UNIVERSITY OF CALIFORNIA SAN DIEGO

Swedish Mutant Nerve Growth Factor **(**NGFR100W): Potential Therapeutic for Neurodegenerative Diseases

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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The Thesis of Sangwon Woo is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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LIST OF ABBREVIATIONS

NGF = Nerve Growth Factor

NGF^{R100W} = Swedish Mutant Nerve Growth Factor

TrKA = Tyrosine Kinase Receptor A

CNS = Central Nervous System

PNS = Peripheral Nervous System

IENF = Intraepidermal nerve fibers

SNP = Corneal subbasal nerve plexus

O.C.T = Optium cutting temperature

DRG = Dorsal Root Ganglion

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ABSTRACT OF THE THESIS

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Master of Science in Biology

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Neurodegenerative diseases are characterized by loss of sensory neurons leading to motor, cognitive, and memory deficits. Due to the lack of a cure for such diseases, these diseases are costly in the United States. Extensive studies in the past showed the potential of nerve growth factor (NGF) as a therapeutic measure due to its trophic capabilities affecting the proliferation and regeneration of neurons. Therefore, NGF has been tried in several clinical trials but all failed because of painful side effects such as site injection hyperalgesia or myalgia.

However, the recent discovery of a mutant NGF (NGF^{R100W}) that does not induce pain allowed us to investigate its potential as an alternative. Due to the blood brain barrier limiting the administration of these NGF, we explored the effect of NGF^{R100W} by injecting it into Charcot Marie Tooth Type 2B mouse models as they closely resemble peripheral neuropathy. We performed a hot plate test for nociception and sensitivity of the peripheral nerves by injecting increasing doses of NGF^{R100W}. NGF^{R100W} injected groups did not show significant pain perception compared to wild-type NGF injected groups even in high doses. Our immunohistochemistry data further indicated that NGF^{R100W} prompted neuronal recovery comparable to that observed in wild-type NGF. We also explored the regenerative aspect of NGF^{R100W} which rescued the sensory perception of the CMT2B mouse. Based on these findings, we validated the capability of NGF^{R100W} in regenerating neurons and sensory function, which can be potentially used to improve the condition of individuals with peripheral neuropathy.

INTRODUCTION

The current understanding of treating peripheral neuropathy derived from injuries in the peripheral nervous system still remains a challenge. The PNS consists of an extensive network of neurons and glial cells which are responsible for transmitting the sensory messages throughout the body. The nociception pathway of the peripheral nervous system is initiated when the noxious or non-noxious stimulus reaches the skin. Based on the type of stimulus the body reacts differently as noxious stimulus is known to cause hypersensitivity while non-noxious stimulus is known to cause allodynia as a response mechanism during the acute phase of the pain syndrome (Liu et al., 2021).

When the stimulus reaches the skin, the sensory message is delivered through myelinated and unmyelinated skin fibers: A-β fibers, A-δ fibers, and C-fibers. The different sensory fiber types, which are characterized by thinly myelinated fibers and loss of myelin, differed in their action potential conductivity and congenital insensitivity which was also observable during clinical experiments testing for anhidrosis (Shim et al., 2019). Once these action potentials are generated, it transmits its message to an area called dorsal root ganglion (DRG). DRG consists of primary sensory neurons and nociceptive sensory neurons serving an important role in mediating inflammation and pain (Liu et al., 2021; Berta et al., 2017). Once the pain signals arrive at DRG, the message will be further traveled to the dorsal horn of the spinal cord and supraspinal structures of the body leading to neuronal sensitization which increases the nociceptive signaling.

A condition known as peripheral neuropathy arises when such acute pain derived from the stimulus has been prolonged for a period of many months. The release of macrophages from the Schwann cells leads to loss of myelin within the sensory neurons (Ramburrun et al., 2014). It could result in permanent damage in the peripheral nervous system as a result of trauma, or diabetic

conditions leading to development of peripheral neuropathy (Hughes 2002). Peripheral neuropathy is a widely known neurodegenerative disease characterized by pain, muscle weakness, and loss of sensory and sympathetic neurons (Hanewinckel et al., 2016; England et al., 2014). Peripheral neuropathy is further classified as mononeuropathies and polyneuropathies based on the number of peripheral nerves causing the neuropathy (Hanewinckel et al., 2016). The degree of severity varies as one progresses with the disease based on how many sensory neurons have been lost; it results in failure in coordinating motor, cognitive, memory, and even sensitivity. However, no effective measures are currently available as the administration of the drugs to the brain is limited.

