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Temporal changes in key developmental transcription factors in dopamine neurons during MPP^+ induced injury and recovery in zebrafish brain

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Poor regeneration of functional dopaminergic neurons (DA) is one of the most common healthcare challenges for Parkinson's Disease (PD) patients. In contrast to mammals, zebrafish has an amazing potential to repair their dopaminergic neurons (DA) after injury. However, the molecular mechanisms that regulate these reparative events remain to be determined. To address this, we compared the temporal changes in key transcription factors (TFs), which regulate the developmental trajectories of DA neurons during injury and regeneration in zebrafish. MPP^+ was exposed to zebrafish embryos between 18 Hpf and 96 Hpf to create a model of DA injury and regeneration after injury (1day, 2days, 3 days, 4 days, 5 days, 6 days, 7 days and 87 days post DA injury). During time series of MPP^+ exposure, we found temporal alterations in the expression patterns of TFs; Nurr1, Foxa1, Lmx1a/b and En1/2 using WISH and RT-PCR. This turning point in expression dynamics coincided with a DA phenotypic turning point, as shown by 50% decline in TH^+/DAT^+ neurons and locomotor activity in the days following the MPP^+ exposure. Using this model, we demonstrate for the first time that zebrafish are capable of regenerating a functional DA phenotype after 50% DA ablation. Following acute DA injury, mRNA levels of most TFs started to increase between 3-8 days after injury which was significantly elevated to normal levels in adult zebrafish brain i.e. at 87^{th} day after injury. Remarkably, the changes in mRNA expression of TFs temporally correlate with corresponding increase in TH/DAT expression and functional recovery. Taken together, this study showed a highly relevant role of TFs for dopamine producing neurons during regeneration of DA neurons following ablation with restoration of normal behavior. This study implies that TFs as potential therapeutic targets for enhancing regeneration of DA neurons in mammalsobserved over.

Keywords: Dopaminergic neurons, Locomotor activity, Regeneration, Transcription factors, Zebrafish

Dopaminergic (DA) neurons regulate a diverse set of behavior's, from the control of movement to modulation of cognition, and are susceptible to degeneration in Parkinson's disease (PD)[1]. The substantial (*i.e.*, >70%) loss of DA neurons in substantianigra causes long term functional deficiency[2] which is devastating because lost neurons are not replaced in the adult mammalian central nervous system (CNS)[3]. In contrast, zebrafish has impressively higher capacity for neurogenesis and CNS regeneration after spinal cord transection, tail amputation or lesioning DA neurons[4]. In adult vertebrates' lifelong neurogenesis relies on neural stem cells (NSCs) producing proliferation zone that generates neural progenitor cells (NPCs) with diverse fates. Proliferation zones in the adult zebrafish brain are located in 16 different zones unlike mammals, which have two main proliferation zones—the sub granular and sub ventricular zones[5]. The NPCs proliferate and migration to injury site in order to integrate the new neurons into existing neural circuits resulting in remodeling the brain in regenerative manner[6]. However, the regeneration efficiency of neurons in the injured mammalian brain is extremely low. Besides studies have reported an activation of immune responses following ablation of DA neurons using 6-hydroxydopamine (6-OHDA) is necessary for regenerative proliferation of ependymo-radial glia progenitor cells (ERGs) and neurogenesis in the adult zebrafish telencephalon[7]. Interestingly, a recent study points that DA neurons regenerate following chemogenetic ablation in the olfactory bulb of adult Zebrafish (*Danio rerio*)[8]. While, the molecular mechanisms relevant for DA regeneration in zebrafish is less explored.

One approach to promote recovery would be to induce or enhance DA neurogenesis[9]. Several signaling

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molecules and transcriptional cascades are known to influence neurogenesis in the developing DAergic system of zebrafish and other vertebrates[10]. Specifically, TFs such as Otx2, Nurr1, Lmx1a and Lmx1b (Lmx1a/b), Foxa1 and Foxa2 (Foxa1/2), Engrailed 1 and Engrailed 2 (En1/2), act co-operatively for the development, survival and physiology of DA ergic system[11]. For instance, during early development, Foxa subfamily of forkhead/winged helix transcription factors, Foxa1/2 are required, in a gene dosage-dependent manner, for specification of mDA progenitor identity[12]. Loss-of-function studies revealed that Foxa1/2 are also vital for maturation of mDA neurons by regulating Lmx1a/b, Nurr1 and En1 in immature mDA neurons. During early and late differentiation of mature mDA neurons, Foxa1/2 regulate the binding of Nurr1 to Tyrosine Hydroxylase (TH) and dopamine transporter (DAT) transcriptional regulatory sequences of the gene leading significant expression of TH and DAT in embryonic and adult DA neurons. Some of these transcription factors continue to be expressed in the adult CNS, might be to mediate the preservation of the dopaminergic phenotype and survival of DA neurons in the adult brain[13]. Loss of function and morpholino-knock down studies of these TFs, demonstrated that establishment of proper patterning and projections of DA neurons during development is pivotal for emergence of a precisely functioning locomotor system[14].

Signals that resemble the developmental programs by which the DA neurons were originally specified and organized may be redeployed during regeneration after injury. For example, Wnt/β-catenin signaling promotes regeneration after adult zebrafish spinal injury[15]. Furthermore, recruitment of developmental signaling such as hedgehog signaling promotes motor neuron regeneration in adult zebrafish[16]. However, it is not known whether TFs associated with developmental trajectories of DA are required for regeneration of DA neurons post injury.

