ORIGINAL ARTICLE

Modulatory Role of Neuropeptide Y in the Development and Degeneration of Dopaminergic Neurons in the Zebrafish Ascending Dopaminergic System

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ABSTRACT

Introduction: Understanding the intricate molecular processes governing dopamine neurogenesis and degeneration is crucial for unraveling the complexities of neurological disorders. This study explores the interplay between Neuropeptide Y (NPY) and dopaminergic neurons, crucial for brain function and implicated in conditions like Parkinson's disease (PD).Using zebrafish as a model organism, we investigate the impact of the neurotoxin MPP+ on NPY gene expression during critical dopamine neurogenesis windows. **Methods:** Focusing on NPY's modulatory function in zebrafish's ventral diencephalon, we employed quantitative real-time polymerase chain reaction (qRT-PCR) and whole-mount in situ hybridization (WISH) to analyze NPY expression during critical dopamine neurogenesis windows (18 hpf-96 hpf). **Results:** Our phylogenetic analysis of NPY homologs across species reveals evolutionary diversity, emphasizing potential functional implications. qRT-PCR analysis indicates a significant reduction in NPY expression upon MPP+ exposure, particularly at 48hpf, 72hpf, and 96hpf, suggesting a link between neurotoxicity and NPY regulation. Sequencing analysis highlights nucleotide variations associated with decreased NPY expression. Whole-mount in situ hybridization confirms a reduction in NPY expression in mesencephalon and telencephalon regions following MPP+ treatment.**Conclusion:** These findings underscore the intricate relationship between NPY, neurotoxicity, and dopaminergic neurons, providing valuable insights for future diagnostic and therapeutic strategies in PD and related disorders.
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Keywords: Dopaminergic neurons, Neuropeptide Y (NPY), Parkinson's disease (PD), Zebrafish, Neurotoxicity

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INTRODUCTION

The human brain, with its intricate web of hormones and neurotransmitters, holds the key to understanding and potentially treating a multitude of neurological and psychiatric conditions. Among these vital players is dopamine, a neurotransmitter produced in various brain regions, including the substantia nigra, ventral tegmental area, and hypothalamus. Dopamine's influence extends far and wide, impacting everything from body movements to mood, motivation, learning, and reward (1). Its pivotal role in brain function has raised hopes of devising crucial therapies for conditions like Parkinson's disease (PD) and numerous psychiatric disorders (2).

In this complex neurochemical landscape, neuropeptides, particularly those from the neuropeptide Y (NPY) family, emerge as significant players. NPY, primarily secreted by the hypothalamus in response to hunger, exerts profound effects on diverse biological processes, from regulating glucose levels and daily rhythms to influencing food intake and neuroendocrine activities (3). Intriguingly, NPY has been implicated in various human diseases, especially psychiatric conditions like depression (4). Among the brain regions where NPY exerts its influence, the nucleus accumbens (NAc) stands out prominently. Importantly, dopaminergic neurons, with their far-reaching effects on brain function, have been found to regulate NPY expression through specific receptors (5). This connection suggests that NPY may play a pivotal role in the development and function of dopaminergic neurons.

To unravel the intricate molecular processes underlying

the degeneration of dopaminergic neurons in mammals, researchers have turned to chemo-ablative models using potent neurotoxic substances like 1-methyl-4-phenyl-pyridinium (MPP^+). Interestingly, the zebrafish, a vertebrate model organism, has proven highly susceptible to MPP^+ exposure during its early developmental stages, resulting in dopamine neuron loss and impaired motility (6). Zebrafish, with their well-established role as a reliable in vivo model for neurodegenerative diseases, offer a unique opportunity to study the dopaminergic system, particularly in the ventral diencephalic area, which bears similarities to the mammalian nigrostriatal pathway (7).

Intriguingly, previous research in our lab unveiled a remarkable connection between MPP^+ exposure during critical dopamine neurogenesis windows and a dramatic 70% degeneration of dopamine neurons by 96 hours post-fertilization (hpf) (8). This discovery prompted our current investigation, where we delve into the influence of MPP^+ on NPY gene expression, shedding light on the intricate interplay between neurogenesis and degeneration in the context of NPY and dopaminergic neurons. Our research seeks not only to expand our understanding of these intricate neurological processes but also to unveil potential avenues for future diagnostics and therapeutics in the realm of Parkinson's disease and related disorders.

