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Visualization of monoaminergic neurons and neurotoxicity of MPTP in live transgenic zebrafish

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Abstract

We describe an enhancer trap transgenic zebrafish line, *ETvmat2:GFP*, in which most monoaminergic neurons are labeled by green fluorescent protein (GFP) during embryonic development. The reporter gene of *ETvmat2:GFP* was inserted into the second intron of *vesicular monoamine transporter 2* (*vmat2*) gene, and the GFP expression pattern recapitulates that of the *vmat2* gene. The GFP positive neurons include the large and pear-shaped tyrosine hydroxylase positive neurons (TH populations 2 and 4) in the posterior tuberculum of ventral diencephalon (PT neurons), which are thought to be equivalent to the midbrain dopamine neurons in mammals. We found that these PT neurons and two other GFP labeled non-TH type neuronal groups, one in the paraventricular organ of the posterior tuberculum and the other in the hypothalamus, were significantly reduced after exposure to MPTP, while the rest of GFP-positive neuronal clusters, including those in telencephalon, pretectum, raphe nuclei and locus coeruleus, remain largely unchanged. Furthermore, we showed that the effects of hedgehog signaling pathway inhibition on the development of monoaminergic neurons can be easily visualized in individual living *ETvmat2:GFP* embryos. This enhancer trap line should be useful for genetic and pharmacological analyses of monoaminergic neuron development and processes underlying Parkinson's disease.

Keywords: Monoaminergic; Transgenic; Zebrafish; MPTP; Parkinson's disease

Introduction

The monoaminergic neurons use dopamine (DA), noradrenaline (NA), adrenaline, serotonin (5-hydroxytryptamine, 5-HT) and histamine (HA) as transmitters, and participate in a variety of physiological and behavioral processes in the vertebrate nervous system (Kandel et al., 1991). Some neurological disorders including the second most common neurodegenerative disease Parkinson's disease (PD) (de Lau and Breteler, 2006) are caused by progressive loss of DA neurons in the midbrain substantia nigra. Genetic analyses of familial PD showed that mutations in alpha-synuclein (SNCA), parkin, leucine-rich repeat kinase 2 (LRRK2), PTEN-induced putative kinase 1 (PINK1) and DJ-1 are associated with this disease (Wood-Kaczmar et al., 2006). In addition, environmental toxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), increase the risk of PD (Langston et al., 1983). Nonetheless, these factors only account for a small portion of PD and pathogenesis of most PD still remains elusive.

MPTP is commonly used as a neurotoxin to induce animal models for PD studies and its underlying mechanism of toxicity has been well established (Beal, 2001; Dauer and Przedborski, 2003). After passing through the blood–brain barrier, MPTP is metabolized to *N*-methyl-4-phenylpyridine (MPP^+) by monoamine oxidases (MAO-B) in glia and 5-HT neurons. The MPP^+ is then released and enters DA neurons through dopamine transporters (DAT). In the DA neurons, it is

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concentrated in the mitochondria to impair complex I of the respiratory electron transport chain and ultimately cause cell death. MPP^+ can also bind to the vesicular monoamine transporter 2 (VMAT2) and is thus sequestrated into synaptic vesicles (Liu et al., 1992; Reinhard et al., 1987; Takahashi et al., 1997).

In addition to being a good vertebrate organism for developmental genetic studies, zebrafish (*Danio rerio*) has emerged as a valuable model to study human diseases (Grunwald and Eisen, 2002). The DA (Guo et al., 1999; Ma, 1994, 2003; Ma and Lopez, 2003; Rink and Wullimann, 2002b), 5-HT (McLean and Fetcho, 2004) and HA (Kaslin and Panula, 2001) systems in zebrafish brain appear well conserved. A genetic screen for mutants with abnormal *th* (tyrosine hydroxylase) expression has been performed (Guo et al., 1999) and several novel genes participating in the development of DA neurons have been discovered (Ryu et al., 2006). Although there are no DA neurons in the midbrain of zebrafish, recent studies (Kaslin and Panula, 2001; Rink and Wullimann, 2001, 2002a) indicated that some DA neurons in the ventral diencephalon (TH populations 1, 2 and 4) have ascending projections to the subpallium of telencephalon, suggesting that these neurons are homologous to the ascending midbrain DA neurons of mammals. Further analyses demonstrated that DA neurons in the ventral diencephalon and pretectum are sensitive to MPTP treatment (Bretaud et al., 2004; Lam et al., 2005; McKinley et al., 2005). In addition, some PD-related genes have been shown to be expressed in ventral diencephalic DA neurons (Bai et al., 2006; Bretaud et al., 2006; Son et al., 2003). These findings suggest that zebrafish has the potential to become a useful model of PD for studying pathogenesis and testing therapeutic drugs.

Although the technology of making transgenic animals with fluorescent reporters has been widely applied in zebrafish (Lin, 2000), no fish line labeling monoaminergic neurons has been reported. Such a transgenic zebrafish line would facilitate the analyses of genetic and environmental factors affecting these neurons in living transparent embryos. In this study, we describe an enhancer trap transgenic zebrafish line, *ETvmat2:GFP*, which has a *GFP* reporter gene linked to zebrafish *gata*-2 minimal promoter inserted in the *vmat2* genomic locus. We showed that the expression of *ETvmat2:GFP* recapitulates the *vmat2* gene expression, allowing visualization of most monoaminergic neurons during embryonic development. More importantly, GFP positive neurons corresponding to TH populations 2 and 4 in the posterior tuberculum (PT neurons) are sensitive to MPTP treatment. TH populations 1 and 6 surrounding the posterior tuberculum, and population 8 in the pretectum, which are not labeled by GFP, are also MPTP-sensitive as expected. In addition, the GFP-positive neuronal groups in the paraventricular organ of the posterior tuberculum and the hypothalamus are also reduced after MPTP exposure. Finally, the effects of cyclopamine, an inhibitor of hedgehog signaling pathway, on the development of monoaminergic neurons can be directly observed in living *ETvmat2:GFP* embryos. These studies suggest that *ETvmat2:GFP* should be useful for studying monoaminergic neuron development and testing PD therapeutic drugs.