Interestingly, the developmental period of dopaminergic system in zebrafish (18–96 Hpf) (Hours post-fertilization) is homologous to those in humans[17]. The earliest differentiation of DA neurons from progenitors occurs approximately at 18 Hpf and by 96 Hpf, the full complement of DA system is present in zebrafish[18]. While DA neurons are conspicuously absent from the ventral midbrain, the ventral forebrain DA neurons ascending to the striatum where ventral midbrain DA neurons in mammals project are likely the functional counterpart of the mammalian midbrain DA neurons[19]. Previously studies have shown that, a neurotoxin 1-methyl-4-phenyl pyridinium (MPP^+) selective for diencephalic population 5,6,11 induced a transient functional deficit and motility disorder in zebrafish larvae[20].

Here we profiled TFs (Nurr1, Lmx1b Foxa1/2 and En1/2), their targets (TH/DAT) and locomotor activity during early stages of zebrafish differentiation of progenitors to DA neurons (18 Hpf, 24 Hpf, 48 Hpf and 96 Hpf) in the presence of MPP^+ (1 mM). Our study showed a time dependent modulation of TFs that correlated with the appearance of 50% loss of DA neurons and motor impairments by 96 Hpf. This treatment paradigm mimics PD pathology without the need to wait for several months to generate age-old treated disease. These findings inspired us to focus on the participation of TFs in the specific process of DA recovery response. RT-PCR was performed to measure the gene expression changes after 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days and 87 days post DA injury. Differently, a time dependent restoration of expression of TFs, allowing functional recovery at 87 days (*i.e*., 3 months old adult zebrafish) was observed.

Transcription factors identified are likely to be critical in inducing neurogenesis in the damaged brain and further investigations warranted to establish regenerative strategies to enhance or induce endogenous repair of human DA neurons.

Methods

Vertebrate animals and collection of eggs

Wild AB type adult Zebrafish (<8 month old) were reared and kept at aquatic research laboratory at Sathyabama Institute of Science and Technology under standard laboratory conditions of temperature 28±0.5°C, pH 7.2±0.2 on a 14:10 Dark/light photoperiod according to the standard breeding protocols[21]. Collected eggs were stored in embryo medium (EM) and fertilized embryos were staged under stereo microscope (ZEISS) according to the description of Sharmili S *et al.* 2015[22].

Embryos showing proper and sequential development when they reached 18 Hpf were used and randomly classified into four groups: control group (Group 1; n=10), MPP^+ lesioned group (Group 2; n=10), 1 day-1 week post- DA injury group (Group 3, n=10),) and 3 months post-DA injury group (Group 4, n=10).

Treatment paradigm for DA neuron injury and regeneration

To determine the optimum point at which DA neurons degenerate and induce functional impairments in response to MPP^+ toxicity, briefly zebrafish embryos were maintained in EM containing 1 mM MPP^+ between 18 Hpf to 96 Hpf. At 96 Hpf, significantly ablated larvae were removed from MPP^+ and allowed to recover in EM. Severely ablated larvae were selected based upon disruption of TH^+/DAT^+ neurons in zebrafish brain and motor impairments. The evidence of regeneration/neurogenesis/functional recovery was documented for 1 week every 24 h or 87 days post injury. For the control group same volume of EM only was added. The solution of each group was disposed of daily and replaced with fresh EM. Exposures were carried out as three independent experiments and results were combined. MPP^+ exposure was performed as described by[23] with slight modifications and disposal according to current safety protocols[21].

Whole mount *in situ* hybridization (WISH)

Whole mount *in situ* hybridization was performed as previously described[24]. Briefly, the embryos were fixed in 4% paraformaldehyde and then digested with proteinase K. Subsequently, the embryos were prehybridized for 5 hr in hybridization solution (50% Deionized formamide, 5% SSC, 0.1% Tween 20, 50µg mL^{-1} of heparin, 500 µg mL^{-1} of RNase-free tRNA and 1 M Citric acid) and then incubated overnight at 70°C in hybridization solution containing 50 ng digoxigenin-labeled probes. Probe detection was performed with alkaline phosphatase-coupled anti-Digoxigenin antibody (Roche) and nitroblue tetrazolium salt/5-bromo-4-chloro-3-indolyl phosphate substrate (NBT/BCIP, Sigma). The NBT/BCIP-stained embryos were mounted on microscope slide and captured using a Zeiss microscope equipped with a digital camera. The following digoxigenin labeled antisense probes were used: Nurr1; Foxa1; En-1a; Lmx1a; Tyrosine hydroxylase (TH) and dopamine transporter (DAT). These probes were synthesized using the Roche Digoxigenin (DIG) labeled Kit (Catalogue number: 1175025) according to the kit manufacture protocol while TH and DAT DIG labeled probes were procured from IDT Technologies (Suppl. Table 1).

RNA isolation and Real Time PCR

Total RNA was extracted using TRIZOL (Invitrogen) reagent according to the standard procedures. One microgram of RNA was converted into the complementary DNA (cDNA) by using high capacity cDNA synthesis kit from Applied Biosystem. The fluorescent real time PCR reaction was carried out in "Applied Biosystem Step one" instrument with Applied Biosystem power SYBR green with specific primers targeting of TH, DAT, Nurr1, Foxa1, En1a, En1b, En2a, En2b, Lmx 1 a/b (Suppl. Table 2) following the PCR conditions, Initial denaturation 95°C for 1 min and followed by 40 cycles of denaturation 95°C for 30 sec, annealing with 52-59.6°C for 45 sec (Suppl. Table 2) and extension with 72°C for 1 min, followed by a final extension at 72°C for 10 min. β-actin was used as internal control, assay was performed in duplicates with 25 µL reaction. Real time PCR data was quantitatively analyzed by using the formation of $2^{-\Delta Ct}$, in which $^{-\Delta Ct}$ represents the ratio between the number of cycles (Ct) of the target genes with the endogenous control.