The blood brain barrier, which is a unique aspect of the human brain, prevents the effective administration of therapeutic drugs that are used to complement the degeneration of neurons. The outer brain is surrounded by the cerebrospinal fluid working as a protective barrier to absorb shock. In addition, the brain is also surrounded by the blood-brain barrier (BBB) which is located at the microvascular wall around the brain limits the transport of proteins and antibodies making it difficult for antibodies to actually reach the target site (Pardridge, 2016). In addition, dismantling the integrity of BBB would cause more problems such as slow delivery of the drug, or rapid degradation of the drug (Furtado et al., 2018), so it requires a careful approach. As a result, overcoming BBB disruption has been one of the greatest obstacles in ensuring the administration of drugs for the brain. Many scientists have been attempting to create antibodies that could effectively bypass the BBB. The discovery of neuropeptides/neurotrophins called nerve growth factors (NGFs) which could bypass BBB through the endothelium of the brain capillary, thus, changed the paradigm of neurodegenerative therapeutics (Jefferies et al., 1984). Despite the many attempts that have been made to rescue the loss of sensory perception and nociception mechanisms, there has not been much progress made until the discovery of NGF.

Scientists Levi-Montalcini and Hamburger initially discovered these NGF neuropeptides which were found to be essential in the survival and modulation of neurons (Montalcini et al., 1951). The transcription factors which alter gene expressions are secreted when NGF binds to its receptors: Tyrosine Kinase Receptor A (TrkA) and p75 (DeFranco et al., 1993; Montalcini et al., 1951). Therefore, NGF binding to its receptor initiates the multiple signal cascades necessary for survival and modulation of sensory and sympathetic neurons of the peripheral nervous system (Aloe et al., 2012; McMahon 1996). The binding relays sensory messages to carry out various functions inside the human body (Aloe et al., 2012; McMahon 1996). Since then, the therapeutic potentials of NGFs have captured the attention of many scientists over the years. Further research also revealed the relationship with the neuronal glial cells with NGF. When NGF binds to the TrKA and p75 receptors, these coupled receptors were also involved in activation of the neuronal-glial cells which secreted factors that mediated inflammation during the immune response (Villoslada 2004). This suggested that regulating the trophic pathway involved in TrKA and p75 receptors by using NGF could possibly prevent inflammation responses initiated by viral factors and infections during the degeneration of sensory neurons. This possibility is promising because the inflammation responses often cause neuron degeneration, which leads to neurodegenerative diseases and pain (Kempuraj 2016). Therefore, such possible roles allowed further research which allowed NGF to be a popular therapeutic agent for treating both peripheral nervous system and central nervous system diseases (Manni et al., 2013). Accordingly, the loss of interaction of the NGF with their receptors TrKA and p75 often led to the rapid degradation of the neurons and often resulted in neurodegenerative diseases (Rocco et al., 2018); therefore, injecting these factors could possibly improve the conditions of the neurodegenerative disease. However, the application of exogenous NGFs or upregulating endogenous NGF receptors was found to cause inflammation of

the sensory neurons leading to pain responses as one of their main side effects (McMahon 1996). In addition, activation of the downstream signal cascades upon binding of NGF to the receptors often led to increased sensitization decreasing the pain threshold of the individuals (Sung et al., 2019). Therefore, these side effects have been raising concerns about their prospective utilization, which highlighted the desperate need to find a way to circumvent them.

Recent genomics studies on NGF signaling have opened a new door for using NGF variants in regenerative medicine with proper pain control. People with hereditary sensory and autonomic neuropathy type IV (HSAN IV) were shown to have the mutation(s) in the TrkA receptor. These patients with HSAN IV have lost nociception to pain, but suffer from mental retardation (Capsoni 2014). However, people with another type of HSAN, HSAN type V, which is caused by a point mutation in NGF, NGF^{R100W}, lost their pain perception without suffering from mental retardation (Sung et al., 2019). NGF^{R100W} extracted from patients with HSAN type V resulted in decreased sensitization of nociceptive ion channels as their p75 receptors were silenced (Yang et al., 2018). As these receptors contribute to the pain sensitization, silencing these receptors results in an increased pain threshold for individuals which would allow administration of NGF^{R100W} for a prolonged period without notable side effects. Therefore, the discovery of NGF^{R100W} which does not induce both mental retardation and pain has allowed scientists to explore the possibility of using this novel NGF^{R100W} to treat diseases such as peripheral neuropathy which caused much distress in those who are affected (Capsoni 2014; Sung et al., 2019).

The discovery of NGF^{R100W} has opened new doors where the regenerative aspect of NGFs can be also applied to other therapeutic purposes involving pain control. If NGF^{R100W} were to be utilized in an assessment plan for patients, physicians can also possibly overcome the current limitation of chemotherapies for cancer patients. Many cancer patients undergo chemotherapy at

some time during their treatment, but they often must stop in the middle of the treatment due to their side effects; one of the side effects is the peripheral neuropathy induced by anti-cancer therapeutics during chemotherapy. If the administration of NGF^{R100W} does not elicit pain, but brings about a similar clinical effect of wildtype NGF in regenerating neurons it can allow prolonged treatment for patients. It could not only bring better prognosis but also vouch better welfare for the patients.

