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# Construction of Zebrafish Mutants of CNTF Gene Using CRISPR/Cas9 Genome Editing Technology

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## Abstract

**Background:** The prevalence of Parkinson's disease (PD), a chronic and progressive neurodegenerative disorder, is projected to increase twofold by 2030. Leucine-rich repeat kinase 2 (LRRK2) is the most commonly observed gene in both familial and sporadic PD cases. Notably, there is a substantial augmentation in motor activity during both larval and adult stages of zebrafish lacking the lrrk2 gene. Nevertheless, the precise genetic abnormalities accountable for eliciting these phenotypes in zebrafish are yet to be elucidated. **Methods:** Real-time polymerase chain reaction (PCR) was conducted on zebrafish larvae at 6 days post fertilization (dpf) belonging to both the wild-type and lrk2(-/-) groups. Guide RNA was designed and subsequently employed in the PCR process. Electrophoresis was performed to facilitate identification. **Results:** The expression of CNTF mRNA was significantly diminished in lrrk2(-/-), in comparison to the wildtype zebrafish larvae. This finding implies that CNTF may have crucial implications in the regulated functioning of lrrk2, which is widely acknowledged as the predominant genetic factor contributing to hereditary PD. The primers for CNTF DNA were meticulously designed, and the electrophoresis results of the PCR product were subsequently presented. The wild type zebrafish embryos were meticulously prepared for micro-injection, and the resulting efficiency identification displayed the presence of the mutant PCR product, which exhibited the presence of several debris. **Conclusions:** The present study demonstrates the successful generation of CNTF mutant zebrafish using the CRISPR/Cas9 genome editing technique. Further investigations are necessary to deepen our understanding of the exogenous CNTF gene's functionality.

**Keywords**

Environment Pollution, Ultraviolet Stabilizer, Parkinson's Disease, Ciliary Neurotrophic Factor, Zebrafish, CRISPR/Cas9

## 1. Introduction

Parkinson's disease (PD) is a chronic and progressive neuron-degenerative disorder that has a significant impact on individuals aged 65 and above, affecting more than 1% of this population [1] [2]. It is projected that the prevalence of PD will double by the year 2030 [2]. The primary characteristic of PD is a disruption in the initiation and maintenance of normal movement, resulting in bradykinesia, resting tremors, and postural instability. The aforementioned symptoms can be attributed to the degeneration of dopamine-producing neurons in the substantia nigra (SNpc) within the midbrain, alongside a reduction in dopamine neurotransmitter levels and dopaminergic terminals within the caudate and putamen nuclei of the striatum [3]. Therapeutic interventions for PD involve the regular administration of either the dopamine precursor levodopa or the dopamine agonist [4]. However, it is important to recognize that a definitive cure for PD has not yet been found. Currently, there are no therapeutic agents that have shown clear evidence of modifying the progression of PD [5]. Therefore, a deeper understanding of the underlying pathogenesis of the disease has the potential to greatly aid in the identification of treatments.

The pathology of Parkinson's disease (PD) is influenced by two significant factors: environmental factors and genetic factors. Long-term exposure to environmental pollutants, including pesticides, heavy metals, organic solvents, air pollutants, and high levels of ultraviolet radiation, has been found to elevate the risk of developing PD [6] [7] [8] [9] [10]. Among the various genes associated with PD, Leucine-rich repeat kinase 2 (LRRK2) is the most frequently observed gene in both familial and sporadic PD patients. The abnormal expression of LRRK2 gene is widely recognized as the most prevalent genetic cause of hereditary PD [11] [12]. In the latest study, Sheng *et al.* observed a significant increase in motor activity in both larval and adult stages of lrrk2 knockout zebrafish [13]. However, the specific genetic abnormalities responsible for inducing these phenotypes in zebrafish remain to be determined. Therefore, we screened the target gene from transcriptome analysis.

Ciliary neurotrophic factor (CNTF), a member of the IL-6 family of multifunctional neurotrophic factors, has been shown to plays a pivotal role in the viability, differentiation, and plasticity of neural cells. CNTF, known for its role in promoting the viability of parasympathetic neurons within the chicken ciliary ganglion in an *in vitro* setting, exerts nutritional and differentiation influences on various peripheral and central neurons, glial cells, and extracellular cells within the nervous system [14] [15]. Recent researches suggested that astrocytes contribute to neuroprotection in PD by generating CNTF via the activation of

transient receptor potential vanillin 1 (TRPV1) [16]. Additionally, CNTF has demonstrated the ability to stimulate dopamine synthesis and release [17]. Masu *et al.* utilized homologous recombination methods to ablate the CNTF gene in fully developed mice, resulting in a gradual decline in muscle mass and motor neuron degeneration, ultimately leading to a modest yet discernible decline in muscular strength [18]. The absence of CNTF hampers the mice's ability to recover from sciatic nerve compression injuries [19]. These findings suggested that CNTF may play crucial roles in the survival, differentiation, and synaptic plasticity of neural cells.