Locomotor Activity

Locomotor activity assays were performed as described previously[18] with minor modifications. At 24 Hpf, the tail movement of the embryos was observed under a stereo zoom microscope. At 48 Hpf–adults, the locomotor activity was studied in a Petri plate marked with grid lines, containing embryo medium. The movement across the grid lines was observed for a period of 1 min, from which the distance covered by the embryos/larvae was calculated. However, the locomotor activity of adult zebrafish was measured as per the protocol followed by[25]. Small experimental tank (30 cm × 10 cm × 15 cm) containing water was used to assess the locomotor activity of zebrafish. A transparent plastic film was placed in front of the tank in order to divide the tank into four segments. Fish were placed individually in the tank and their behavior was recorded for 5 min after a 10 min habituation period. Spontaneous swimming activity was measured by recording the distance.

Statistical analysis

Statistical analysis was performed using SPSS for multi group analysis, Student's *t*-test and one-way analysis of variance (ANOVA) was used followed by post hoc tests for multiple comparison. Regression and Pearson correlation was used for correlation analysis and the *P*-values were adjusted with the Benjamini and Hochberg method to control the false discovery rate. Quantitative data was represented in

Mean ± SEM and Statistical significance was defined at 95% level (P<0.05).

Results

MPP$^+$ elucidates significant degeneration of DA neurons at 96 Hpf

First, we determined the optimum time point that MPP$^+$causes significant DA neuron degeneration. Two phenotypic markers of DA:TH, the rate-limiting enzyme in dopamine synthesis and DAT, which up takes dopamine from the extracellular space to terminate dopamine neurotransmission were used to determine the loss of DA neurons using WISH and RT-PCR.

In controls, mild TH signals were detected in ventral diencephalic cells at 24 Hpf. Later, between 48 Hpf to 96 Hpf a considerable increase in TH expression was identified in the ventral telencephalon and in several diencephalic areas as well as in the inner nuclear layer of the retina[26]. In the cellular response to MPP$^+$, WISH detected a time specific decrease in TH/DAT mRNA in DA neurons across the four time points analyzed in this study. By 96 Hpf, this protocol produces a 50% DA loss within 3 days of MPP$^+$ exposure (for all comparisons; Fig. 1A).

To further validate the accuracy of WISH results, we measured the expression changes for TH and DAT using RT-PCR at 24 Hpf, 48 Hpf, 72 Hpf and 96 Hpf (summarized on Suppl. Table 3 and illustrated on (Fig. 1B) (DAT and TH). Consistent with WISH, TH/DAT expression was down regulated in a time dependent manner between stages 24 Hpf and 48 Hpf,