Expanding our research after clinically proving the effect of NGF, our next step was to understand its efficacy in treating chemotherapy-induced peripheral neuropathy which can open more doors for treatments. There are different types of drugs used during chemotherapy leading to an increased survival rate of many survivors. However, use of these drugs such as cisplatin or paclitaxel on patients led to patients suffering from adverse side effects (Farquhar-Smith and Brown, 2016; Starobova et al., 2017). For instance, the mechanism of the cisplatin has been known to cause sensory axonal peripheral neuropathy in sensory fibers which lead to differences, especially in their nociception (Han and Smith 2013, Starobova et al., 2017).

Therefore, I hypothesized NGF^{R100W} could restore the peripheral neurons which will subsequently restore the sensory perception. To test the hypothesis, wildtype NGF and NGF^{R100W} was injected intradermally at the left hind paw of the CMT2B mouse for a span of 6 weeks. To understand the regenerative capabilities of NGF^{R100W}, we compared the differences in their paw withdrawal latency as a measure of pain/sensory perception through the hot-plate test. In addition, we observed whether increased doses of NGF^{R100W} injection would elicit pain responses compared to the wildtype-NGF. Lastly, we witnessed the regeneration of small sensory fibers in the CMT2B mouse models by sectioning these skin paws and loading them onto the confocal microscopy. Based on the microscope images, we established their sensory perception has been rescued.

MATERIALS AND METHODS

NGF^{R100W} knock-in mouse model:

Our experiments utilized the NGF^{R100W} knockin mice model used in previous literature (Yang et al., 2018). The same NGF^{R100W} knockin mouse model used in Yang et al., 2018 paper was used. NGF^{R100W} knockin mice were generated by the Model Animal Research Center of Nanjing University (Nanjing, Jiangsu, China). At position 100 of mature mouse NGF, the arginine (R) was mutated to tryptophan (W) by gene targeting with the Neo cassette removed from the mutant allele (Yang et al., 2018). Genotyping identification of NGF^{R100W} knock-in mice was carried out by PCR using the primer pairs that recognize sequences near the loxP sites: 5-GGGGAAGGAGGGAAGACATA-3 for forward primer and 5-GATTCCCTTAGGAAGGTTCTGG-3 for reverse primer (Yang et al., 2018). The following amplification protocol (95 °C 5 min; 95 °C 30 s; 58 °C 30 s; 72 °C 45 s; 35 cycles; 72 °C 5 min; 15 °C hold) was used for genotyping; the expected PCR products were marked for +/+, +/fln or fln/fln (Yang et al., 2018).

Rab7V162M knock-in mouse model:

Our experiments utilized the Rab7V162M knock-in mouse models used in previous literature (Gu et al., 2021). This knock-in mouse model (C57BL6) for CMT2B was created through the mutation from G to A at position 484 (V162>M) in Rab7 Exon 5 (Gu et al., 2021). Of the selection markers NeoR and LoxP introduced into the mouse genome together with the mutated allele, LoxP sites were used to facilitate the selective deletion studies necessary by using Cre. Genotyping was performed using the PCR primer pair (Gu et al., 2021). Three genotypes: wt (+/+), heterozygote (fln/+), and homozygote (fln/fln) with a typical Mendelian segregation ratio was obtained. Both the fln/+ and fln/fln pubs survived to full adulthood (Gu et al., 2021).

Animal housing conditions:

All mice were housed in individual cages on a 12/12 h light/dark cycle at 21 ± 2 ℃. All animals were provided with free access to water and foods containing no ad libitum. The colonies were regularly monitored by trained staff at the UCSD vivarium. All tests were carried out in a quiet room between 10 AM and 4 PM.

Nociceptive hot plate test:

In the nociceptive hot plate test, mice were placed on a metal hot plate at 55 ℃ (Analgesia Hotplate, Columbus Instruments, Columbus, OH). The latency time to a discomfort reaction (jumping, licking, or shaking hind paws) was recorded and the mice were immediately removed from the hot plate and were returned to their home cages. Decreased thermal threshold was calculated by subtracting the differences between the 2 data values we have obtained. The cut-off time was 20 seconds.

Mouse paw skin PGP 9.5 Staining Protocol:

The mouse was anesthetized using isoflurane. Then they were sacrificed using spine dislocation and their paw skins were collected. Once the paw skins were collected, they were fixed using methanol/acetone (1:1 dilution) for 30 minutes at a temperature of -20 °C. After 30 minutes, they were washed three times with 1x PBS. Once the washing process was complete, they were dehydrated with 30% sucrose and embedded with Tissue-Tek O.C.T compound overnight at 4 °C in the fridge.

The sections were taken out from the fridge and were sectioned into 20 μm cryo-sections using a Leica Cryostat (Model# CM1900). The cryo-sections were embedded on the glass-slide, and they were washed with PBS to melt OCT for a few minutes. They were incubated in 50mM glycine for 45 minutes, were washed with PBST (0.2%) three times, and blocked with PBST

(0.5%) + 10% goat serum +1 % BSA for one hour. Once they were blocked, they were incubated with primary antibodies overnight at 4 °C. Primary antibodies (UCHL1/PGP9.5 Proteintech #14730-1-AP) were diluted in 10 % goat serum, 1 % BSA, and 1:300 dilution in PBST (0.2%).

