other *th1* cell groups in diencephalon (Suppl Fig. 3). Furthermore, the *th2* positive signal in caudal hypothalamus (Hc, group 10B, definition based on th2 cell groups as described earlier by Chen et al., 2009. was reduced in MANF-deficient morphants compared to that in the ctrl MO group (Fig. 4M, N and R). At this early stage, the other *th2* cell groups were not sufficiently developed to allow reliable evaluation. The effect was apparently specific because co-injecting zebrafish *manf* mRNA with antisense *manf* MOsp was able to rescue the decrease of *th1*, *dat* and *th2* cell groups at 3 dpf (Fig. 4F, I, L and O). qPCR results also confirmed that *th1* and *th2* transcript levels were significantly lower in the MANF knockdown group than in the control and *manf* mRNA rescue groups (Fig. 4S and T). Immunocytochemistry using TH1 antibody on 3 dpf MANF deficient fish revealed the same defect as that shown by *in situ* hybridization (Fig. 4A and B). Moreover, *manf* mRNA injection rescued the decrease of the TH1-containing cells (Fig. 4C). Importantly, the dopamine level was significantly decreased in MANF morphants (Fig. 4U), and the decrease of dopamine level was restored by *manf* mRNA injection. During early development, the number of *th1*-containing cells was lower than control and *manf* rescue groups at 24 hpf and 36 hpf (Suppl Fig. 2). Over-expression of *manf* mRNA at early embryonic stages did not affect dopaminergic neuron development (Suppl Fig. 2). Taken together, lack of MANF resulted in a significant decrease of *th1*, *dat* and *th2*-containing cells in the prethalamus and TPp region, suggesting that MANF may play an important role in regulation of the dopaminergic neuron development during zebrafish embryonic development.

Knockdown of manf does not alter 5-HT, GABA and epinephrine levels or hdc and hcrt expression

To demonstrate that *manf* MOsp does not exhibit a general effect on all hypothalamic neuron populations, the serotonergic and GABAergic systems were analyzed by 5-HT and GABA immunostaining because 5-HT and GABA synthesis is mediated by proteins encoded by several genes, the histaminergic system by *hdc* mRNA level (the diagnostic marker of hypothalamic histaminergic neurons) and hypocretin/orexin system by *hcrt* mRNA level (hypocretin neuropeptide precursor) were quantified by qPCR because these transmitters are produced by proteins encoded by single genes. The expression pattern of 5-HT and GABA immunoreactivity on 5 dpf brains was not altered in MANF morphants (Fig. 5A–D). In addition, the serotonin and epinephrine levels measured by HPLC (Fig. 5E and F) and *hdc* and *hcrt* expression did not differ in 3 dpf MANF morphants from that of control MO treated fish (Fig. 5G, H; $p > 0.05$, $n=3$ Student's *t*-test). These data indicated that MANF is at least to a significant degree specifically involved in regulation of dopaminergic neuron development.

The expression patterns of Notch1a, zash1a and BrdU labeled cells are not altered

Lack of MANF in embryonic stages caused a significant decrease of catecholaminergic neurons in group 5,6,11 and 12 of *th1* clusters located in the prethalamus and TPp and group 10b of *th2* cells in the caudal hypothalamus. Since MANF was mostly expressed in HuC/D positive cells, we hypothesized that MANF may have function on regulation of differentiation and maturation of dopaminergic neurons during development. In order to examine this hypothesis, several zebrafish genes involved in early neurogenesis were first analyzed (Mueller and Wullimann, 2002, 2003; Wullimann and Mueller, 2002). *In situ* hybridization was performed on 2 dpf fish using *Notch1a* (a proliferative cell marker, Fig. 6A–C) and *zash1a* (a proneural marker, Fig. 6D–F). The BrdU saturation labeling was performed to detect all proliferative cells at 5 dpf (Fig. 6G–I). In comparison with those of the control group, the signals in MANF morphant groups showed no difference, suggesting that MANF may not be involved in the early proliferation phase but possibly in the later differentiation and maturation stage or in the maintained survival of the progenitor neuron cells in the dopaminergic neuronal lineage.