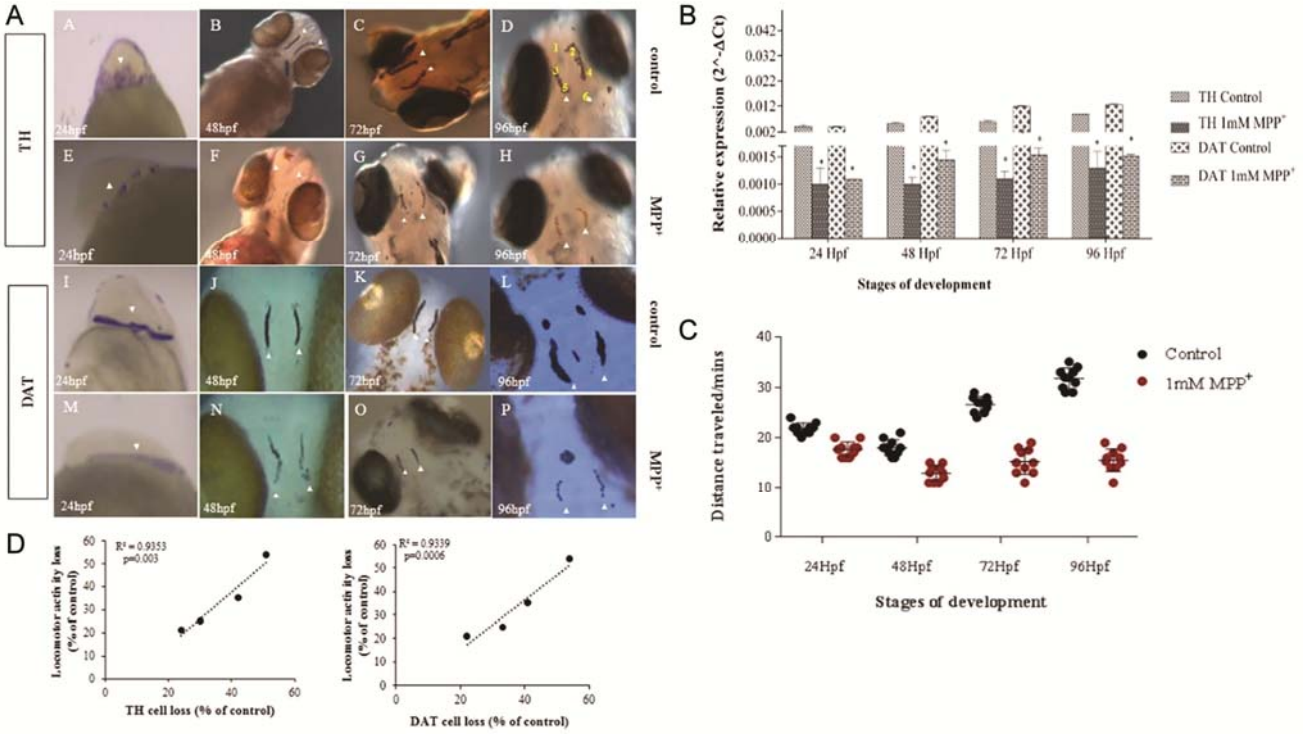

Fig. 1 — (A) Effects of MPP$^+$ on TH and DAT expression *via* Whole mount *In situ* Hybridization: TH-reactive neurons in ventral diencephalon can be divided into DC: diencephalic catecholaminergic cluster at 24 Hpf; catecholaminergic clusters in ventral diencephalon of 4 dpf embryos can be divided 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). The DA neurons in the ventral diencephalon are shown by arrowheads. Exposure of 1mM MPP$^+$ for different time periods reduced TH$^+$ /DAT$^+$ neurons in the ventral diencephalon in a time dependent manner. All images are shown as 500 μM bar; (B) Effects of 1mM MPP$^+$ exposure on TH and DAT mRNA expression by qRT-PCR. MPP$^+$ induced a significant reduction in transcripts of TH/DAT in a time dependent manner (ANOVA,*P<0.05 relative to age matched controls), Data are presented as Mean ± SEM, Gene expression was normalized with beta actin gene; (C) The dynamics of behavioral response to continuous embryonic MPP$^+$ (1 mM) exposure. Exposure of MPP$^+$ for different time periods (24 Hpf-96 Hpf) significantly reduced the distance traveled in a time dependent manner (ANOVA, *P<0.05 relative to control), Data are presented as Mean ± SEM (n = 10 per each group); and (D) Temporal Correlation between TH$^+$/DAT$^+$ neuron loss and Locomotor activityThere was a significant correlation of TH$^+$neuron loss tolocomotor activity (R^2 = 0.9533, P = 0.0031 by Pearson's coefficient) and DAT$^+$neuron loss tolocomotor activity (R^2 = 0.9339, P = 0.0006 by Pearson's coefficient)

24 Hpf and 76 Hpf and 24 Hpf and 96 Hpf in the presence of MPP^+. The assessed genes reported a strong difference in fold change between control and MPP^+ embryos (change >3-fold and $P< 0.03$; Suppl. Table 4)

MPP^+ induced time course locomotor deficits correlatewith DA loss

In order to characterize the locomotor activity of the zebrafish embryos during the course of DA degeneration, the current study observed the mean distance travelled during the exposure period between 24-96 Hpf (Fig. 1C). In comparison to controls, locomotor activity reduced in a time-dependent manner over the course of MPP^+ exposure. At 24 Hpf, locomotor activity was measured as spontaneous tail coiling and it was observed 21.28% reduction in MPP^+ exposed group. At 48 Hpf, 72 Hpf and 96 Hpf the locomotor activity was significantly reduced by 25.38%, 35.26%, 54.35%, 54.72% in MPP^+ exposed groups ($P< 0.05$).

Further, the association between the loss of DA neurons and locomotor activity in the MPP^+ embryos was investigated by performing linear regression. The % TH^+/DAT^+ neurons loss within the DA system was positively correlated with MPP^+-induced motor deficits ($R^2 = 0.93$, $P< 0.03$; Fig. 1D).

MPP^+ modifies major developmental TFs in DA neurons

To investigate the correlation between TFs and DA phenotype, we first looked at how normal developmental processes progressed over the course of MPP^+ exposure. First we used WISH and RT-PCR to analyze the MPP^+ expression patterns in relation to developmental TFs in DA neurons.

Spatiotemporal expression patterns of the 4 TFs specifying the DA phenotype: Nurr1, Lmx1a, Foxa1 and En1a were analyzed using WISH. While, transcript abundance of the 8 TFs: Nurr1, Lmx1a/b, Foxa1, En1a/b and En2a/b were quantified by RT-PCR.

The Nurr1 hybridization signal was detected in the ventral telencephalon, diencephalic areas, mid- and hindbrain tegmentum[27], as well as in the medulla. Irrespective of treatment, mRNA expression of Nurr1 was considerably increasing over the course of exposure. Comparisons between control and MPP^+ expression patterns of NURR1 show a time specific decrease in the above areas (for all comparisons; Fig. 2A).