After overnight incubation, they were washed 3 times with PBS for 5 minutes each and were incubated with a secondary antibody (Alexa 568) in 1:500 dilution PBS for one hour. Another wash was done 3 times with PBS for 5 minutes and they were stained with DAPI at 1/10,000 for 5 minutes in PBS. Last set of 3 washes were done with PBS. They were mounted on the confocal microscopy (Leica TCS SP8 Confocal Microscope) and were analyzed to collect an image series. Image stacks were collected at a 0.5 μm step size by examining 10-15 sections with 200 μm in length/section. Maximal projection images were generated from these stacks and the IENF density was quantified using ImageJ.

Chemicals, antibodies:

Our experiments utilized the same chemicals used in previous literature (Yang et al., 2018). Unless specified, all chemicals were purchased from either Sigma or Fisher. Mouse hind paw skin sections were stained with the UCHL1/PGP9.5 rabbit polyclonal antibody (Cat. No. 14,730-1-AP, Proteintech Group, Inc, Rosemont, IL) at a dilution of 1/300 to 1/500 (Yang et al., 2018). Biotin-conjugated donkey anti-rabbit IgG (Cat. No 711-065-152, Jackson ImmunoResearch, West Grove, PA, USA) was used with dilution of 1:100 (Yang et al., 2018). Goat secondary anti-rabbit or anti-mouse IgGs conjugated to Alexa 488 or Alexa 568 (ThermoFisher) were used at 1/800 dilution (Yang et al., 2018).

CHAPTER 1

NGF^{R100W} does not elicit pain response even at high doses in vivo

To illustrate the therapeutic potentials of mutant NGF (NGF^{R100W}) on neurodegenerative diseases compared to the wild-type NGF, the control mouse groups were injected with wild-type NGF while the experimental mouse groups were injected with different doses of NGF^{R100W} (Figure 1A,1B). Then these mouse groups were placed on a hot plate at 55 °C to measure their sensory perception at specific periods of time. The control group injected with wild-type NGF showed increased paw withdrawal latency compared to any doses of NGF^{R100W} injected into experimental groups indicating increased pain perception of the mouse (Figure 1A). However, as opposed to the control group injected with the wild-type NGF, the experimental groups injected with NGF^{R100W} maintained a similar level of paw withdrawal latency indicating no significant pain perception at different time intervals (Figure 1A). At specific time intervals, the control group showed the highest decreased thermal threshold from baseline compared to any of the experimental groups (Figure 1B). Increasing the doses of the NGF^{R100W} decreased the thermal threshold compared to the baseline, creating a downward trend (Figure 1B). At 20 minutes compared to the baseline, mouse groups injected with NGF^{R100W} seemed to elicit pain while still showing. At 45 minutes compared to the baseline, the dose of NGF^{R100W} ten times that of wildtype NGF did not induce no significant pain in the mouse groups (Figure 1B). Overall, since the doses of NGF^{R100W} were 10 times the doses of wildtype-NGF, NGF^{R100W} still induced no significant pain after a longer time in the mouse groups showing its therapeutic potential.