MATERIALS AND METHODS

Zebrafish Maintenance and Embryo Collection

Wild-type adult zebrafish, under 8 months old, were raised and housed in a controlled environment at Sathyabama Institute of Science and Technology. Standard laboratory conditions were maintained, including a constant temperature of 28±0.5°C, pH levels at 7.2±0.2, and a 14:10 dark/light photoperiod following established breeding protocols (9). Fertilized embryos were collected within 0.5 hours of spawning and cultured in embryo medium. Embryos were staged at 24 hpf, 48 hpf, 72 hpf, and 96 hpf using a ZEISS stereo microscope.

Treatment Paradigm for Dopaminergic Neuron Injury

To investigate the NPY gene, we utilized MPP^+ toxin to selectively ablate dopamine neurons in zebrafish embryos. Embryos were exposed to 1 mM MPP^+ in embryonic medium (EM) from 18 hpf to 96 hpf. For the control group, an equivalent volume of EM without MPP^+ was administered. Daily, the solution for each group was replaced with fresh EM. MPP^+ exposure followed the method described by Nellore et al. (8) with minor adjustments.

Phylogenetic tree and percentage identity analysis of NPY homologs

Amino acid sequences of NPY from zebrafish and other animals were aligned using the CLUSTALW multiple sequence alignment program to determine homologies. Evolutionary distances were calculated using Tree-Puzzle version 5.0, and a phylogenetic tree was constructed via Neighbor-Joining with PHYLIP version 3.6a3 (10-12).

Whole mount in situ hybridization (WISH)

WISH was conducted following the protocol outlined by Thisse et al. (13). Embryos were fixed in 4% paraformaldehyde, treated with proteinase K, and prehybridized in a hybridization solution. Subsequently, embryos were incubated overnight at 70°C in hybridization solution containing 50 ng digoxigenin-labeled probes. Detection of probes was achieved using alkaline phosphatase-coupled anti-Digoxigenin antibody (Roche) and NBT/BCIP substrate (Sigma). Stained embryos were mounted on microscope slides and imaged using a Zeiss microscope equipped with a digital camera.

RNA isolation and Real-Time PCR

Total RNA was extracted with TRIZOL reagent according to standard procedures (14). One microgram of RNA was reverse-transcribed into complementary DNA (cDNA) using the Applied Biosystem high capacity cDNA synthesis kit. Real-time PCR (qRT PCR) was performed on an Applied Biosystem Step One instrument using SYBR green with NPY primers (Table I and Table II). β-actin served as an internal control, and assays were conducted in duplicates. Data were quantitatively analyzed using the 2-ΔΔCt method (15).

Table I. Primer Sequences and Annealing Temperatures. F: Forward R: Reverse Ta: annealing Temperature.

Primer name	Sequence	Primer length	Ta (°C)
QPCR *NPY* F	TCTCTTGTTCGTCTGCTTG	149	55
QPCR *NPY* R	TCCCATACCTCTGCCTTGT		
Seq *NPY* F	ATTCCAAGACCTCATTCAT	2116	63.5
Seq *NPY* R	GACACAACACGCATACAC		

Table II: Thermal profile for qRT PCR

Stage	Condition	Time	No. of cycles	Remarks
Stage 1	98°C	3 min	1	Enzyme activation
Stage 2	95°C	30 sec		Denaturation
	63.5°C	30 sec	35	Annealing
	72°C	30 sec		Primer extension
Stage 3	95°C	30 sec		Melt curve
	60-95°C	30 sec	1	
	95°C	30 sec		

NPY gene sequencing

Sequencing of gel-purified products was carried out with bidirectional primers (Forward and Reverse) using gene-specific primers. This sequencing was outsourced to Xcelris Labs Ltd, Ahmedabad, utilizing an automated terminated cycle sequencing method on a DNA sequencer (ABI 3500, Applied Biosystem).

Statistical Analysis

Comparative statistical analysis, were performed by ANOVA, to assess differences between the MPP^+ exposed group and the control group concerning developmental stages, emphasizing the impact of MPP^+ treatment on NPY gene regulation.

RESULTS

Sequences, Phylogenetic, and Percentage Analysis

In our study, we initiated our investigation by conducting a comprehensive analysis of NPY (Neuropeptide Y) homologs across various species. This analysis allowed us to gain insights into the evolutionary relationships and genetic divergence of NPY genes.