Materials and methods

Zebrafish

ETvmat2:GFP transgenic zebrafish was identified from a large scale enhancer trap screen (unpublished data) using a *Tol2* vector (Kawakami et al., 2004) containing a 249 bp zebrafish *gata*2 minimal promoter (Meng et al., 1997) linked to a *GFP* reporter gene. The fish line was maintained in a recirculating aquaculture system of the fish facility of Peking University at 28.5 °C.

Cloning of vmat2 cDNA

The cDNA sequence of zebrafish *vmat2* has been deposited in the UniProtKB/TrEMBL database (Primary accession number: Q5CZP4). To generate a probe for detecting *vmat2* expression, a 629 bp cDNA fragment was amplified from 72 hpf (hours post fertilization) embryonic RNA by RT-PCR (primers: 5′-CCTGTACACGGTGGACGACGAG-3′ and 5′-TGCCAGCACTGCCAGGATAAGA-3′). The fragment was cloned into pGM-T (TianGen) vector and sequenced. The construct was digested with the restriction enzyme *Aat*II and an antisense riboprobe was transcribed with Sp6 RNA polymerase. For making *th* probe, a 927 bp fragment was amplified using the forward primer 5′-ATGCCGAATTCAAGCAGCTCCAC-3′ and the reverse primer 5′-TAATACGACTCACTATAGGGAGAAGCGTGCCGTATGTACTGTGTGC-3′, based on the published *th* cDNA sequence.

In situ hybridization and immunofluorescence

RNA *in situ* hybridization was carried out following standard protocols (Thisse et al., 1993). For double immunofluorescence staining, embryos were treated with 0.25% trypsin and then incubated simultaneously in a mouse monoclonal anti-Th antibody (Chemicon) and a rabbit polyclonal anti-GFP antibody (Zhongshan Goldenbridge) at 4 °C overnight. This was followed by several washes and then overnight incubation with a TRITC-coupled anti-mouse IgG antibody and a FITC-coupled anti-rabbit IgG antibody.

Image acquisition and analysis

For general examination, GFP-positive embryos or larvae were viewed under an Axioimager Z1 fluorescence microscope (Zeiss), equipped with 5, 10 and 20× lenses, and filter set 10 for detection of GFP (excitation: 450–490 nm, barrier: 510 nm, emission: 515–565 nm). For more detailed examination, anesthetized embryos were mounted in 1.2% agar on a cover glass and observed using a Leica laser-scanning confocal imaging system equipped with a GFP filter (488 nm excitation and 505–550 nm bandpass) and 20 and 40× lenses. Serial optical sections were taken, and 3-D images were reconstructed from the stacked confocal images by the supplied software.

MPTP and cyclopamine treatment

The MPTP (Sigma-Aldrich) was dissolved in distilled water to 10 mg/ml and was used at a final concentration of 10–40 μg/ml for the treatment. Embryos were obtained from outcross between the *ETvmat2:GFP* transgenic fish and wild-type ones. At 22–24 hpf, GFP-positive embryos were collected and dechorionated. Ten embryos were then transferred into a six-well plate containing 4 ml Holtfreter's buffer with 0.003% phenylthiourea (PTU, Sigma-Aldrich) and MPTP. The MPTP-containing buffer was changed once after 2 days and at 5 days post-fertilization (dpf), the embryos were examined under a fluorescence microscope, and then fixed in 4% paraformaldehyde after extensive washing. The MPTP manipulation was performed with appropriate safety precautions (Przedborski et al., 2001) and all MPTP-containing water was bleached.

Cyclopamine (BIOMOL) was dissolved in DMSO at 8 mg/ml and diluted in Holtfreter's buffer to 2.5 to 20 μg/ml for treatment. Embryos were soaked in cyclopamine solutions from 4 hpf on and then observed under a fluorescence microscope at 48 hpf.

Results

Identification of the enhancer trap vector insertion in vmat2 gene

The *ETvmat2:GFP* transgenic fish line was selected for its neuronal specific expression of GFP from our ongoing enhancer trap screen (unpublished data). Both 5′- and 3′-flanking genomic sequences near the *Tol2* insertion site were obtained via linker-mediated PCR (Wu et al., 2003) and the repeats of 8-bp genomic sequence resulted from transposition were found as expected (Fig. 1A). Blast search using the flanking sequences through Ensembl database showed that the enhancer trap vector was inserted in the second intron of *vmat2* gene with the transcription direction of the *GFP* reporter opposite to that of the *vmat2* gene (Fig. 1A). The *vmat2* gene is located on chromosome 17, extending for about 36 kb and containing 16 exons. Phylogenetic analysis indicated that this gene was closely related to the *VMAT2* genes in human and mouse (Fig. 1B). Continuous observation showed that homozygous *ETvmat2:GFP* transgenic adult fish were viable and fertile, and both of their morphology and behavior were indistinguishable from their wild-type and heterozygote siblings.

GFP expression pattern of the ETvmat2:GFP transgenic zebrafish

From 22 hpf onward, GFP-positive neurons of the *ETvmat2:GFP* line become highly specific and visible. At 22 hpf, a small number of GFP-positive neurons appear in the posterior tuberculum of ventral diencephalon and spinal cord (Fig. 2A). From 22 to 30 hpf, the GFP-positive neurons in the posterior tuberculum, designated as PT neurons, extend axonal fibers