Increase of nr4a2b and pax2a transcripts in manf MOsp morphants at 3 dpf

pax2 and *pax5* are reported to be involved in the differentiation of dopaminergic progenitors, and Nr4a2 plays an important role in both the survival and differentiation of mesencephalic dopaminergic precursor cells (Blin et al., 2008; Luo et al., 2008; Simon et al., 2003). To confirm our hypothesis that MANF may participate in later differentiation of the dopaminergic development, zebrafish Nr4a2b, pax2a and pax5 were studied in 3-dpf larvae. Compared to the control group, *manf*-deficient larvae showed an increase of *nr4a2b* positive signals in the posterior tuberculum (15 individuals out of 15, white rectangle in Fig. 7A and B), rhombencenphalic area (white arrow in Fig. 7A and B) and spinal cord (Fig. 7D and E). The *pax2a* transcript was also increased in midbrain hindbrain boundary, hindbrain (15 individuals out of 15, Fig. 7G and H) and spinal cord (Fig. 7J and K) in *manf*-deficient larvae compared to that of the control group. Zebrafish *manf* mRNA co-injected with the *manf* MOs prevented the increase of *nr4a2a* expression in the posterior tuberculum and spinal cord as well as *pax2a* expression in rhobencephalic and spinal cord in *manf* morphants (Fig. 7C, F, I and L). The *in situ* hybridization result was further validated by qPCR. The transcript level of *nr4a2b* in *manf* morphants showed a tendency towards increase compared to ctrl MO and *manf* mRNA co-injected groups although the increase of *nr4a2b* was not statistically significant (Fig. 7M; $p > 0.05$, $n=3$, Student's *t*-test). It is possible that the highly localized increase is not evident in qPCR done on whole embryos due to expression in many other sites. The *pax2a* qPCR analysis result, a significant increase ($p < 0.05$, $n=3$, Student's *t*-test), was in accordance with the *in situ* hybridization result. The *pax5* transcript level showed no difference between control, *manf* morphants and *manf* mRNA co-injected groups (Fig. 7O). In short, knockdown of MANF resulted in up regulation of *nr4a2b* and