Zebrafish, possessing a comparable genome and nervous system to that of humans, is frequently employed as a model organism in the investigation of neurodevelopment and neurodegenerative disorders. Moreover, the utilization of the zebrafish model facilitates drug screening and gene modification experiments, rendering it an invaluable instrument for assessing potential therapeutic strategies and comprehending the underlying mechanisms of diseases.

To the best of our knowledge, there is currently a lack of comprehensive understanding regarding the precise function and mechanism of CNTF in zebrafish. Therefore, it is crucial to utilize genome editing technology to create CNTF gene mutants in zebrafish, which will enable further investigation into their role in zebrafish development and nervous system functionality.

## 2. Material and Methods

### 2.1. Zebrafish Maintenance

Zebrafish (*Danio rerio*) were maintained according to methods described in The Zebrafish Book [20]. All animal procedures were performed according to the requirements of the local ethics committee of the Hangzhou Normal University.

### 2.2. Real-Time PCR

Total RNA was extracted from 6-day post fertilization (dpf) zebrafish larvae (wild-type and lrk2(-/-) groups) using TRIzol reagent (Invitrogen). A pool of 25 zebrafish larvae constituted one sample. Three replicate samples for each group. Complementary DNA (cDNA) was synthesized using SuperScript™ II Reverse Transcriptase (Invitrogen). Real-time polymerase chain reaction (PCR) was performed via 7300 Real-Time PCR System (Applied Biosystems) using 2 μL of cDNA/20 μL of SYBR reaction mixture. The forward and reverse primers of real-time PCR are in exon2 and exon3, respectively. The product length is 140 bp. The sequences of real-time PCR primer were shown in **Table 1**.

**Table 1.** The primer sequences.

| Gene name | Forward primer (5' → 3') | Reverse primer (5' → 3') |
|---|---|---|
| CNTF mRNA | ACAAACGGTCGTCATGGGTT | TGACCTCCTTCCACTGGTGA |
| CNTF DNA | CCTACGGGGGTCTTTACCTATT | TATGACTTGAGGAGTTGGATGC |
| CNTF gRNA | ATAATACGACTCACTATAGAGCTGCGCTTGTCCCT GCGGTTTTAGAGCTAGAAATAGC | AGCACCGACTCGGTGCCACT |

### 2.3. Guide RNA and PCR Identification

We choose the gRNA (GAGCTGCGCTTGTCCCTGCGCGG) as the target sequence. In order to identification the efficiency of the gRNA, we designed the identification PCR primer by the genomic DNA templates. The PCR product contain the cas9 target sequence and the length is 177 bp. The sequences of guide RNA and PCR primer were shown in Table 1.

The double-stranded DNA used for the synthesis of specific gRNA was amplified through PCR. The reaction system employed was as follows (20 μl):

| | |
|---|---|
| 5X first strand buffer | 4 μl |
| DTT | 2 μl |
| T7 Polymerase | 0.5 μl |
| 10 mM NTP | 1 μl |
| Template | 1 μg |
| Nuclease-free $H_2O$ | up to total volume to 20 μl |

Mix the system well and put them in 37˚C for 2 hr - 3 hr.

### 2.4. Micro-Injection

About 1 nl of cas9 mRNA and gRNA were co-injected into 1-cell-stage wild type embryos.

The stock volume final concentration employed was as follows:

| | |
|---|---|
| Phenol red | 0.4 μl |
| Cas9 mRNA | 500 ng/μl |
| gRNA | 300 ng/μl |
| Nuclease-free $H_2O$ | up to total volume to 3 μl |

### 2.5. Check the Efficiency

Firstly add 20 μl solution I (25 mM NaOH, 0.2 mM EDTA, PH 12.0) at 95 for 30 min, and then add 20 μl solution II (40 mM Tris-HCl, PH5.0) to lysis the zebrafish embryos. The genomic region surrounding the CRISPR target site for each gene was PCR amplified and digested by the enzyme which you have choose for the cas9 target. The product of PCR cannot be digest by the enzyme should be the representation of the mutation efficiency.

## 3. Results and Discussion

The expression of CNTF was found to be significantly diminished in lrrk2(-/-) zebrafish larvae, in comparison to the CTL group (wildtype zebrafish larvae), as depicted in Figure 1. This finding implies that CNTF may have crucial implications in the regulated functioning of lrrk2, which is widely acknowledged as the predominant genetic factor contributing to hereditary PD. Consequently, our research endeavors were directed towards CNTF as the focal gene for generating CNTF mutant zebrafish through the utilization of CRISPR/Cas9 genome editing technology.