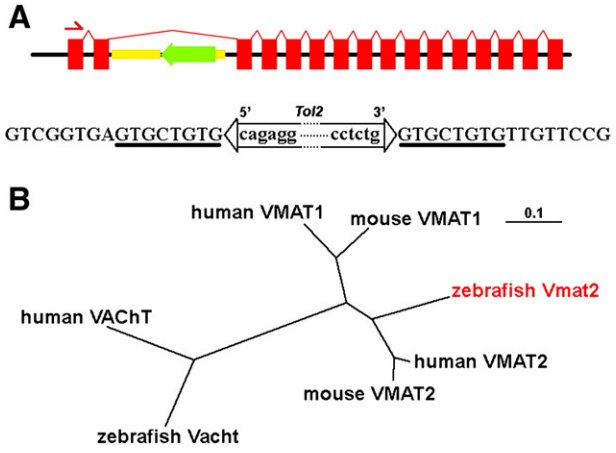

Fig. 1. The *Tol2* enhancer trap vector is inserted into zebrafish *vmat2* gene. (A) Schematic map of *vmat2* gene structure containing 16 exons (red bars) with *Tol2* insertion in the second intron, as well as partial flanking sequences around the insertion site. *GFP* reporter gene is illustrated as a green arrow and the 8-bp repeats of genomic DNA flanking the *Tol2* element are underlined. (B) An unrooted phylogenic tree (Cluster×program) based on full length protein sequences suggests that zebrafish Vmat2 is closely related to its mammalian homologs. VAChT: vesicular acetylcholine transporter.

both anteriorly to the telencephalon and posteriorly to the midbrain (Fig. 2B). The number of these neurons increases from 4–5 at 30 hpf (Fig. 2B) to 8–9 at 5 dpf on each side (Fig. 2I). From 48 hpf on, they are separated into an anterior and a posterior group. The neurons of both groups have large pear-shaped cell bodies and prominent single neurites extending dorsolaterally (Fig. 2I), with the anterior neurons appearing larger and more compact.

By 22 hpf, a small group of neurons also appear outside the neural tube lateral to the rostral hindbrain (Fig. 2A), and from 24 to 72 hpf, they migrate anteriorly to fuse with each other at the midline, rostral to the heart (Fig. 2D). These neurons seem to correspond to the arch-associated neurons. By 24 hpf, a few GFP-expressing neurons appear in the ventrolateral region of rhombomere 1, corresponding to the locus coeruleus (LC) (Fig. 2A). Their number increases from 4–6 at 36 hpf (Fig. 2C) to 6–7 at 48 hpf (Fig. 2E). At the same stage, 1–2 GFP-positive neurons are also detected in the ventral midbrain, and in the larvae of 4 dpf, this group expands to about 6 neurons (Figs. 2A, H). The identity of these neurons is unknown.

Two groups of neurons emerge in the telencephalon (Fig. 2H) and the medulla oblongata (Fig. 2G) by 36 hpf. Also, another group begins to appear in the midline of the ventral hindbrain, corresponding to the raphe nuclei (Fig. 2E). This group can be divided into an anterior cluster and a smaller posterior cluster.

By 60 hpf, several more neuronal groups appear, including those in the paraventricular organ of posterior tuberculum (Figs. 2H, I), hypothalamus (Figs. 2G, H), pretectum (Figs. 2E, G), sympathetic neurons (Figs. 2F, G), retina and epiphysis (data not shown). The cluster in the paraventricular organ looks like an inverted “V” in appearance and is enclosed by the anterior and posterior groups of PT neurons (Fig. 2I). GFP-positive neurons of the hypothalamus contain a smaller group in the intermediate hypothalamus and a larger group in the caudal hypothalamus. In addition to neurons, the anterior artery also shows GFP expression in the *ETvmat2:GFP* fish line. This should facilitate the identification of the sympathetic neurons, which are located just beneath the artery.

We further examined the GFP expression in live *ETvmat2:GFP* larvae at 4 dpf under a confocal microscope. Under higher magnification, the fine processes of the neurons are readily visible (Fig. 2I and Supplementary Video 1).

Expression patterns of vmat2 and comparing with that of GFP and th

To verify that the GFP expression pattern of *ETvmat2:GFP* recapitulates that of *vmat2* gene, we performed RNA whole-mount *in situ* hybridization for *vmat2* gene, and we also performed *in situ* hybridization of *th* for further comparison (Fig. 3). Similar to the GFP expression, the *vmat2* gene becomes first detectable at 22 hpf in two groups of neurons, one in the ventral diencephalon and the other being lateral to the rostral hindbrain (data not shown). The number of the ventral diencephalic neurons increases to 8–10 on each side at 48 hpf and these neurons separate into an anterior and a posterior