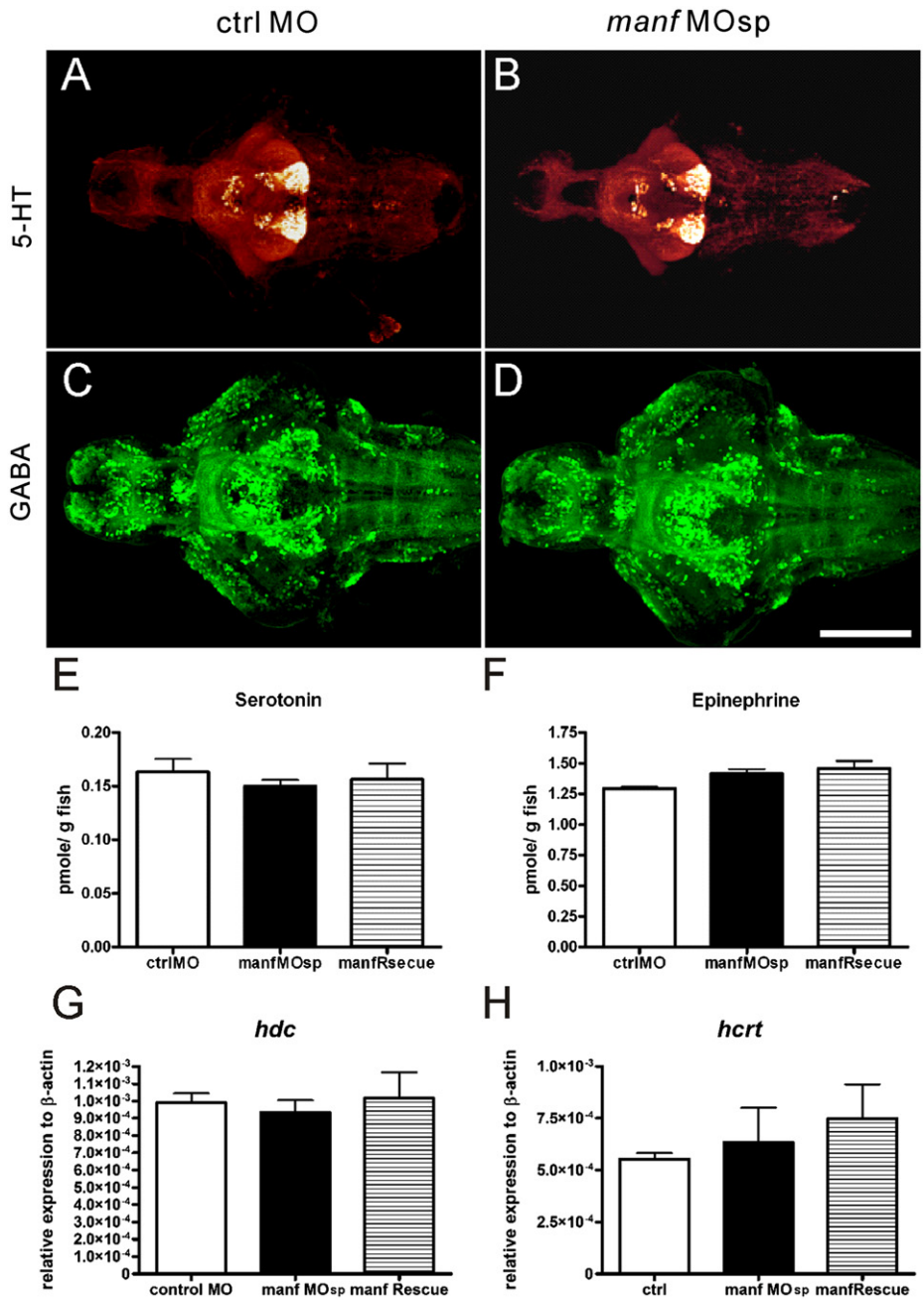

Fig. 5. Knockdown of *manf* did not alter the serotonergic system, GABAergic system, *hdc* and *hcrt* expression in *manf* deficient embryos. (A and B) 5-HT and (C and D) GABA immunostainingin control MO and *manf* MOsp morphants in 5 dpf brains. (E) The serotonin level and (F) the epinephrine level measured by HPLC. qPCR analysis of *hdc* (G) and *hcrt* (H) transcript levels in 3 dpf larvae ($p > 0.05$, $n=3$, Student's *t*-test). Scale bar=100 μm.

pax2a whereas overexpression of *manf* mRNA restored this impairment, suggesting that MANF may interact with Nr4a2 and pax2a in regulation of the differentiation of dopaminergic development.

MANF partially rescues the decrease of th1 containing cells in Nr4a2 morphants

Two homologs of mammalian *nr4a2* genes, *nr4a2a* and *nr4a2b*, are found in zebrafish (Blin et al., 2008; Filippi et al., 2007; Luo et al., 2008). Both *nr4a2* genes play an important role in regulation of differentiation of dopaminergic neurons in the posterior tuberculum region in developing zebrafish brain (Blin et al., 2008; Luo et al., 2008). Knocking down both *nr4a2* expression was associated with a significant decrease of *th1* positive cells in amacrine neurons, preoptic area, prethalamus, and the posterior tuberculum region in Nr4a2 morphants (Fig. 8A and B). This *th1* defect in the Nr4a2 morphants in this study was in agreement to the findings of Blin et al., 2008 and Luo et al., 2008. A similar decline in *th1* expressing neurons was evident also in the *manf*-deficient larvae in the present study in the prethalamus and TPp (population 5,6,11 and 12) (Fig. 4G–I). We hypothesized that MANF and Nr4a2 may regulate dopaminergic development in the same pathway in certain brain areas. To test this hypothesis, *manf* mRNA was overexpressed in the Nr4a2 morphants. Strikingly, the *th1 in situ* hybridization and qPCR analysis showed that *manf* mRNA prevented the decrease of *th1* expression in selective diencephalic regions, population 5,6,11 and 12 (white rectangle,