Percent Identity and Phylogenetic Analysis

The phylogenetic analysis of NPY homologs across various species presented in Figure 1A and 1B provides valuable insights into the evolutionary relationships of NPY genes. Our analysis revealed that NPY sequences exhibit varying degrees of similarity among the studied species. Human (*Homo sapiens*), rhesus macaque (*Macaca mulatta*), and rabbit (*Oryctolagus cuniculus*) share a common ancestor, forming a clade with significant gene similarities. Chickens (*Gallus gallus*) appear to have a more distinct evolutionary path, sharing fewer gene similarities with the other species. Zebrafish (*Danio rerio*) displayed a notably lower degree of genetic similarity, suggesting a more distant evolutionary relationship with the NPY homologs of other organisms.

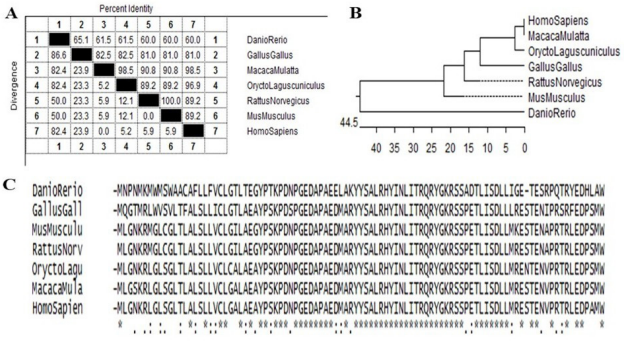

Figure 1. A) Percent identity, B) Phylogenetic analysis, C) Amino acid alignment of NPY homologs of Danio rerio, Gallus gallus, Macaca mulatta, Oryctolagus cuniculus, Rattus Norvegicus, Mus musculus, and Homosapiens. NPY sequences are aligned to the top sequence (from Danio rerio); residues identical to the top sequence are indicated by stars, whereas dots indicate gaps introduced for alignment.

Rats (*Rattus norvegicus*) and mice (*Mus musculus*) share a common ancestor but exhibit a moderate genetic divergence. This phylogenetic information underscores the evolutionary diversity within the NPY gene family across species, with potential implications for their functional roles.

Amino Acid Alignment

The amino acid alignment shown in Figure 1C highlights regions of identity and divergence among NPY homologs. Residues identical to the zebrafish sequence were marked with stars, while dots represented alignment gaps. The sequence alignment provided insights into conserved regions and regions where variations occurred, offering valuable information for future functional analyses.

qPCR Analysis of NPY Gene Expression

We examined the expression of the NPY gene across various developmental stages (24hpf – 96hpf) using qPCR (Figure 2). In the control group, NPY gene expression increased during developmental stages. Exposure to MPP^+ resulted in a significant reduction in NPY gene expression at 48hpf, 72hpf, and 96hpf, suggesting an impact of MPP^+ treatment on NPY gene regulation. The lack of NPY gene expression assessment at 24hpf may indicate a potential disruption caused by the initial exposure to MPP^+.

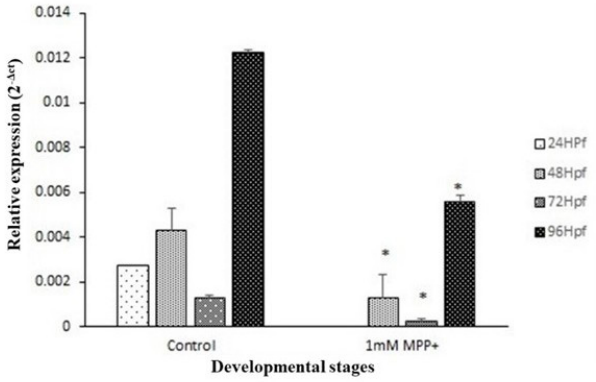

Figure 2. mRNA expression of the NPY gene at various developmental stages (24hpf – 96hpf) was quantitatively analyzed using qPCR. Data are presented as Mean ± SEM, Gene expression was normalized with beta-actin gene. (ANOVA, *P<0.05 relative to control) n=3.

Sequencing Analysis

To further characterize the NPY gene, we conducted sequencing analysis (Figure 3 and Table III). Comparing the nucleotide differences between the 1mM MPP^+ group and the control 96hpf group, we identified minimal nucleotide alterations, including transitions and transversions. These nucleotide alterations may be associated with the decreased expression of the NPY gene induced by MPP^+ treatment.