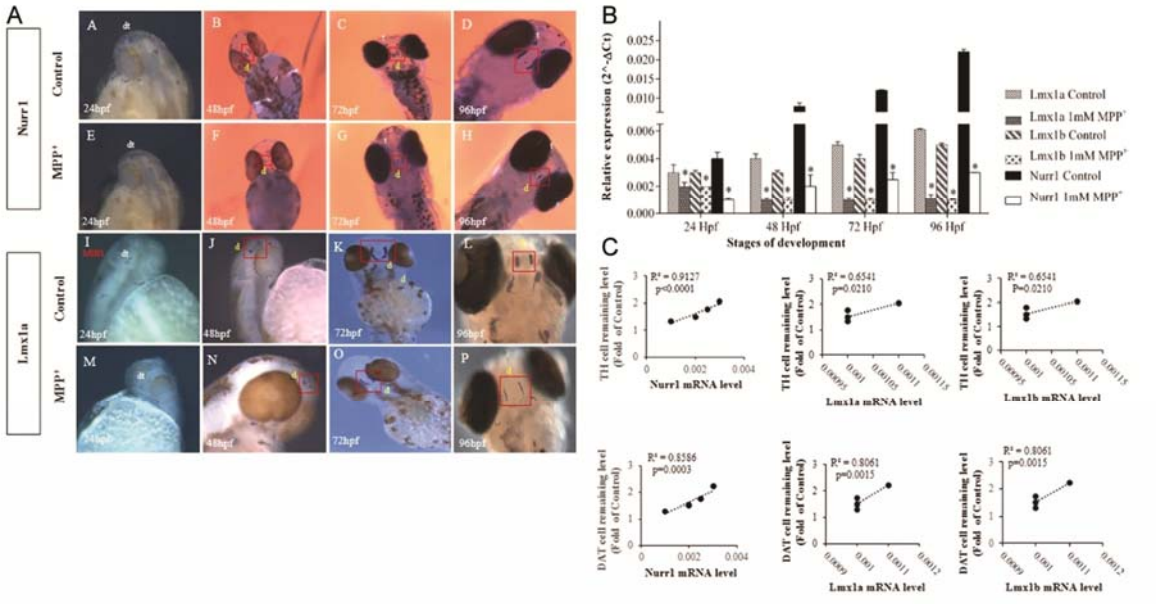

Fig. 2 — (A) Effects of MPP^+ on Nurr 1 and Lmx1a expression *via* Whole mount *In situ* Hybridization whole mount *in situ* hybridization. Exposure of 1 mM MPP^+ for different time periods reduced Nurr1 (E-H) and Lmx1a (M-P) expression in the ventral diencephalon in a time dependent manner relative to control treatment for Nurr1 (A-D) and Lmx1a (I-L). The ventral diencephalon area is framed and the images are in ventral views captures. All images are shown as 500 µM bar. *Abbreviations*: dt, dorsal thalamus; d, diencephalon; t, telencephalon; MHB, Midbrain Hindbrain Boundary; (B) Effects of 1 mM MPP^+ exposure on Nurr1 and Lmx1a/b mRNA expression by qRT-PCR. MPP^+ induced a significant reduction in transcripts of Nurr1 and Lmx1a/b in a time dependent manner (ANOVA, $P<0.05$ relative to age matched controls. Data are presented as Mean ± SEM (n = 3 per each group); Gene expression was normalized with beta actin gene; and (C) Temporal Correlation between TH^+/DAT^+ neuron remaining and transcript levels of Nurr1 and Lmx1a/b There was a positive correlation of TH^+/DAT^+ neuron remaining with Nurr1/Lmx1a/b mRNA level ($P<0.05$)

The results for the mRNA expression of Lmx1a showed a localized expression within broad diencephalic domain at 24 Hpf; diencephalon, midbrain–hindbrain boundary and the amacrine layer at 48Hpf and from 72 to 96 Hpf[26], lmx1a expression considerably increased in the diencephalon and non-adrenergic neurons of locus coeruleus (Fig. 2A). In MPP^+ exposure up regulated expression was observed in diencephalon at 24 Hpf. Furthermore, analysis of lmx1a expression at 48 Hpf detected down regulated expression in midbrain–hindbrain boundary with no detectable changes in the amacrine layer. There was relatively mild loss in expression in the diencephalon, whereas in the non-adrenergic neurons of locus coeruleus a significant down regulated expression was detected at 72 Hpf in MPP^+ exposure. In contrast, a significant down regulated expression in the posterior group of catecholaminergic cluster in ventral diencephalon at 96 Hpf was noted in MPP^+ exposure.

During embryonic development En 1a expression was demonstrated in telencephalon and mesencephalon regions[28]. In contrast to increase in En 1a expression in the midbrain–hindbrain junction at 24 Hpf, a slight down regulated expression in the diencephalon with no effect in midbrain–hindbrain boundary and the amacrine layer at 48 Hpf[25] was observed in MPP^+ exposure. Moreover, from 72 Hpf to 96 Hpf a significant down regulated expression in the ventral diencephalon and midbrain–hindbrain boundary was elicited in MPP^+ exposure (Fig. 3A).

Regardless of MPP^+ exposure, Foxa1 expression visibly increased in the diencephalon and non-adrenergic neurons of locus coeruleus during the course of exposure. When compared with controls, MPP^+ exposure induced increased FOXA1 expression in the floor plate region at 24 Hpf. Although FoxA1 expression did not disappear in ventral diencephalon region, it was clearly reduced from 48 Hpf to 96 Hpf (Fig. 3A).

The RT-PCR results confirmed the dynamic changes in the expression of TFs observed on WISH across the four time points for both control and MPP^+ embryos (Summarized on Suppl. Table 3 and illustrated on (Fig. 2A) (Nurr1); (Fig. 2A) (Lmx1a/b); (Fig. 3B) (Foxa1) and (Fig. 3B) (En1a/b, En2a/b). The assessed genes reported a strong difference in fold change between control and MPP^+ embryos as shown in (Suppl. Table 4). Very similar to WISH analysis all these genes were down regulated in a time dependent manner during the course of MPP^+