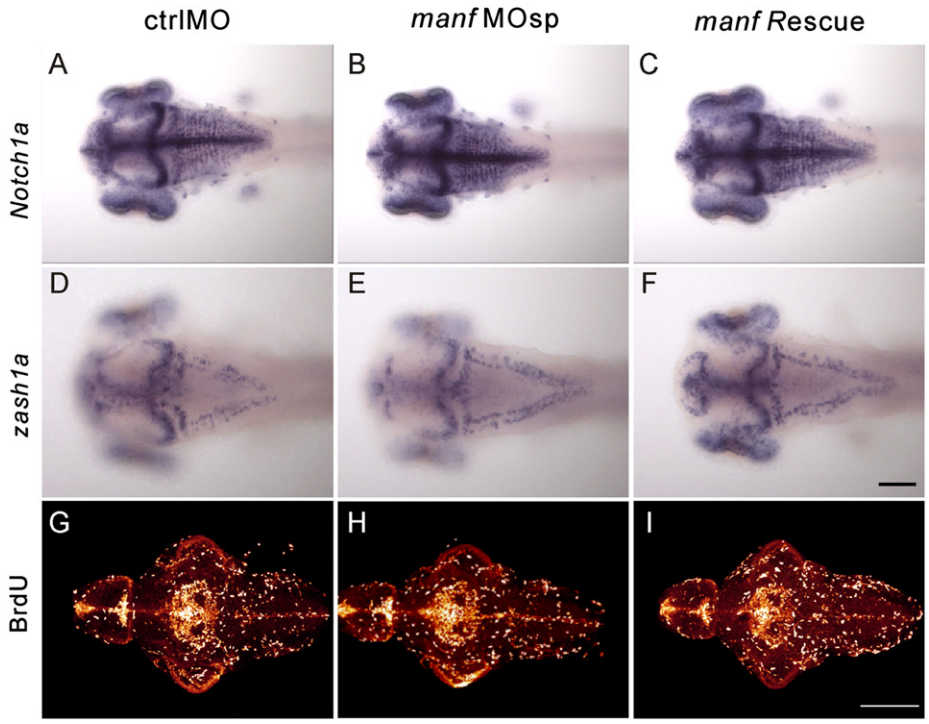

Fig. 6. Expression pattern of *Notch1a, zash1a* and BrdU labeled cells did not alter. Spatial distribution of *Notch1a* and *zash1a* was revealed by whole mount *in situ* hybridization at 2 dpf control MO, *manf* MOsp and *manf* RNA rescued morphants. BrdU saturation label analysis was performed in 5 dpf fish. (A–C) The dorsal view of *Notch1a* expression pattern. (D–F) The dorsal view of *zash1a* expression pattern. (G–I) The dorsal view of BrdU labeled cells. Scale bar=100 μm.

Fig. 8C–E), but not in preoptic area or amacrine neurons in retina. On the other hand, the expression level of *th2* was significantly reduced in Nr4a2 morphants compared to the ctrl MO group whereas *manf* mRNA could not restore the decreased level of *th2* transcripts in Nr4a2 morphants (Fig. 8F). *manf* transcript level showed a tendency towards a decline in Nr4a2 morphants compared to that of the control group (Fig. 8G; $p=0.06$, $n=3$, Student's *t*-test).

Discussion

In this report, we specifically abolished MANF expression by means of antisense morpholino oligonucleotides in zebrafish. The knockdown effect was verified using a specific antibody against MANF, and sequencing of the aberrant transcripts. The effect appeared to be specific, because no characteristic abnormal morphological phenotype was shown, and co-injection of *manf* mRNA with *manf* MOsp rescued the defects observed. Despite this apparent lack of gross phenotype, the knockdown of MANF caused a decrease of catecholaminergic neurons in the certain diencephalic region and led to a significant decrease of dopamine during zebrafish embryonic development. Remarkably, *manf* mRNA enabled a rescue of the decrease of *th1* positive cells in the diencephalon in the Nr4a2 deficient larvae. These results reveal for the first time in a vertebrate model that MANF interacts with Nr4a2 and plays a crucial role in regulation of dopaminergic neuron development and maintenance.