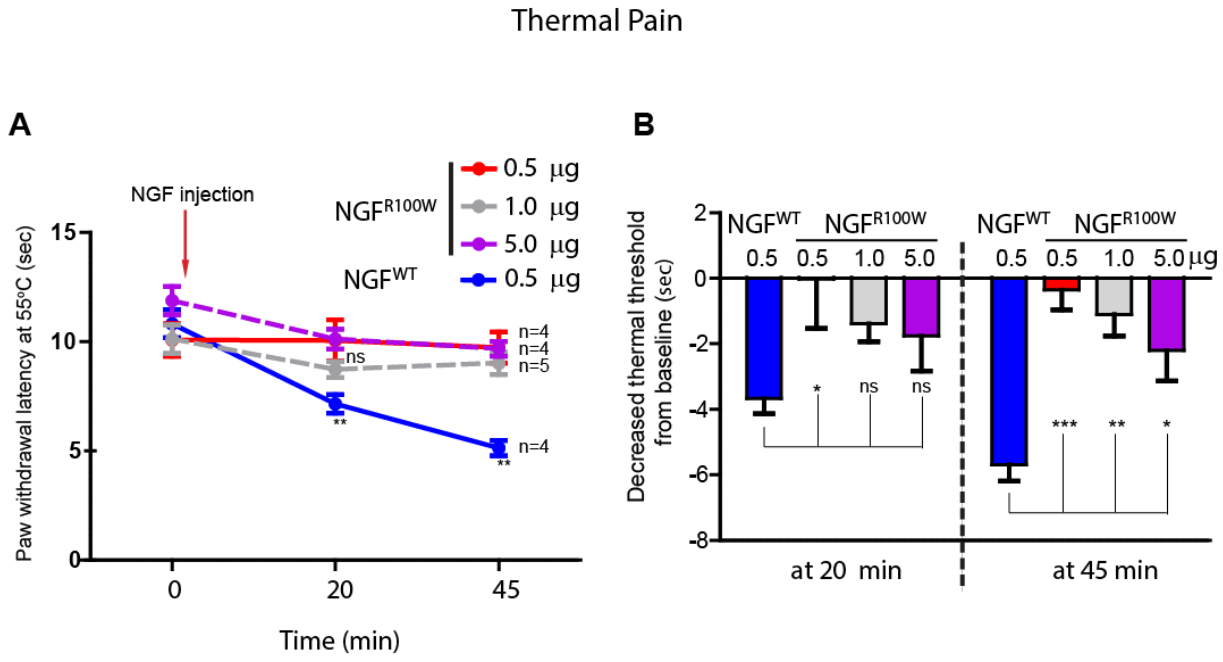

Figure 1. Mouse groups injected with NGF^{R100W} did not elicit pain even in high doses compared to the mouse groups injected with wild-type NGF measured at 45 minutes against the baseline.

Thermal pain sensitization in mice was measured by setting mice injected with nerve growth factor (wild-type NGF) as control and mice injected with mutant nerve growth factor (NGF^{R100W}) as the experimental group. They were placed on a hot plate to measure their pain perception. A) Paw withdrawal latency of both control (blue) and experimental groups (red, gray, purple) compared to the baseline (0 minutes) at 20 minutes, and 45 minutes. T-test was performed against the baseline for both groups injected with NGF^{R100W} and wildtype-NGF. B) Thermal threshold of control and experimental groups were compared from baseline at 20 minutes and 45 minutes to record pain sensitization. Statistically significant effects of the thermal threshold from baseline were determined by a one-way ANOVA test with Dunnett's post-test.

(NS = Not significant, * = <0.05, ** = < 0.005, * = <0.0005, N= Number of mouse)**

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Chapter 1 contains unpublished material co-authored with Dr. Kijung Sung. Dr. Kijung Sung is the primary author of this chapter.

CHAPTER 2

NGF^{R100W} promotes robust regeneration of small sensory fibers in the CMT2B mouse model

To further validate the clinical potential of NGF^{R100W} in regenerating peripheral nerves, skin samples were collected from different mouse groups. Following subcutaneous injections of NGF or NGF^{R100W} into the Charcot Marie Tooth Type 2B (CMT2B) mouse model, their paw skins were sectioned, embedded in O.C.T and mounted on the confocal microscope to obtain the microscope images. They had a mutation in Rab7 which plays a critical role in maintain the function and morphology of the mitochondria. These mouse groups develop CMT2B which leads to increased risk of degeneration of neurons leading to symptoms similar to peripheral neuropathy we were trying to look at. Accordingly, these mouse models were used as they developed loss of small sensory fibers along with loss of pain sensation. The skin tissues were collected both contralateral and ipsilateral (Figure 2A (A-F)), Compared to the wild-type mouse, the CMT2B mouse shows fewer traces of neurons indicating nerve degeneration (Figure 2A & 2B). Then, the relative density of PGF 9.5 IENF (Antibody) was quantified to address the differences normalized on confocal microscope pictures (Allgeier et al., 2022). For the CMT2B mouse groups that were not injected with any of the nerve growth factors, the density of IENF was rather low showing a low level of peripheral sensory neurons (Figure 2B). The mouse groups injected with either wildtype NGF or NGF^{R100W} showed a higher density of IENF when injected in an ipsilateral method compared to the contralateral method (Figure 2B). CMT2B mouse groups injected ipsilaterally in contrast to mouse groups that were injected contralaterally showed less density of PGP 9.5 degrees IENF (Figure 2B).