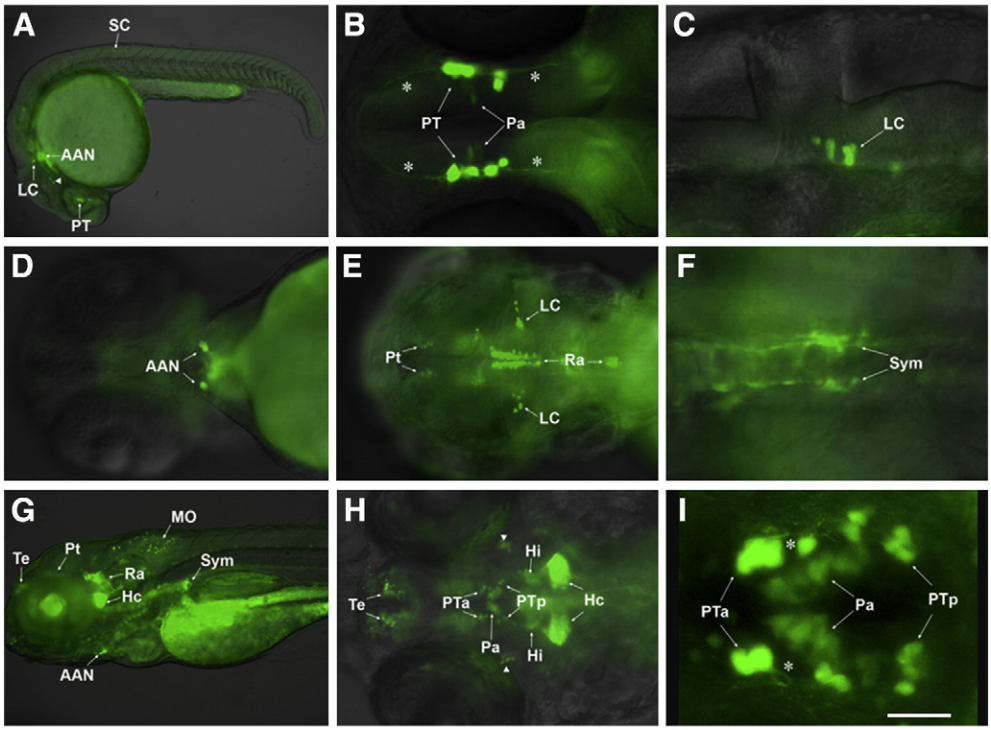

Fig. 2. GFP expression pattern of *ETvmat2:GFP* embryos. Photos were taken under a fluorescence microscope in lateral (A, C, G), dorsal (B, E, F, H) and ventral views (D), or under a confocal microscope in dorsal view (I), rostral to the left. (A) A 24 hpf embryo showing the first GFP-positive (GFP^+) neurons that appear in the nervous system; (B) a 30 hpf embryo showing GFP^+ neurons in posterior tuberculum (PT) and their early axons projecting both anteriorly and posteriorly (asterisks), and some faintly labeled neurons in the paraventricular organ (Pa); (C) a 36 hpf embryo showing GFP^+ neurons in the locus coeruleus (LC) located in the ventral lateral region of rhombomere 1; (D) a 54 hpf embryo showing the GFP^+ arch-associated neurons (AAN) rostral to the heart; (E) a 60 hpf embryo with GFP^+ neurons in the LC, the raphe nuclei (Ra) and the pretectum (Pt); (F) a 72 hpf embryo with GFP^+ sympathetic neurons (Sym) under the artery; (G, H) 5 dpf embryos showing GFP^+ neural groups in the central and peripheral nervous systems; (I) a 4 dpf embryo showing GFP^+ neurons in PT and Pa, with the PT neurons sending out prominent single lateral neurites (asterisks). Scale bar: 25 μm. AAN: arch-associated neurons; Hc: caudal hypothalamus neural cluster; Hi: intermediate hypothalamus neural cluster; LC: locus coeruleus; MO: medulla oblongata neural cluster; Pa: neural cluster of paraventricular organ; Pt: pretectal neural cluster; PTa: anterior group of the posterior tubercular neurons; PTp: posterior group of the posterior tubercular neurons; Ra: raphe nuclei; SC: spinal cord; Sym: sympathetic neurons; Te: telencephalic neurons. Arrowheads in panels A and H: GFP-positive neurons in midbrain.

group. At this stage, the distribution and number of *vmat2*- and *th*-positive ventral diencephalic neurons are similar. However, the number of *vmat2*-positive ventral diencephalic neurons remains the same at 4 dpf, while the number of *th*-positive neurons continues to increase.

The *th*-reactive ventral diencephalic cluster at 4 dpf can be divided into seven populations based on classification by Rink and Wullimann (2002b). Among them, populations 1, 5 and 6 are located at anterior or posterior boundary of the posterior tuberculum, while populations 2, 3 and 4 lie within the posterior tuberculum (Fig. 3H). There is a gap between populations 2 and 4, which are positioned more distal from the ventricle than other populations (Fig. 3H). The *vmat2*-reactive ventral diencephalic neurons seem to correspond to TH populations 2 and 4, since both the anterior and posterior *vmat2*-positive neuronal groups are distal from the ventricle and a similar gap is found between them (Fig. 3D).

At 4 dpf, *vmat2* expression is also found in a group of pretectal neurons, which are also clearly visible as GFP-positive neurons in the *ETvmat2:GFP* fish line. Although a group of *th*-positive neurons can be found at a similar location, it seems that the *vmat2*-positive pretectal cluster is not identical to the *th*-positive one (compare Figs. 3C with G).

Vmat2 is expressed in the raphe nuclei of 48 hpf embryos, as revealed by both *in situ* hybridization and *GFP* expression in *ETvmat2:GFP* line. Other GFP-positive and putative serotonergic neuronal clusters, including those in the paraventricular posterior tuberculum and hypothalamic regions, also express *vmat2*. The LC and sympathetic neurons are faintly labeled. However, *vmat2* expression is not detected in telencephalon, retina or medulla oblongata, although GFP expression is apparent in these regions in the *ETvmat2:GFP* line. Also, *vmat2* is not expressed in artery that is labeled by GFP expression. These expanded and ectopic expressions of GFP probably reflect the complex transcriptional regulation of *vamt2* gene or the existence of a long distance enhancer of another gene.

GFP-positive neurons in posterior tuberculum express Th

To determine whether the PT neurons labeled by GFP in the *ETvmat2:GFP* line are catecholaminergic, we performed double immunofluorescence staining of GFP and Th in