MANF distribution in fly and rodents has been characterized (Lindholm et al., 2008; Palgi et al., 2009). It is widely expressed in central nervous system and in peripheral organs at all development stages and in adult animals. In the fruitfly, it is detectable in glial cells, but not co-localized with any known neuronal markers. In mammalian brains, it is largely expressed in cells with neuronal morphology, and a few MANF positive dopaminergic neurons can be found in substantia nigra pars compacta (Lindholm et al., 2008). Likewise, zebrafish MANF has widespread expression at various stages in the central nervous system and peripheral organs. Before the gastrula stage *manf* mRNA is distributed in the axis notochord, floor plate and hatching gland. Moreover, zebrafish *manf* RNA has the highest expression during pharyngula stage, but the expression declines in the brain at later stages. Both in mice and zebrafish, *manf* mRNA was detected at early embryogenesis and expressed in brain and peripheral organs. Based on the high similarity of spatiotemporal distribution and predicted protein structures across the species, MANF may play a role during developmental stages. In adult zebrafish brain, the main expression regions are largely restricted in ventricular zones along the entire anteroposterior axis. These regions have been defined as proliferation zones that include neural stem cells involved in neurogenesis in zebrafish brain (Grandel et al., 2006; Kaslin et al., 2008). Interestingly, most MANF-positive cells are Hu/D positive cells surrounding TH-positive cells indeed but lack GFAP expression, suggesting that MANF is expressed in neuronal rather than glial cells in zebrafish brain, a clear difference from the expression pattern in fly. These results illustrate that zebrafish MANF is mostly expressed in the neuronal cells, and provide useful evidence that MANF plays a crucial role in maintenance or regulation of dopaminergic, but not e.g. histaminergic, hypocretin/orexin, or GABAergic neurons in central nervous system.

The MO knockdown technique provides a powerful tool to temporally knockdown gene expression in zebrafish. Although MOs may cause some unexpected side effects, under appropriate controls such as mRNA rescue experiments and p53 diagnosis of off-targeting effect, it enables specific and effective knockdown of genes of interest (Eisen and Smith, 2008; Robu et al., 2007). In this study, two types of MOs against MANF expression were applied, a translation-blocking MO disturbing the translation mechanism of maternal and zygotic mRNA (data not shown) and a splice-blocking MO perturbing the zygotic pre-RNA splicing process.