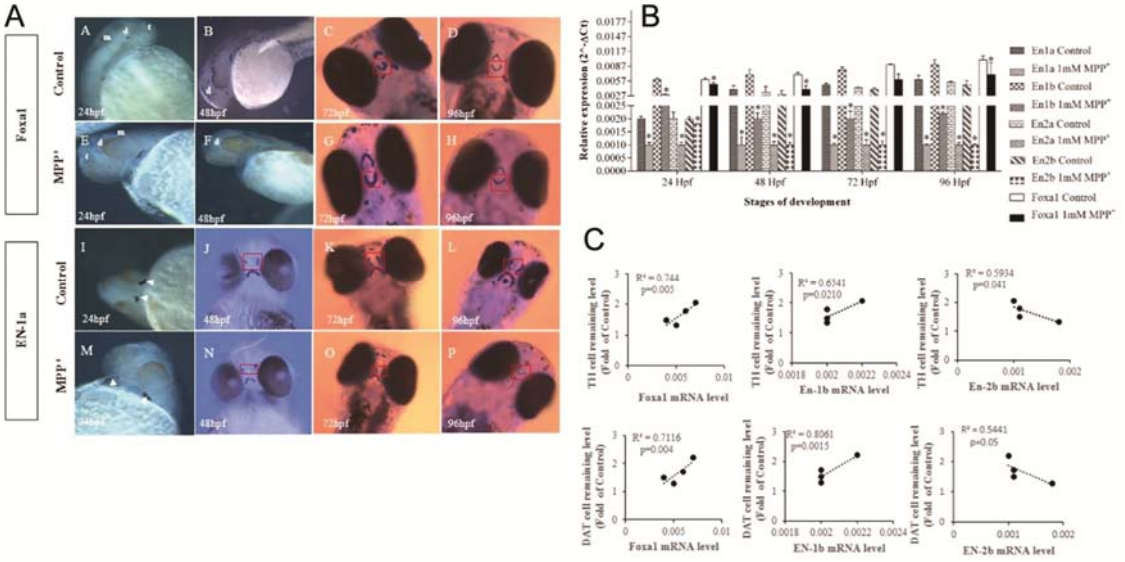

Fig. 3 — (A) Effects of MPP^+ on Foxa1 and EN-1a expression *via* Whole mount *In situ* Hybridization. Exposure of 1 mM MPP^+ for different time periods reduced Foxa1 (E-H) and EN-1a (M-P) expression in the ventral diencephalon in a time dependent manner relative to control treatment for Nurr1 (A-D) and Lmx1a (I-L). The ventral diencephalon area is framed and the images are in ventral views captures. All images are shown as 500 μM bar. Arrows shows the EN-1a expression at 24 Hpf. *Abbreviations*: m, mesencephalon region; d, diencephalon; t, telencephalon; (B) Effects of 1 mM MPP^+ exposure on Foxa1 and En1a/b and En 2a/b mRNA expression by qRT-PCR., MPP^+ induced a significant reduction in transcripts of Foxa1 and En 1b and En2b in a time dependent manner (ANOVA, $P< 0.05$ relative to age matched controls). Data are presented as Mean ± SEM (n = 3 per each group); Gene expression was normalized with beta actin gene; and (C) Temporal Correlation between TH^+/DAT^+ neuron remaining and transcript levels of Foxa1 and En1a/b and En 2a/b A positive correlation was observed with TFs; En 1b & Foxa1 while a negative correlation was observed with TF; En 2b (*$P< 0.05$). No correlation was seen with En1a expression.

exposure *i.e*., between 24 Hpf and 48 Hpf, 24 Hpf and 76 Hpf and 24 Hpf and 96 Hpf embryos.

Correlation between altered TFs expression and loss of DA phenotypes

To investigate the correlations between changes in transcript abundance of TFs with TH/DAT expression at four different time periods during MPP^+ exposure, we used Pearson linear regression. The analysis suggests that TH/DAT expression significantly correlated with mRNA expression levels of TFs. Pearson correlation values between TH/DAT and Nurr1 and Lmx1a and Lmx1b and Foxa1 and En 1b and En 2b are represented in (Fig. 2C & 3C), respectively. The tightest correlation appears to exist between TH and Nurr1 (R^2 = 0.9127, P< 0.001) and the weakest correlation between TH/DAT and En1b (R^2 = 0.54-0.59, P< 0.05). An interesting significant negative correlation was observed between the expression of TH/DAT and En2b (R^2=0.54-0.59, P< 0.05).However, no correlation exists between TH/DAT and En1a and En2a (data not shown). Overall, the significant decrease observed in mRNA expression of eight different TFs in DA neurons led to the substantial loss of DA phenotypes, highlighting the importance of analyzing TFs expression in DA regeneration post injury.

Progression of gene expression changes post MPP^+ induced DA injury

Next we profiled the temporal changes in gene expression after DA injury and related these changes to the locomotor activity.

Using RT-PCR, we profiled the expressions changes post injury for the following 10 genes: phenotypic markers of DA neurons (TH/DAT) and TFs specifying the DAergic phenotypes (Nurr1, Lmx1a/b, Foxa1, En1a/b and En2a/b) for 1 week every 24 h or 3 months, where males and females were sampled separately.

Our RT-PCR showed that the expression of Nurr1, Lmx1a/b, Foxa1, En1a/b, and En2a/b was unchanged over 3 days but a sustained up regulation was noted from 4 to 8 days reaching normal levels in adult zebrafish brain (summarized on Suppl. Table 5) and illustrated on (Fig. 4A) (DAT and TH); (Fig. 4B)