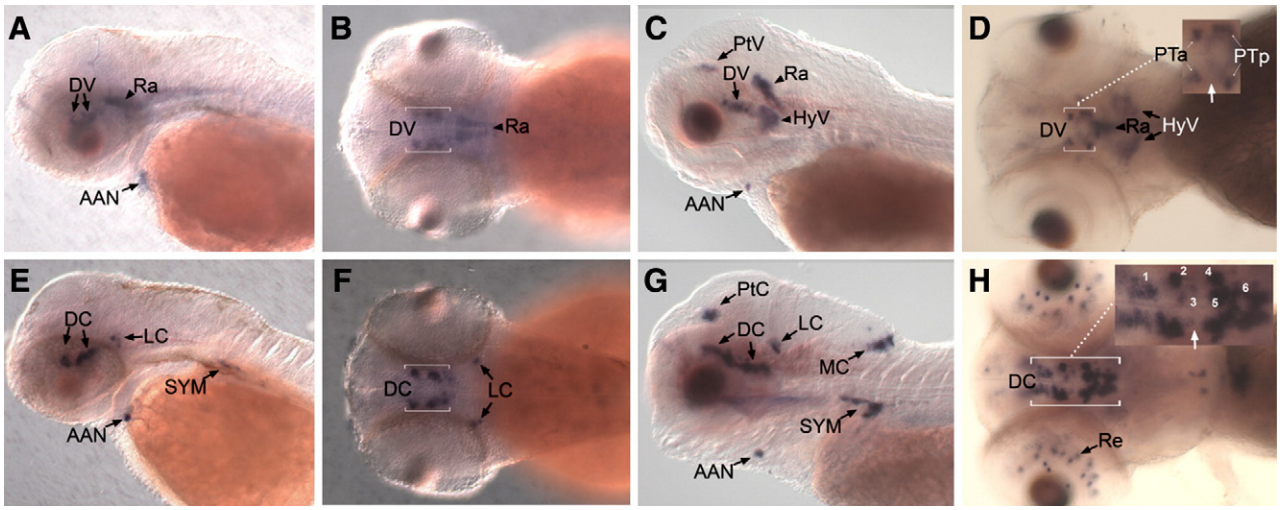

Fig. 3. Comparison of *vmat2* and *th* expression patterns. (A–H) Whole-mount *in situ* hybridization detecting *vmat2* (A–D) and *th* (E–H) expression in 48 hpf (A, B, E, F) and 4 dpf (C, D, G, H) embryos. (A, C, E, G) Lateral views, (B, D, F, H) dorsal views, rostral to the left. *Vmat2*-reactive neurons in ventral diencephalon can be divided into anterior group of posterior tuberculum (PTa), posterior group of posterior tuberculum (PTp) and group of paraventricular organ (asterisk), and there is a gap (white arrow) between PTa and PTp; (H) catecholaminergic clusters in ventral diencephalon of 4 dpf embryos can be divided into several groups from anterior to posterior: population 1 (between ventral thalamus and posterior tuberculum), population 2 (anterior group of posterior tuberculum), population 3 (paraventricular organ), population 4 (posterior group of posterior tuberculum) and populations 5 and 6 (between posterior tuberculum and hypothalamus); there is also a gap (white arrow) between populations 2 and 4. AAN: arch-associated neurons; DC: diencephalic catecholaminergic cluster; DV: diencephalic *vmat2*-expression cluster; HyV: hypothalamic *vmat2*-expression cluster; LC: locus coeruleus; MC: medulla catecholaminergic cluster; PTa: anterior group of the posterior tubercular neurons; PTp: posterior group of the posterior tubercular neurons; PtC: pretectal catecholaminergic cluster; PtV: pretectal *vmat2*-expression cluster; Ra: raphe nuclei; Sym: sympathetic neurons.

ETvmat2:GFP embryos. The result showed that all the GFP-positive PT neurons are also Th-positive (Figs. 4C, F, I) and thus corresponding to TH populations 2 and 4 (Fig. 4I). Since Th is a cytoplasmic enzyme and GFP is distributed in both cytoplasm and nucleus, we are able to show that Th- and GFP-positive cytoplasm are yellow and GFP-positive nuclei are green in fluorescent signals (Figs. 4F, I).

Effects of MPTP treatment on GFP-positive neurons

To examine the effect of MPTP on the GFP-positive cells of the *ETvmat2:GFP* fish line, we exposed embryos to MPTP for 4 days starting at 24 hpf. The GFP expression of 5 dpf embryos was viewed under a fluorescence microscope and *th* expression was visualized using *in situ* hybridization. Given that the apoptotic GFP positive cells would be quickly cleared by circulating cells (Lopez-Schier and Hudspeth, 2006), disappearance of GFP could be a readout of cell death. We found that the GFP-positive groups in the paraventricular organ, hypothalamus and PT are relatively sensitive to MPTP exposure. In embryos treated with 20 μg/ml MPTP or more, the paraventricular and hypothalamic groups are significantly reduced (Figs. 5E, I). The PT neurons remain present at 20 μg/ml MPTP but are not well organized compared with that of controls (Fig. 5E). In embryos treated with 40 μg/ml MPTP, most of these neurons are lost, except a few are remained in the anterior group (Fig. 5I). In contrast to these three groups, other GFP-positive neuronal clusters, including those in telencephalon, pretectum, raphe nuclei and LC, are mostly intact even treated at 40 μg/ml MPTP (Fig. 5J). Among these neurons, the GFP-positive pretectal group is probably not catecholaminergic, as discussed later in this paper.

We also examined *th* expression to compare our MPTP experiments with those reported previously (Bretaud et al., 2004; Lam et al., 2005; McKinley et al., 2005). The *th*-positive neurons in the pretectum (population 8) were confirmed to be most sensitive to MPTP and became no longer detectable after exposure to 20 μg/ml MPTP (Figs. 5G, H). This effect could also be observed in embryos treated with as low as 10 μg/ml MPTP (data not shown). Similar sensitivity was found in TH populations 1 and 6 in the anterior and posterior boundary zone of posterior tuberculum (Fig. 5G). Populations 2 and 4 in PT were not as sensitive as those three populations, which were relatively intact at 20 μg/ml MPTP (Fig. 5G) but greatly reduced at 40 μg/ml MPTP (Fig. 5K). Reduction of both *th* and GFP in the PT neurons suggests that such reduction is most likely due to cell death other than reduction of gene expression. In contrast, we observed reduction in the expression of *th* (Figs. 5H, L) but not *GFP* (Figs. 5F, J) in LC neurons at 20 and 40 μg/ml MPTP, which is consistent with previous report (McKinley et al., 2005), and these data suggest that MPTP treatment led to reduction of *th* transcription rather than cell death in these neurons. The MPTP-sensitivity of different GFP- and *th*-positive neuronal clusters was summarized (Fig. 5M).