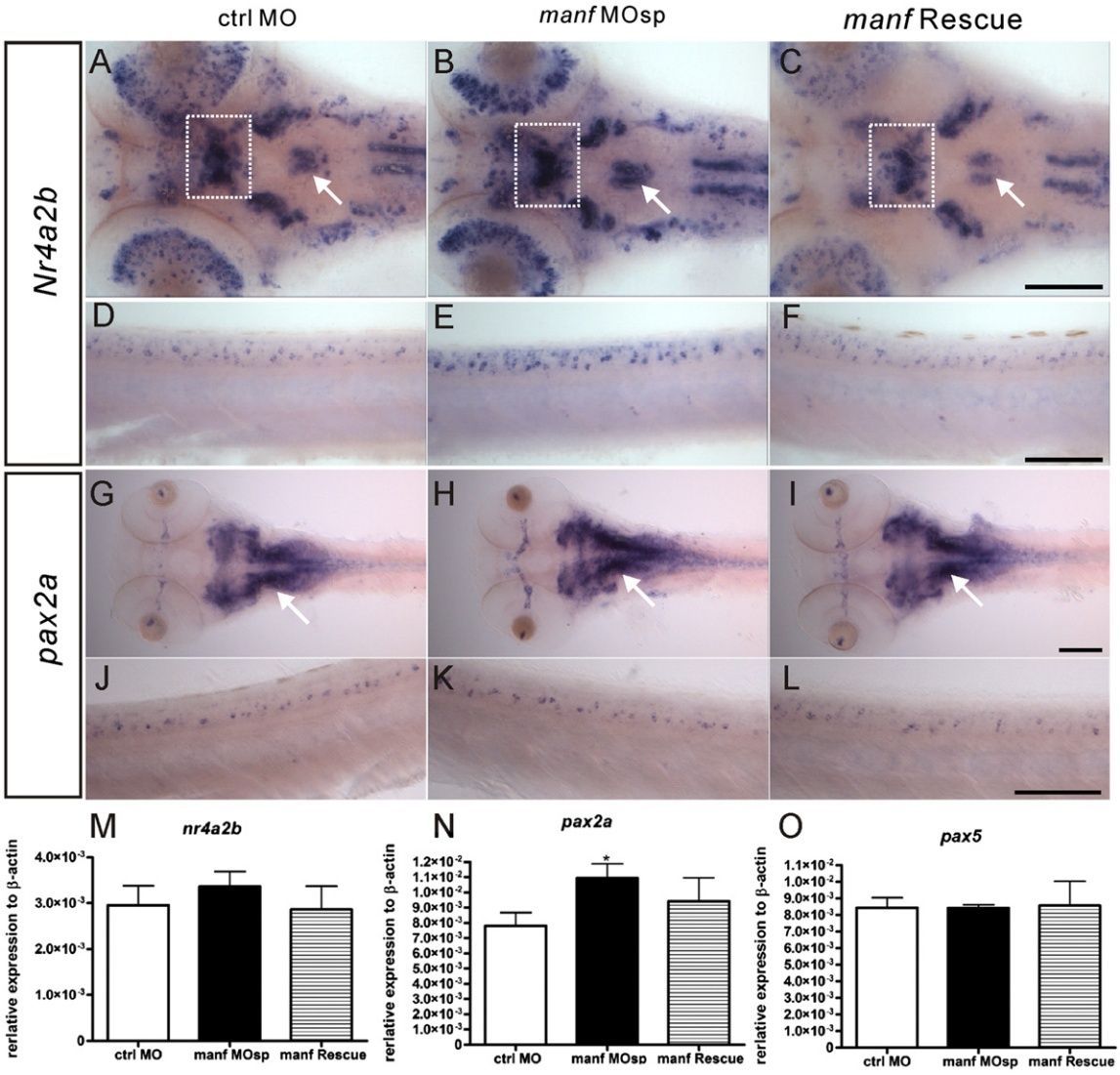

Fig. 7. Increase of *nr4a2b* and *pax2a* expression in *manf* MOsp morphants at 3 dpf. The spatial distribution of *nr4a2b* and *pax2a* was revealed by whole mount *in situ* hybridization. (A–C) dorsal views of *nr4a2b* expression pattern in the head and (D–F) lateral views of the spinal cord. (G–H) dorsal views of *pax2a* expression pattern in the head and (J–L) lateral views of the spinal cord. qPCR analysis of *nr4a2b* (M), *pax2a* (N) and *pax5*(O) ($^*p < 0.05$, $n=3$, Student's *t*-test). White rectangles indicate the increase of *nr4a2b* expression in the posterior tuberculum and white arrows indicate the increased *nr4a2b* and *pax2a* expression in rhombencephalon in *manf* MOsp morphants. Scale bar = 100 μm.