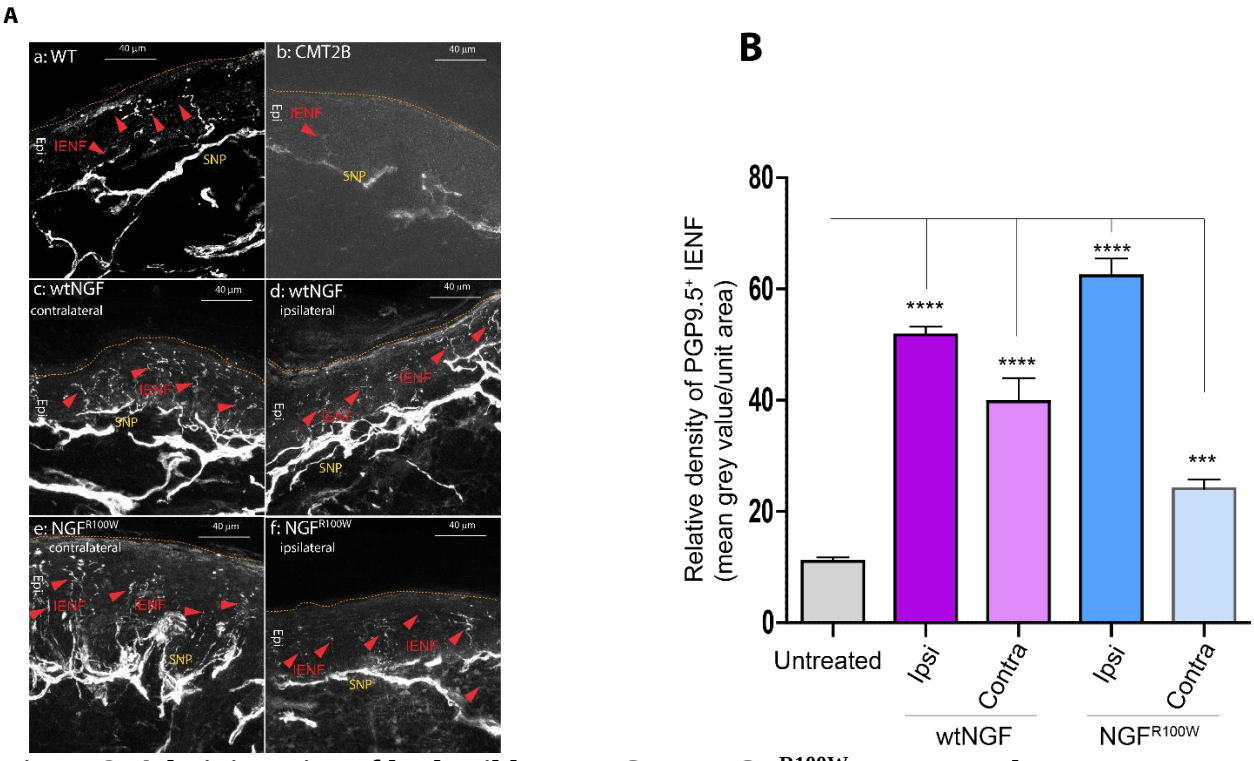

Figure 2. Administration of both wild-type NGF or NGF^{R100W} regenerated sensory neurons and even showed systematic regeneration of neurons in CMT2B mouse groups.
Tissue staining of skin neural fibers: Skin samples were collected after 6 weeks of injection of either wild-type NGF or NGF^{R100W}. These skin samples were stained with DAPI and visualized on a Leica confocal microscope at 40x magnification. The density of intraepidermal nerve fibers (IENF) and corneal subbasal nerve plexus (SNP) was used as biomarkers to assess the development of peripheral neuropathy. One way ANOVA and Dunnett's post comparison test was used to assess the statistical significance of relative density of PGP9.5 IENF.

A. **(A-F) Skin tissues were stained for neural fibers collected from the WT and CMT2B mouse either injected with wildtype NGF or NGF^{R100W}.**
B. **The relative density of PGP 9.5 IENF(Antibody) was quantified for all skin samples discriminating between injection methods.**

(NS = Not significant, * = <0.05, ** = < 0.005, * = <0.0005, **** = < 0.00005)**

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Chapter 2 contains unpublished material co-authored with Dr. Kijung Sung. Dr. Kijung Sung is the primary author of this chapter.

CHAPTER 3

NGF^{R100W} promotes functional recovery of peripheral sensory nerve fibers in CMT2B mouse model

A thermal pain test was conducted only once for the wild-type mice that were not injected with any of the NGF. Compared to CMT2B mutant mice who were tested before and after the injection, the paw withdrawal latency was the lowest for the non-injected wild-type mouse groups. Before the injection of either wildtype NGF or NGF^{R100W}, both mouse groups had similar paw withdrawal latency. Similarly, when these mouse groups were tested after 6 weeks of injection of neurotrophin, paw withdrawal latency was almost identical.

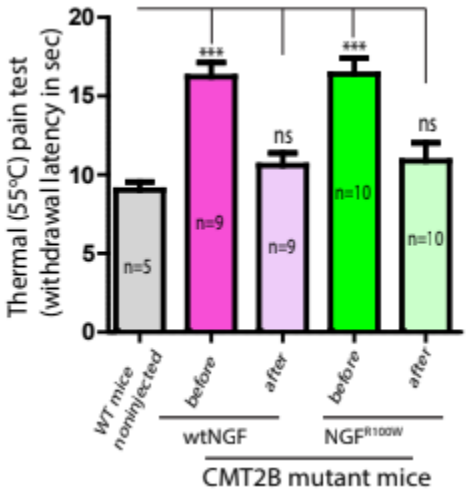

Figure 3. Injection of both NGF and NGF^{R100W} rescued the function of peripheral sensory nerve fibers in CMT2B mouse groups.
A thermal pain test was conducted to measure the paw withdrawal latency of mouse groups. The wild-type mouse groups which did not receive any injections was measured once to serve as a threshold. For CMT2B mutant mice groups, they were injected with either wildtype NGF or NGF^{R100W}. Then a thermal pain test was conducted before and after the 6-week long injection to compare difference in the paw withdrawal latency. T-test was performed for paw withdrawal latency each of the before and after of the CMT2B mouse against the paw withdrawal latency of the non-injected wildtype mouse.
(NS = Not significant, * = <0.0005, N= Number of mouse)**

Acknowledgements:

Chapter 3 contains unpublished material co-authored with Dr. Kijung Sung. Dr. Kijung Sung is the primary author of this chapter.