Effects of Hh pathway inhibition on GFP-positive neuronal groups

We also examined the development of the GFP-positive neuronal groups in the absence of Hh signaling pathway by incubating *ETvmat2:GFP* embryos from 4 hpf with 20 μg/ml cyclopamine, a Hh signaling inhibitor (Cooper et al., 1998; Incardona et al., 1998). The embryos showed U-shaped somites

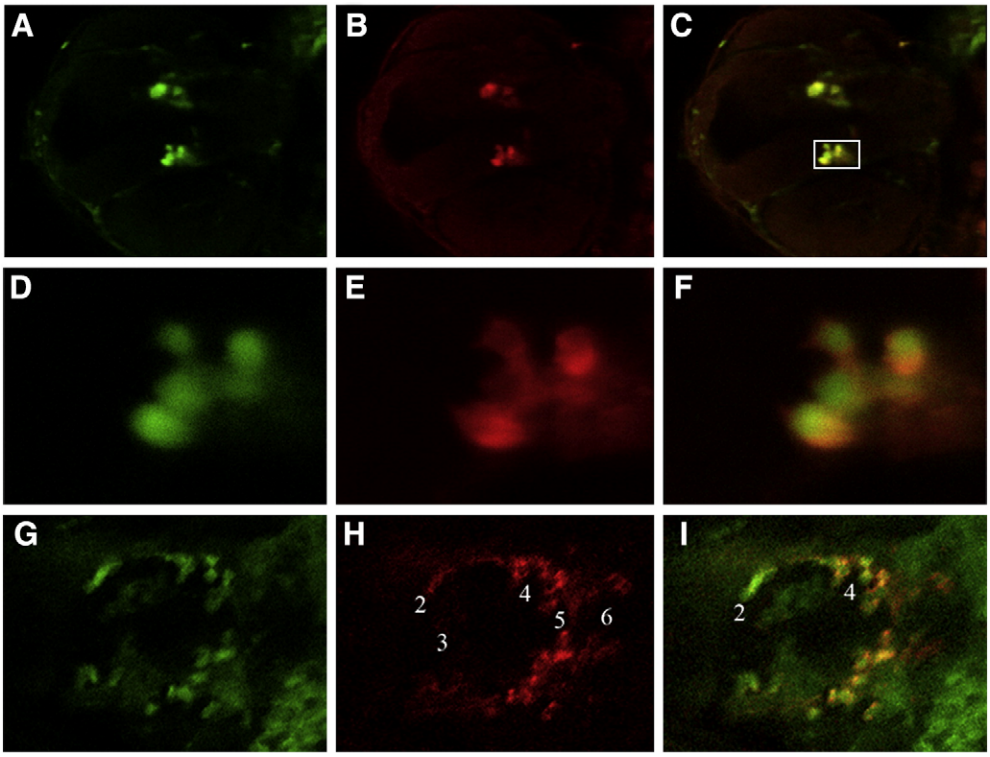

Fig. 4. Double immunofluorescence staining for detection of GFP and endogenous Th. Simultaneous immunofluorescence staining was generated using a polyclonal anti-GFP antibody (A, D, G) and a monoclonal anti-Th antibody (B, E, H) for 30 hpf (A–F) and 3 dpf (G–I) *ETvmat2:GFP* embryos. Composite photos were made from stacked confocal images, focusing on catecholaminergic neurons of the ventral diencephalon. Merged pictures of Th and GFP staining are shown in panels C and F. Higher magnification images of the posterior tubercular neurons (D–F) correspond to the framed region in panel C.

and curved bodies at 24 hpf (Supplementary Fig. 1), which are typical phenotypes of loss of Hh signal. We found that different sub-groups of GFP positive neurons respond to Hh inhibition differently. In cyclopamine treated embryos, the GFP positive neurons in raphe nuclei are absent (10/10 larvae), while those in the LC, the medulla oblongata and the telencephalon are not affected (Figs. 6B, D). The PT neurons are not reduced but relocated in a dorsoventral direction (Fig. 6B), unlike the anteroposterior direction seen in wild-type embryos (Fig. 6A). We also checked the effects of cyclopamine at lower concentrations. The loss of GFP positive neurons in raphe nuclei is already prominent for 48 hpf embryos at 2.5 μg/ml cyclopamine, when the phenotypes of U-shaped somites and curved bodies are not obvious (data not shown).

Discussion

The large and pear-shaped GFP-positive neurons in the posterior tuberculum of ventral diencephalon (PT neurons) in *ETvmat2:GFP* embryos can be divided into an anterior and a posterior group, which form "parentheses" around neurons of the paraventricular organ and send out prominent single dorsolateral neurites (Fig. 2I). From these characteristics and the results from *in situ* hybridization (Figs. 3D, H) as well as co-immunofluorescence (Fig. 4I) comparing the expression patterns of *vamt2*, GFP and TH, we propose that these neurons are TH populations 2 and 4 as classified by Rink and Wullimann (2002b). These two neural populations send ascending projections to the ventral telencephalon and are thought to be functional equivalents to the meso-striatal and meso-limbic systems (A9–A10 catecholaminergic cell groups) in mammals (Rink and Wullimann, 2001, 2002a). They are also suggested to be related to the A11 group with descending projections according to their morphology (Kaslin and Panula, 2001) and requirement for Orthopedia (Otp) homeodomain protein (Ryu et al., 2007). Indeed, both ascending and descending fibers of these neurons are found as early as 30 hpf (Fig. 2B). Moreover, it appears that the number of these PT neurons at 4 dpf (8–9 on each side) are maintained through adulthood (7.2±1.0 on each side shown by Ma and Lopez, 2003).

We further showed that GFP expression of the PT neurons overlap with *vmat2* expression (Figs. 3A–D). Since *vmat2* expression indicates that the neuron is able to accumulate dopamine into synaptic vesicles and thus becomes fully functional, it is interesting that the PT neurons are among the earliest neurons to express both *vmat2* and *th*, which may suggest the importance of their functions. The TH populations 1, 6 and 8, which are not *vmat2*-reactive even at 5 dpf (see the following discussions), may either eventually express *vmat2* later, or belong to the *th*-positive but *vmat2*-negative neurons found previously in mammalian brains (Weihe et al., 2006).