Moreover, proper control experiments were carried out to prove the specificity of the working *manf* MOs. The MANF-deficient fish had no apparent phenotypic defects within 5 dpf and morphant larvae swam normally (data not shown) although shortage of MANF caused a reduction of tyrosine hydroxylase positive cells during embryogenesis. This phenotype, however, is contradictory to the MANF mutant fly (Palgi et al., 2009). In Drosophila, lack of MANF has a lethal effect, characterized by a massive degeneration of cells, disappearance of axon bundles, and developmental abnormalities of the nervous system and cuticular elements. Nevertheless, both MANF deficient animal models are characterized by the decrease of dopamine and dopamine neurons. The different outcomes may be due to significant differences in the organization of neurotransmitter systems between invertebrates and vertebrates, even if MANF is an evolutionarily conserved neurotrophic factor. Interestingly, the species-related phenotype differences among human, zebrafish and fruitfly also appear in some other PD-related genes such as parkin and pink1 (Botella et al., 2009; Flinn et al., 2009; Kitada et al., 2007; Park et al., 2006; Pienaar et al., 2010; Sallinen et al., 2010). In those cases, the mutant zebrafish share more phenotypic identities with primates compared to those invertebrate models (Pienaar et al., 2010). On the other hand, MOs work effectively only within the first few days of fish life because of dilution during embryogenesis, so that the phenotype difference may be in part due to the incomplete perturbation. Further studies using a stable MANF mutant zebrafish line, when one becomes available, may provide a solution to confirm the phenotype observed in this study.

Due to the gene duplication in the vertebrate lineage, there are two *th* genes in zebrafish, *th1* and *th2* (Candy and Collet, 2005; Chen et al., 2009; Filippi et al., 2010; Yamamoto et al., 2010). Since zebrafish has evolved as a significant model animal for studying human neurodegenerative diseases, it is important to understand the difference of catecholamine system between zebrafish and mammals (Panula et al., 2010; Schweitzer and Driever, 2009). Studies of *th1* have been carried out for a long time (Kaslin and Panula, 2001; Ma, 1997; Panula et al., 2010; Rink and Wullimann, 2001; Schweitzer and Driever, 2009) while the *th2* expression pattern in larval zebrafish was first revealed by Chen et al., 2009, yet the biological function of *th2* is still unclear.

