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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