While the function of Vmat2 is to accumulate monoamines from the cytoplasm into synaptic vesicles, previous studies demonstrated that it can also incorporate MPP^+ and thus sequester the MPP^+ toxicity (Liu et al., 1992; Takahashi et al., 1997), which could explain the cellular- and species- specificity

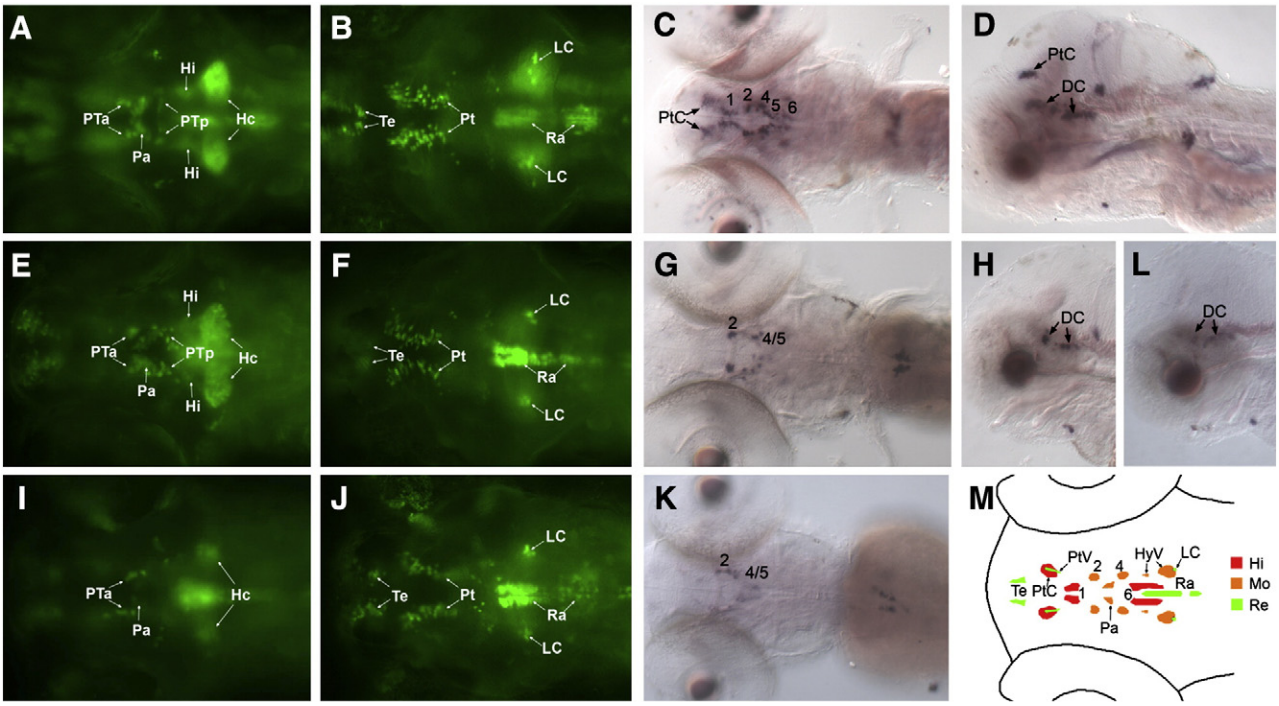

Fig. 5. Effect of MPTP on zebrafish embryos. *ETvmat2:GFP* embryos untreated (A–D) or treated with 20 μg/ml (E–H) or 40 μg/ml (I–L) MPTP from 24 hpf to 5 dpf were examined at the last day. Photos were taken under a fluorescence microscope in ventral views (A, B, E, F, I, J), and whole-mount embryos after *th in situ* hybridization are in dorsal (C, G, K) and lateral (D, H, L) views, with rostral to the left. The neural clusters of ventral diencephalon and hypothalamus at a deep focal plane reduced significantly after MPTP exposure (E, I), while neural clusters located more dorsally and appeared at a shallow focal plane were almost unaffected (F, J). Similar reduction of ventral diencephalic and pretectal catecholaminergic clusters after MPTP treatment was observed with TH staining (G, H, K, L). Note that TH population 1 and 6 (G) and the pretectal cluster (G, H) were already eliminated after exposure to 20 μg/ml MPTP. (M) A schematic diagram representing the MPTP-sensitivity of different monoaminergic neuronal groups. 1, 2, 4, 5, 6: ventral diencephalic TH populations; DC: diencephalic catecholaminergic cluster; Hc: caudal hypothalamus neural cluster; Hi: intermediate hypothalamus neural cluster; HyV: hypothalamic *vmat2*-expression cluster; LC: locus coeruleus; Pa: neural cluster of paraventricular organ; Pt: pretectal neural cluster; PtC: pretectal catecholaminergic cluster; PtV: pretectal *vmat2*-expression cluster; PTa: anterior group of the posterior tubercular neurons; PTp: posterior group of the posterior tubercular neurons; Ra: raphe nuclei; Te: telencephalic neurons. Hi, highly MPTP-sensitive; Mo, moderately MPTP-sensitive; Re, MPTP-resistant.

of MPTP sensitivity (Speciale et al., 1998). Indeed, the PT neurons (TH populations 2 and 4) are not as MPTP-sensitive as other three TH populations: population 8 within the pretectum, populations 1 and 6 at anterior and posterior boundary of posterior tuberculum (Fig. 5G, also see summary in Fig. 5M). No *vmat2* expression is found in populations 1 and 6 (Fig. 2D). Although there is a *vmat2*-positive group at the pretectal location similar to the TH population 8, it is nearly unaffected when the TH group is completely ablated after MPTP exposure (compare Figs. 5C, G with B, F). This group is likely serotonergic, based on its more ventral location comparing with the *th*-reactive pretectal cluster (Figs. 3C, G) and *vmat2* expression (Kaslin and Panula, 2001; McLean and Fetcho, 2004). These data suggest that the high MPTP-sensitivity of TH populations 1, 6 and 8 is likely due to their lack of *vmat2* expression.