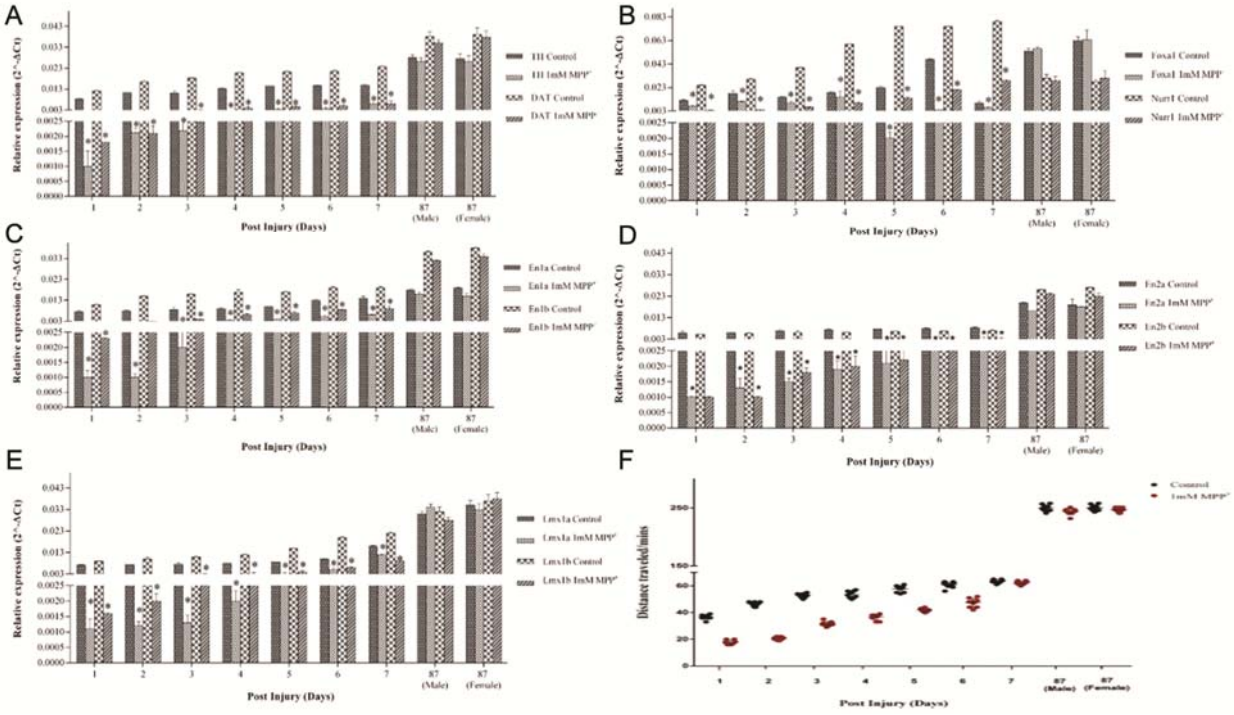

Fig. 4 — Changes in gene expression and locomotor activity after DA injury. The figure shows RT-PCR analysis for (A) TH/DAT; (B) Nurr1/Foxa1 (C) En-1a/b; (D) En-2a/b; (E) Lmx1a/b. Each graph represents the relative expression of the corresponding gene after 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days and 87 days post injury. mRNA levels of most genes started to increase between 3-7 days after injury (ANOVA,*P<0.05 relative to age matched controls) which was significantly elevated to normal levels in adult zebrafish brain *i.e*. at 87th day after injury. Gene expression was normalized with beta actin gene. Data are presented as Mean ± SEM; and (F) Changes in locomotor activity at different time points after acute DA injury. All data expressed as Mean ± SEM and statistical significance * denotes P<0.05 relative to age matched controls

(Nurr1/ Foxa1); (Fig. 4C) (En1a/b); (Fig. 4D) (En2a/b)) and (Fig. 4E) (Lmx1a/b). The fold changes of each gene was calculated at different phases and summarized in (Suppl. Table 6).

Subsequently, 54.73%, 56.28%, 38.90%, 32.03%, 29.83%, 20.88% and 0.4% reduction in locomotor activity was demonstrated at 1Day, 2 Days, 3 Days, 4 Days, 5 Days, 6 Days and 7 Days post-injury, respectively, ($P< 0.05$). Furthermore, levels of locomotor activity were found to rapidly return to values comparable to those observed in control adult zebrafish at 87th day post injury. In adult zebrafish both genders showed an identical recovery when the distance traveled was considered.

Correlating mRNA expression levels of TFs with TH/DAT mRNA expression during functional recovery

Aside from the expression levels of TFs and TH/DAT, we used Pearson linear regression to define further the associations between mRNA expression patterns of the 8 TFs with the expression of TH/DAT and expression of TH/DAT with locomotor activity across each time point analyzed. The 18 correlation coefficients found to be statistically significant ($R^2>$ 0.559, $P< 0.001$) are indicated in (Table 1). The tightest correlation appear to exist between TH/DAT and En2a ($R^2 = 0.9917$, $P< 0.001$) and the weakest correlation between TH/DAT and Nurr1 ($R^2 = 0.56$-0.59, $P< 0.05$). TH/DAT expression also significantly correlated with En2b, En1b, En1a, Lmx1b, Foxa1 and Lmx1a expression. This shows that the timing of up regulation of TFs expression relates to the activation of TH/DAT expression.

Next we investigated whether the timing of activation of TH/DAT expression correlated with the restoration of locomotor activity in post-DA injury model of MPP^+. We observed that the magnitude of expression of TH/DAT that has increased at specific time points correlates positively with the locomotor activity (R^2=0.9976, P<0.0001, R^2 = 0.9947, $P< 0.0001$).

Table — Correlating mRNA expression levels

	TH	DAT
Nurr1	0.5881**	0.5599**
Foxa1	0.9253*	0.9356*
Lmx1a	0.8222*	0.8646*
Lmx1b	0.9616*	0.9703*
EN-1a	0.9623*	0.9513*
En-1b	0.9891*	0.9914*
En-2a	0.9907*	0.9986*
En-2b	0.9917*	0.9931*
Locomotor Activity	0.9976*	0.9947*

Discussion

Transcriptional regulation is central to specification and differentiation of neural progenitor cells to DA neurons[11]. Previous studies in human and animal models has led to the hypothesis that measuring the developmental trajectory of functional connectivity deficits in PD models is useful to understand the factors driving the pathology progress and may be crucial for therapeutic purposes, such as regenerative medicine

Here, we tested this hypothesis by administrating MPP^+, in zebrafish model commonly used in PD research, during critical DA developmental periods between 18 Hpf and 96 Hpf. Remarkably we found that the number of TH^+/DAT^+ neurons progressively decreased from 24 Hpf manifesting 50% loss of DA neurons at 96 Hpf. When linear regression analysis was performed, MPP^+-induced progressive TH^+/DAT^+ neuron loss exhibited a positive correlation with the decrease in locomotor activity. In humans, clinically noticeable functional deficits occur at least after 60-70% of DA neurons in substantia nigra are lost[2] and our MPP^+ treatment regimen mimics specific aspects of age-old related neurodegenerative condition in zebrafish larvae.