DISCUSSION

Chronic pain caused by nerve growth factors (NGFs) in both the central and peripheral nervous systems is a well-known side effect of NGFs (Latremoliere & Woolf, 2009). Such side effects have become one of the significant barriers to administering neurotrophin in patients as a possible treatment option (Latremoliere & Woolf, 2009). Our data also confirmed the side effect of NGFs observed in the previous studies as the mouse groups injected with the wild-type NGF elicited thermal pain and showed a significant decrease in thermal threshold compared to the baseline measured at 20 mins and 45 mins (Figure 1A & 1B). In this study, we examined whether a new type of mutant NGF (NGF^{R100W}) which elicits reduced pain compared to wildtype NGF could be used as an alternative. Our results demonstrated that injection of NGF^{R100W} did not elicit much pain in mice compared to when the mice were injected with wild-type NGF after 45 minutes compared to the baseline (Figure 1A). Even more, we were able to increase the doses of NGF^{R100W} up to 10 times compared to the wild-type NGF dose (Figure 1A). While the dose is still not high enough to be considered a potential treatment option, our data allows us to expect the utilization of this improved/modified neurotrophin as a therapeutic measure to treat neurodegenerative diseases in the future.

Our data also partially confirmed the previous hypothesis that NGF^{R100W} could be used to regenerate sensory neurons which could improve the conditions of peripheral neuropathy in individuals. As expected, the thermal threshold of mouse groups injected with wild-type NGF showed hypersensitization as their thermal threshold was significantly decreased compared to the baseline (Figure 1B). Hyperalgesia, increased sensitivity upon losing sensory neurons, is one of the early symptoms observed in people with neuropathies as a result of nerve damage (Theodosiou 1999 et al.,). The peripheral nerve injury due to loss of C-fiber terminals in the dorsal root ganglia

often leads to synaptic plasticity which evokes pain sensation observed during hot plate tests (Woolf 1983; Zhang et al., 2006). Our data showed that the decrease in thermal threshold from the baseline in the NGF^{R100W} treated group was significantly smaller compared to that in the wild-type NGF-treated group at 25 min and 45 min of treatments (Figure 1B). The use of NGF^{R100W} in mice groups did not disturb sensory perceptions nor elicited much pain in mice groups as opposed to when wild-type NGF was used (Figure 1A & 1B). Therefore, we could conclude that the use of NGF^{R100W} did not extensively cause hypersensitivity in peripheral sensory neurons of mice groups by preventing the accumulation of pain receptors that lead to greater pain perception.

However, we did see a trend of decreasing thermal thresholds for NGF^{R100W} when we increased the concentration from 0.5 μg to 1.0 μg and 5.0 μg (Figure 1B). This could be concerning as our experimental results showed increasing doses of NGF^{R100W} increased pain sensitization in mice groups. However, we were able to increase the amount of NGF^{R100W} injected by 10 times which still elicited much less pain in mouse groups compared to when injected with control. Therefore, if we were to continue our research and obtain similar results in future studies with higher doses by either driving more mutations in the current NGF^{R100W} or making modifications in the delivery system, the use of these modified NGF in clinical studies would soon be possible.

In addition, there was a need to test for the efficacy of the NGF^{R100W} in regenerating peripheral sensory neurons. The efficacy of the NGF^{R100W} in regenerating the peripheral nerves was further validated from the skin sample microscope pictures collected. Compared to the wildtype mouse, the Charcot-Marie-Tooth Type 2B (CMT2B) mouse suffered from nerve loss. This can be clearly seen in the degeneration of intraepidermal nerve fibers (IENF) (Figure 2A & 2B). The density of IENF is used as a biomarker to assess the progression of peripheral neuropathies, therefore, CMT2B mice are great candidates for studying peripheral neuropathy as

these mice exhibit sensory loss, and motor deficits analogous to the symptoms that patients with neurodegenerative diseases exhibit (Saveri et al., 2020). As expected, the tissue samples taken from the wildtype groups had a higher density of IENF compared to the tissue samples taken from the CMT2B mouse groups (Figure 2A & 2B). The low density of IENF of CMT2B mouse groups suggests the progression of neuropathy leading to a degeneration of neurons. However, when these CMT2B mice were injected with either wildtype NGF or NGF^{R100W}, we were able to witness the level of IENF to be restored as much as the wildtype mouse which did not suffer from loss of neurons (Figure 2A). Interestingly enough, the density of IENF was higher in groups injected with NGF^{R100W} which proved not only the efficacy of the neurotrophic factor in restoring neurons but also has better potential as therapeutics compared to the wildtype NGF. Therefore, we could possibly conclude that it serves similar purposes to the neurotrophic factor NGF allowing us to apply this NGF^{R100W} as future therapeutics.