There are two other factors that play important roles in the mode of MPTP toxicity: MAO-B and DAT (Beal, 2001; Dauer and Przedborski, 2003), which may also contribute to the variable MPTP-sensitivities of the TH cells. Only one *mao* gene is found in the genome of zebrafish (Anichtchik et al., 2006), whose expression may lead to the lack of degeneration of *th*-positive cells after systemic MPTP administration to adult zebrafish (Anichtchik et al., 2004, 2006). Nevertheless, the MPTP toxicity to larval zebrafish is shown to be mediated by MAO activity since its inhibition by deprenyl can prevent reduction of dopaminergic cells after MPTP exposure (Lam et al., 2005; McKinley et al., 2005). The distribution of *mao* mRNA seems to be uniform in the brain of larval zebrafish as detected by whole-mount *in situ* hybridization (Rauch et al., 2003), suggesting that variable MPTP-sensitivities of the TH neurons are not due to different distribution of MAO activity. However, detailed expression and activity analyses on sections are needed to further exclude this possibility. The gene *dat* is also suggested to be responsible for the MPTP toxicity to zebrafish dopaminergic neurons since DAT inhibition has protective effects (McKinley et al., 2005). The expression patterns of *dat* and *vmat2* apparently match well with MPTP-sensitivities of TH populations. Two most sensitive populations, 6 and 8, express *dat* but not *vmat2*, while two less sensitive populations, 2 and 4, express both genes (Holzschuh et al., 2001), suggesting that *vmat2* counteracts with *dat* in the latter cells. It is interesting that the population 1 appears to be *dat*-negative (Holzschuh et al., 2001) while it is still highly sensitive to MPTP exposure, suggesting the existence of a *dat* paralog or *dat*-independent mechanism for transporting MPP^+ into these cells.

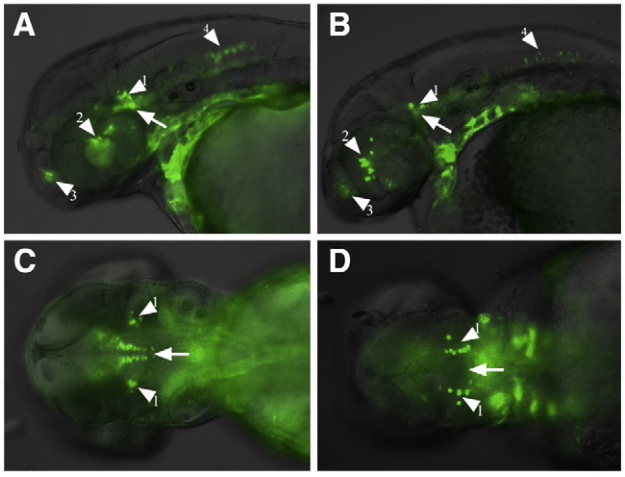

Fig. 6. Effect of cyclopamine on *ETvmat2:GFP* embryos. Lateral (A, B) or dorsal (C, D) views of 48 hpf *ETvmat2:GFP* embryos after treatment with cyclopamine at 0 μg/ml (A, C) or 20 μg/ml (B, D) from 4 hpf to 48 hpf. Note that the raphe nuclei cluster (arrow) was abolished. Arrow: raphe nuclei cluster; arrowhead 1: locus coeruleus cluster; arrowhead 2: posterior tubercular cluster; arrowhead 3: telencephalic cluster; arrowhead 4: medulla oblongata cluster.

We found that the GFP-positive neurons in paraventricular organ and hypothalamus, most of which are presumably serotonergic, are also reduced after MPTP treatment. Although MPTP-sensitivity of these 5-HT neurons is not as great as TH populations 1, 6 and 8, it is specific since the GFP-positive clusters in telencephalon, pretectum, raphe nuclei and LC are unaffected. Effects of MPTP on 5-HT neurons have been reported previously. Studies in mice (Speciale et al., 1998) and monkeys (Herkenham et al., 1991) have demonstrated that MPP^+ is accumulated in 5-HT neurons using an autoradiographic method. Moreover, MPTP has the ability to cause a profound loss of 5-HT neurons (Namura et al., 1987; Perez-Otano et al., 1991; Takada et al., 1987) and affect serotonin metabolism (Hara et al., 1987; Mitra et al., 1992). Overall, our transgenic line offers a much more sensitive way to map out MPTP effects on DA neurons.

Since both catecholaminergic and serotonergic neuronal groups are easily visualized in individual living *ETvmat2:GFP* embryos, it is possible to analyze their development *in vivo*. Our examination of development of these neurons in the absence of Hh signal pathway validates this utility. Experimental embryology studies indicated that Hh is required for development of both serotonergic and dopaminergic neurons (Hynes et al., 1995; Ye et al., 1998). Two recent studies examined serotonergic (Teraoka et al., 2004) and catecholaminergic (Holzschuh et al., 2003) neurons in zebrafish mutants of Hh signaling pathway, including *slow-muscle-omitted* (*smu*), in which the Hh signaling transducer *smoothened* is mutated and nearly all Hh activity is blocked (Chen et al., 2001; Varga et al., 2001). In *smu* mutant embryos, serotonergic neurons in raphe nuclei are completely absent (Teraoka et al., 2004), while catecholaminergic neurons in ventral diencephalon are morphologically altered and those in LC, medulla oblongata and telencephalon remain unaffected (Holzschuh et al., 2003). All these changes can be observed directly in individual living *ETvmat2:GFP* embryos treated with cyclopamine which interrupts Hh signal transduction (Cooper et al., 1998; Incardona et al., 1998). These data indicated that the *ETvmat2:GFP* line could serve as a good model for the analysis of serotonergic and catecholaminergic neuron development.

The *ETvmat2:GFP* line can be applied as a useful tool for the study of PD and is helpful in dissecting the development, organization and function of monoaminergic neurons. The PT neurons, particularly, provide a good model system with their potentially important functions, low numbers and large cell bodies (Ma and Lopez, 2003). It should be interesting to examine lesions of the PT neurons caused by genetic disturbance, including overexpression or loss-of-function of PD genes such as neurotoxic alpha-synuclein and DJ-1 (Bretaud et al., 2006). Such a model together with fluorescent PT neurons will allow for chemical genomics screening for drugs with protective activity. In addition, with GFP expression, physiological recording and laser ablation experiments become feasible. Finally, the transgenic fish line will facilitate genetic analyses of the development of serotonergic and catecholaminergic neurons.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.11.012.

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