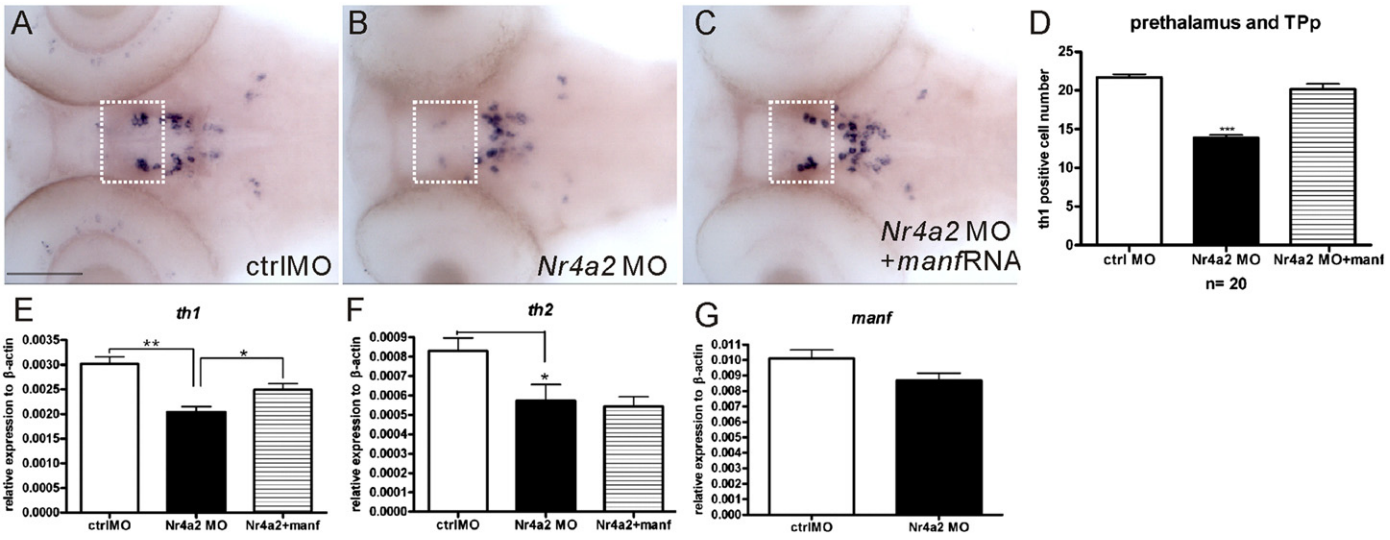

Fig. 8. MANF partially rescued the loss of *th1* cells in 3 dpf *Nr4a2* morphants. (A–C) *th1* expression pattern was revealed by whole mount *in situ* hybridization at 3 dpf control MO, *Nr4a2* MO and *Nr4a2 MO* co-injected with *manf* mRNA embryos, anterior to the left and dorsal to the top. (D) Quantitative analysis of the number of th1-containing cells in prethalanus and TPp dopaminergic population 5,6,11 and 12 defined by Sallinen et al., 2009b; DC1 and DC2 group defined by Rink and Wullimann, 2002. (***$p < 0.001$, one-way ANOVA with Dunnett's test) (E and F) qPCR analysis of *th1*, *th2* and *manf* transcript levels (*$p < 0.05$, $n = 3$, Student's *t* test). White rectangles indicate the loss of *th1* cell population in population 5,6,11 and 12. TPp: periventricular nucleus of posterior tuberculum. Scale bar = 100 μm.

We report here that shortage of MANF diminish the cell distribution of both *th1* and *th2* in the diencephalic groups 5,6,11 and 10b (see Sallinen et al., 2009b and Chen et al., 2009) during embryogenesis, suggesting that MANF may be involved in regulation of the development of both *th1* and *th2* containing neurons, but not GABAergic, serotonergic or histaminergic neurons which also reside in caudal hypothalamus close to the *th2* cell group 10b. MANF is largely expressed in postmitotic neuronal cells in proliferation zones in zebrafish brains. Since the expression pattern of several proliferation markers shows no change in MANF deficient embryos, it is possible that MANF is involved in differentiation of the dopaminergic progenitors into mature dopaminergic neurons.

A number of regulators and neurotrophic factors have been identified to control or maintain the developmental fate of dopaminergic neurons (Andressoo and Saarma, 2008). For instance, transcription factors pax2/5 participate in the late proliferation of dopaminergic progenitor cells (Simon et al., 2003). The orphan steroid receptor Nr4a2 is essential in differentiation of dopaminergic precursor cells and maintenance of dopamine neurons (Jankovic et al., 2005; Kadkhodaei et al., 2009). Loss of Nr4a2 leads to a decline of certain dopaminergic populations. On the other hand, it is possible that overexpression of Nr4a2 could cause the impairments of differentiation and maturation of dopaminergic cells. In *manf*-deficient larvae, a higher expression level of *pax2a* and *nr4a2b* transcripts was observed while this upregulation was prevented by *manf* mRNA overexpression, indicating that MANF may have an inhibitory effect on *nr4a2* and *pax2a* to direct dopaminergic precursors differentiating into mature dopamine neurons. Moreover, in Nr4a2 morphants, *manf* mRNA enabled a rescue of the decrease of *th1* expression in the prethalamus and TPp area, indicating that MANF may compensate the loss of Nr4a2 in order to maintain the regular development of dopaminergic neurons. That MANF and Nr4a2 function in the same pathway to regulate the dopaminergic development in particular regions of the zebrafish brain is possible. It is still unclear whether MANF and Nr4a2 regulate dopaminergic neuron development in the same mechanism or compensate each other. However, this study provides direct *in vivo* evidence that MANF is a relatively specific neurotrophic factor that is required for the development, differentiation or maintenance of dopamine neurons in embryogenesis.

In summary, this work indicates that zebrafish MANF is involved in regulating the development of certain groups of dopaminergic neurons in developing brains, and enables to rescue the loss of dopamine neurons in Nr4a2-dificient fish. Our MANF-deficient fish may provide a good animal model to study how MANF and other factors regulate the development and protection of dopaminergic system.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.07.030.

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