Another important point that needs to be discussed would be the regeneration of neurons in unexpected sites (Figure 2A). For 6 weeks, we administered the injection intradermally only in the left hind paw. Therefore, when these tissue samples were taken both from the contralateral and ipsilateral paws, it was logical for us to expect to see the regeneration of peripheral nerves mostly in the ipsilateral paws. However, results showed that injecting in the same site still ended up regenerating the nerves in both paws. The IENF density was higher for the ipsilateral paws compared to the contralateral paws, but contralateral paws from the injected groups were indeed higher than the untreated groups (Figure 2B). Since the IENF density of contralateral paws was indeed higher than the IENF density of untreated groups, one might conclude that this might not be a problem as the IENF density of the ipsilateral paws was still higher than the control lateral paws. However, this could be critical as one does not know how the unexpected regeneration of

nerves at unwanted sites would affect the mechanisms of the immune system. For instance, the increased nerve at a specific site could lead to hypersensitivity providing unexpected pain for the patients. While there needs to be more research that has to be conducted to investigate the actual reason for how the injection at one site led to regeneration in the opposite, it was fortunate for us to witness such unexpected results at an early stage.

In addition to the paw withdrawal test conducted using wild-type mice injected with NGF and NGF^{R100W}, we performed additional studies using CMT2B mice as experimental groups for injection of both NGFs. Compared to the mouse groups that were only tested for paw withdrawal latency after injection, we also compared the paw withdrawal latency test before and after completing all the injections (Figure 3). The paw withdrawal latency was the shortest for the wild type. Similarly, the paw withdrawal latency was not significantly different for CMT2B mice after the administration of wild-type NGF and NGF^{R100W} (Figure 3). Moreover, compared to the pre-injection for both wildtype NGF and NGF^{R100W}, the withdrawal latency decreased significantly from 17 seconds to 12 seconds (Figure 3). The most significant finding was the difference in latency between before and after the injections. Therefore, CMT2B mice groups did show an increase in paw withdrawal latency after injection of NGF, but not a significant difference in latency indicating regeneration of neurons leading to restoration of sensory perception. This again proved the capability of NGF in contributing to peripheral nerve regeneration. It also highlighted the fact that NGF^{R100W} has similar clinical efficacy compared to wild-type NGF (Figure 3). Therefore, we concluded that NGF^{R100W} has the ability to not only regenerate neurons but also could serve as a better/alternative version of wildtype NGF without previously known side effects.

Our study showed that the use of NGF^{R100W} did not elicit pain. We plan to expand our study in different areas such as using NGF^{R100W} to reduce side effects induced by cisplatin which is a chemical used in chemotherapy. We have shown the potential of Swedish mutant NGF to be used as an alternative to the wild-type NGF, where it restored the sensory perception and regenerated the peripheral sensory neurons in CMT2B mouse groups. Therefore, reducing the effects of peripheral neuropathy during cancer treatment by using NGF^{R100W} could be promising as patients can continue receiving their treatment. Further research on the application of NGF^{R100W} will enhance the possibility of developing novel therapeutics for chemotherapy induced peripheral neuropathy.

Once the effect of NGF^{R100W} has been proved, future studies will be conducted where the cisplatin will be administered to the mouse models to mimic the side effects of chemotherapy drugs especially peripheral neuropathy accompanied by sensory neuron degeneration. Along with the cisplatin injections, we will conduct a set of behavioral tests to look at their motor and nociception deficits over 3 weeks. We will also address the effect of NGF^{R100W} in minimizing sensory neuron degeneration and observe the cellular anatomy of the skin fibers in the presence or absence of the NGF^{R100W}. With the results. I expect to look at improved measures of motor movement and restoration of the nociceptive threshold, with the ultimate goal of sensory neuron regeneration which will improve sensory propagation to a level of transgenic fare. Moreover, future research could improve the side effects observed in chemotherapy allowing the continuation of the treatment for cancer patients. Also, increased application of NGF^{R100W} will provide more options and possibly expect the neurotrophin as a potential therapeutic measure for neurodegenerative diseases.

Currently, we are in the final stage of administering cisplatin with nerve growth factors, as we have established the effect of cisplatin in inducing CIPN in mouse groups. We expect to see improvements not only in their sensory perception but also in the regeneration of their neurons once we have obtained their tissue samples. If our future results provide improved clinical outcomes for the mouse groups, we can expect to move onto the next stage of our project which would be testing for different doses of NGF as we cannot ever expect how the drug might act differently in human's bodies. Therefore, our aim would be to provide the same clinical results with reduced doses. We could possibly expect to expand our research subject into other areas as it has shown to be effective in peripheral neuropathy. There are more areas where neurotrophin can potentially be applied, therefore I believe further research would provide benefits not only to current patients but also prevent the onset of diseases.

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