WISH

We performed WISH to visualize the expression patterns

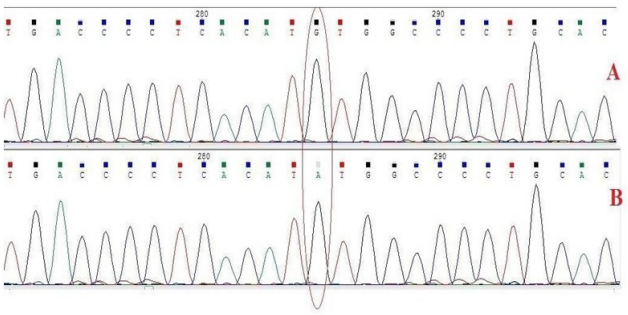

Figure 3. DNA sequence chromatograms demonstrating mutations at NPY gene. A) The reference sequence derived from control B) Sequence derived from 1mM MPP+ treated zebrafish embryo at 96hpf shows homozygous c.729G>A

Table III. Nucleotide Variations with respect to the DNA position and location

S.No	Nucletide change	Position	Intron or Exon
1	T>C	125	**Exon**
2	C>A	356	**Exon**
3	G>A	789	**Intron**
4	A>G	1202	**Intron**
5	C>T	1500	**Intron**

of NPY in zebrafish embryos at 96hpf (Figure 4). In the control group, we observed robust NPY gene expression primarily within the mesencephalon and telencephalon regions of the zebrafish brain. This expression pattern suggests that NPY is actively transcribed in these brain areas under normal conditions.Following the induction of dopamine neuron ablation using MPP^+ treatment, we observed a substantial reduction in NPY gene expression within the same brain regions compared to the control group. This finding is consistent with our quantitative PCR (qPCR) expression analysis, confirming that MPP^+ treatment leads to a significant decrease in NPY expression in the mesencephalon and telencephalon regions of zebrafish embryos.

DISCUSSION

Our investigation yields valuable insights into the evolutionary conservation and divergence of NPY genes across species. For instance, the observation that zebrafish (Danio rerio) displayed a lower degree of genetic similarity with other species is consistent with their evolutionary position. Zebrafish are considered a more distant relative in comparison to mammals like humans, rats, and mice. This result supports the use of zebrafish as a model organism to study evolutionary aspects of genes related to neurodegeneration, as previously suggested in the literature (16).

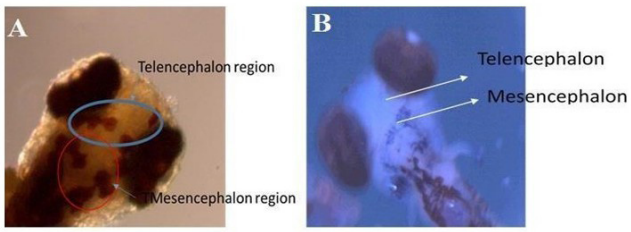

Figure 4. NPY expression Visualized Through Whole-Mount In Situ Hybridization. A) Control B) MPP+ treatment

Alignment analyses conducted provide a preliminary understanding of the functional conservation of NPY genes. Conserved regions often signify functional significane, and while our alignment studies offer initial insights, delving into the functional implications of these conserved and divergent regions in future research, perhaps through mutagenesis studies or functional assays, is imperative. Past research has emphasized the pivotal role of conserved regions in NPY for receptor binding and signaling (17,18).

Moreover, our exploration of NPY gene expression through qPCR and sequencing underscores the impact of MPP^+ treatment on zebrafishNPY gene. The substantial reduction in NPY gene expression following MPP^+ exposure suggests a potential link between dopaminergic neuron injury and NPY regulation (19). The identified nucleotide alterations in the sequencing analysis may may contribute to this observed downregulation (20).
The WISH results provide spatial insights into NPY expression patterns in zebrafish embryos. The robust expression within the mesencephalon and telencephalon regions in the control group aligns with the known roles of NPY in neuroendocrine activities. The significant reduction in NPY expression following MPP^+ treatment corroborates the qPCR findings, reinforcing the impact of dopamine neuron ablation on NPY regulation. These spatial expression changes offer a glimpse into the intricate interplay between dopamine signaling and NPY expression(21, 22).

CONCLUSION

In conclusion, our study elucidates the intricate relationship between Neuropeptide Y (NPY) and dopaminergic neurons in response to neurotoxicity, using zebrafish as a model organism. Through phylogenetic analysis, gene expression studies, sequencing, and spatial expression mapping, we unveil the evolutionary diversity, genetic regulation, and spatial distribution of NPY under dopaminergic neuron injury induced by MPP^+ exposure. Our findings underscore NPY's importance in neurogenesis and degeneration processes, providing valuable insights for potential therapeutic interventions in neurodegenerative disorders like Parkinson's disease. This clear understanding of NPY's role enhances our grasp of neurochemical interactions, paving the way for targeted treatments and diagnostic strategies in neurological conditions.

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