Importantly, current studies imply that PD pathophysiology is associated with dysregulation of TFs. It is interesting to point out that many interconnections between En1/2, Nurr1, Lmx1a/b, Foxa1/2, and PD-linked genes such as α-synuclein, Pink1 or Parkin have been recognized [29]. Conversely, decreased expression of Nurr1 reported in PD patients could transcriptionally induce expression of α-synuclein[30]. Haubenberger *et al*., data suggest that variations of the engrailed-2 gene are implicated in the development of young-onset PD[31]. Laguna *et al*., reveal that Lmx1b dysfunction is associated with PD pathogenesis[32]. Previous findings from our lab endorse the fact that common haplotype variation in Nurr1 and Foxa1 might have important and clinically relevant associations with PD[33]. Interestingly, a selective ablation of Nurr1 in adult DA neurons using a tamox-ifen (TAM)-inducible Cre/LoxP recombination system leads to the fiber pathology of DA neuronal population and loss of striatal dopamine, recapitulating early stages of PD developments[34]. An asymmetrical degeneration of SNpc DA neurons has been observed in about 30% of aged mice bearing a global heterozygous deletion of Foxa2 gene allele[35]. A tissue-specific ablation both the Foxa1 and Foxa2

genes selectively in adult DA neurons resulted in DA neurons losing their dopaminergic phenotype, which was reflected by the decline in expression of DAT and TH, as well as reduced striatal dopamine leading to the development of locomotor abnormalities[36].

Our results are in concurrence with these discussed findings, suggesting a general role of TFs in DA neuron physiology and functional impairment consequent to its impairment. This study is first to report MPP^+ induced changes in TFs transcript levels during the critical time periods of DAergic development in zebrafish. The key TFs measured by WISH and qRT-PCR showed temporal and spatial pattern of altered mRNA levels by MPP^+, over differentiation times. Apparently, a certain level of TFs is maintained for survival of remaining DA neurons, particularly during stress[37].Interestingly, the progressive loss of TH^+/DAT^+ positively correlated with the degree of MPP^+ induced changes in transcript abundance of most TFs over time. These trajectories demonstrated that cells exposed to MPP^+ during differentiation presented a phenotype similar to PD with altered transcriptional program.

Some researchers have proposed that brain has an intrinsic ability to undergo neuroanatomical changes that lead to reorganization of remaining tissue following injury likely by certain events related to normal development[38]. However, this recover has behavioral consequences that need to be considered while determining whether recovery has occurred. Another way experimental researchers define recovery is to achieve a particular end point following injury which is similar to how it would be performed in the intact animal[39]. Our study is consistent with these literatures on recovery following damage that 2 days post injury; do not exhibit significant impact on locomotor deficits. Over time, recovery in locomotor activity was noted from 3 to 7 days reaching to normal levels in male and female adult zebrafish (91Dpf). This functional recovery may be the result of the regenerative proliferation of progenitor cells and replacement of lost neurons after DA injury[40]. Central nervous system (CNS) regeneration in Newts has mostly been studied after spinal cord transection, tail amputation, by removing a piece of brain tissue or lesioning DA neurons[4]. But adult zebrafish has an amazing potential in the control of progenitor cells to repairs to injuries to their dopaminergic neuron populations in the brain[9]. In previous ablation experiment in adult zebrafish, observations were made that loss of Th^+ cells lead to regenerative proliferation of progenitor cells and replacement of specific dopaminergic neuron populations[41]. This is mainly achieved through a tight regulation of progenitor/stem cell proliferation and differentiation *via* a complex network of signaling pathways that get activated in response to injury[42]. Here we profiled the expressions of TFs involved in DA neuron death which might provide gene targets for reducing the loss of DA neurons and improving their survival following injury. We observed a regenerative response after ablation of DA neurons, defined by an increased expression of TH and DAT. This regenerative neurogenesis depends on sustained activation of TFs, Foxa1/2, Lmxa1/2, Nurr1 and En1/2. There were some reports that demonstrate a rapid increase of Nurr1 expression in the substantia nigra after 6-hydroxydopamine lesion in the striatum of the rat in order to normalize the dopamine levels [43]. Sang-mi Kim *et al*., demonstrated that repeated synthetic mRNA transfection of Nurr1 and Foxa2 in the delayed schedule is an efficient and safe procedure to generate DA neurons from rat neural progenitor cells[44]. The immune system has also been shown to play a critical role in influencing the regenerative response[7] and Nurr1 influences the inflammatory properties of immune cells[45]. To our knowledge, we provide the first evidence of correlation between the dynamic changes in the expression of Foxa1/2, Lmxa1/2, Nurr1 and En1/2 with establishment of TH^+/DAT^+ expression with clearly detectable DA neuronal activities after injury in any model system. This gene expression change may contribute to new research of further understanding the role of these TFs in the stimulation of multiple regenerative processes in the neurogenic area in this animal model of PD. The mechanisms underlying pathology of DA neuron injury and repair in zebrafish may provide critical insights into improving treatment of PD in human's mechanisms and therapeutic targets for PD by inducing new DA neurons through TF-mediated cell fate reprogramming even in adult brain.

Conclusion

In conclusion we have developed a treatment paradigm that mimics PD pathology without the need to wait for several months to generate age-old treated disease. Remarkably, DA neuronal populations in adult zebrafish showed an unexpected regenerative

capacity. Here, neuronal regeneration led to the formation of functional neurons capable of restoring a previously described behavioral impairment. This system shows here that redeploying the developmental transcriptional cascades is critical for DA regeneration. Ultimately, targeting TFs may be used to activate pro-regenerative mechanisms also in mammals to lead to generation and functional integration of new dopaminergic neurons.

Conflict of interest

All authors declare no conflict of interest.

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