The position of these primers for CNTF DNA and CNTF RNA were showed in Figure 2, respectively. The electrophoresis outcome of the PCR product is

presented in Figure 3. The marker, listed from top to bottom as 5 K, 3 K, 2 K, 1.5 K, 1 K, 750, 500, 200, and 100 bp, is displayed on the right, while the PCR product of CNTF (177 bp) is shown on the left. Figure 4 shows the wild type zebrafish embryos prepared for micro-injection. The efficiency identification result was presented in Figure 5. The first list on the left consisted of markers, arranged from top to bottom as follows: 5 K, 3 K, 2 K, 1.5 K, 1 K, 750, 500, 200, and 100 bp. The second list represented the negative control, while the third list displayed the mutant PCR product, which contained several debris. The fourth list shows the PCR product of CNTF, measuring 177 bp.

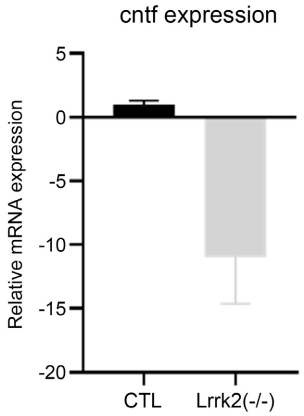


**Figure 1.** Real time PCR.

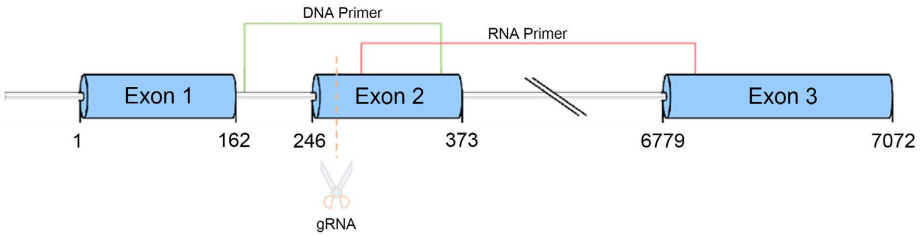


**Figure 2.** Schematic presentation of CNTF gene locus and targeting site.

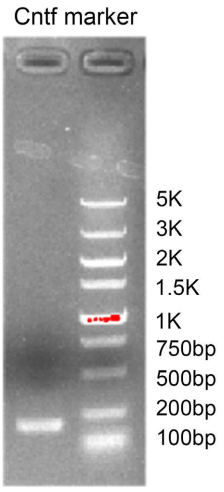


**Figure 3.** Electrophoresis of PCR product.

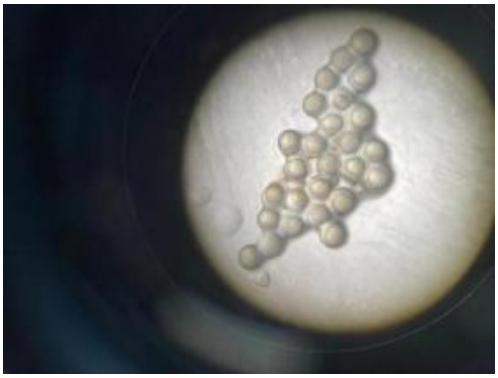

**Figure 4.** Micro-injection.

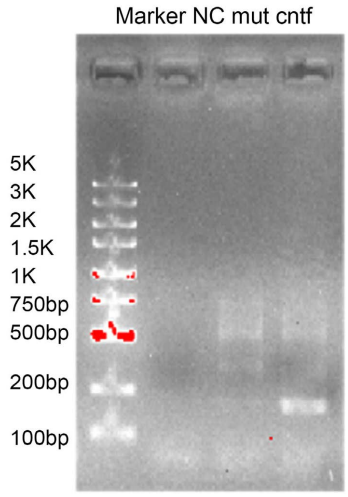


**Figure 5.** Identification of the efficiency.

In summary, the present study demonstrates the successful generation of CNTF mutant zebrafish using the CRISPR/Cas9 genome editing technique. CNTF may play crucial roles in the survival, differentiation, and synaptic plasticity of neural cells. A comprehensive comprehension of the underlying pathogenesis of Parkinson's disease possesses the potential to significantly facilitate the identification of therapeutic interventions. Further investigations are necessary to deepen our understanding of the exogenous CNTF gene's functionality, with the ultimate goal of optimizing its therapeutic efficacy.

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## Credit Author Statement

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## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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