# 論　文　要　旨

Role of JNK in late nerve regeneration monitored by *in vivo* imaging of thy1-YFP transgenic mice

（thy1-YFP トランスジェニックマウスを用いた *in vivo* イメージングによる後期神経再生における JNK の役割に関する研究）

関西医科大学医化学講座
（指導：伊藤　誠二 教授）

NGUYEN HUU TU

## Introduction

Peripheral nerve injury causes many molecular changes in injured neurons and their surrounding Schwann cells. These changes result in successful target reinnervation or apoptosis depending on the response of neurons and their supportive cells to the injury. For the neuron to respond adequately to the injury at its axon, injured information needs to be sent to the cell soma and neurons need permissive environment to regenerate. It means that the local injured signal transportation to the soma, i.e. axon-soma communication, and local environment are important for nerve regeneration. At the site of axon injury, there are many molecules get involved. Among them, mitogen-activated protein kinases (MAPKs) become phosphorylated and have a role in an early phase of nerve regeneration *in vitro*, but the role of MAPKs in nerve regeneration *in vivo* remains largely unclarified.

Neurotrophic factors are produced from Schwann cells and target organs such as muscles and skin distal to the cutting sciatic nerve. These factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glia cell line-derived neurotrophic factor (GDNF), are retrogradely transported and promote neurite extension of the injured nerves. However, which one(s) accelerate nerve regeneration *in vivo* is unclear.

In the present study, I attempted to elucidate the molecules involved in nerve regeneration by using the sciatic nerve transection-regeneration model which has been established in our department in thy1-yellow fluorescent protein (thy1-YFP) mice which express YFP in the nervous system.

## Methods

*1. Sciatic nerve transection-regeneration model and behavioral testing*

Male thy1-YFP transgenic and littermate wild-type mice of 8 to 10 week-old, weighing from 22 to 27 g were subjected to the sciatic nerve transection-regeneration model as recently reported (Unezaki et al., 2009, 2014). In brief, the right sciatic nerve was transected at the mid-thigh level and the two nerve stumps were sutured to the inner wall of a silicone tube, leaving a 5-mm gap and filling with physiological saline. A catheter tube was attached to an osmotic pump pre-filled with medication or phosphate-buffered saline (PBS). The drugs were also applied to the intrathecal space.

To check the functional recovery, we measured withdrawal responses to mechanical stimulus on the hind paw, using calibrated von Frey filaments starting from 0.4 g. Behavioral tests were conducted before and every week after axotomy for 8 weeks. The mechanical force of 4 g was used as a cut-off value of withdrawal thresholds. Behavioral testing result was done simultaneously with *in vivo* imaging analysis.

*2. Assessment of nerve regeneration*

The number of regenerated proper plantar digital nerves in their toes of thy1-YFP mice was counted under a fluorescent stereomicroscope before and every week after sciatic nerve axotomy.

Fluoro-ruby was applied distally or proximally to the silicone tube to mark peripheral neuron regeneration or survival, respectively. Fluoro-ruby-labeled dorsal root ganglion (DRG) neurons and motor neurons were counted.

*3. Culture of DRG neurons*

To examine the effect of neurotrophic factors on neurite extension and its involvement of JNK, cultured DRG neurons were prepared from L4, L5 and L6 DRGs. When the cells extended their neurites longer than 2 cell-body diameters, they were counted as neurons that bear neurites.

**Results:**

1. *Characterization of sciatic nerve transection-regeneration model.*

The right sciatic nerve of thy1-YFP mice was transected; and the proximal and distal nerve stumps were sutured to a silicone tube, leaving a 5-mm gap. YFP fluorescence was observed in the proximal side of the sciatic nerve at 1 week after the operation, and it reached to the distal side through the silicone tube at 4 weeks after the operation. To investigate neuronal survival and nerve regeneration after axotomy, we examined whether motor neurons in the spinal cord and sensory neurons in the DRG would be labeled by Fluoro-ruby, and found that considerable amounts of transected motor and sensory axons coordinately reached the distal side beyond the 5-mm gap at 3 weeks and that 20 - 30 % of sensory and motor neurons died at 8 weeks after axotomy. Some proper plantar digital nerves began to be weakly observed *in vivo* as early as 4

weeks after axotomy, and almost all digital nerves were observed in the foot at 6 weeks. There was a positive correlation between nerve regeneration *in vivo* and the improvement of functional recovery after axotomy.

*2. Role of JNK, but not ERK or p38, in nerve regeneration.*

After axons get injury, MAPK family members including ERK, p38 and JNK become phosphorylated and retrogradely transported to the cell body to trigger regeneration process. JNK inhibitors, but not ERK inhibitor or p38 inhibitor, delayed functional recovery to 1 week compared to control mice. The reinnervation of proper plantar digital nerves in the skin was significantly delayed by the JNK inhibitor, whereas that of the muscle was not affected by it.

The intensity of phosphorylated JNK (p-JNK) immunoreactivity increased first in Schwann cells and then in axons. Six hours after axotomy, p-JNK significantly increased in the proximal site of the transected sciatic nerve and transported to the soma. Expression of phosphorylated c-Jun, a down-stream molecule of pJNK, was significantly lower in L5 DRG treated with the JNK inhibitor than in the control DRG. The JNK inhibitor retained significantly higher expression of ATF3, a marker of injured neurons, over the entire experimental period of 8 weeks.

*3. Role of neurotrophic factors in nerve regeneration and its mediation by JNK*

Neurotrophic factors are released from the target organs and Schwann cells and considered to be transported to the lesion site of axotomy. Antibodies against NGF, GDNF, and BDNF applied to the transection site delayed the functional recovery in this order of potency. These neurotrophic factors enhanced neurite outgrowth from cultured DRG neurons, and the JNK inhibitor reversed their stimulatory effects.

**Discussion**

Although the first description of causalgia in 1864, there has been no definitive treatment for this intractable burning pain until recently. Dr. Y. Inada (part-time lecturer of Kansai Medical University) first succeeded in the treatment by *in situ* tissue engineering with a collagen tube in 2005. To elucidate the mechanisms of nerve regeneration, our laboratory established the sciatic nerve transection-regeneration model.

I characterized the model in thy1-YFP mice and obtained the following findings.

1. While it took 4 weeks for regenerated axons to pass though the 5-mm gap of silicone chamber, it took only another 2 weeks to reach fingers of the toe that are 40 mm in length from the distal site. It clearly demonstrated important roles of the environment distal to the sciatic nerve cut in nerve regeneration. The distal environment such as basal lamina formed by Schwann cells may provide nutrients such as lactate and neurotrophic factors for nerve extension in the late phase of nerve regeneration.

2. I investigated reinnervation of sensory and motor neurons to the skin and muscle simultaneously in the same mice for a long period and demonstrated the difference in the involvement of JNK in nerve regeneration between sensory and motor neurons.

3. The interaction of JNK and neurotrophic factors (NGF, GDNF, BDNF) suggested that JNK is involved in the late phase of nerve regeneration though pathways triggered by neurotrophic factors.

In conclusion, the transection-regeneration model allowed me to non-invasively monitor the functional recovery and peripheral nerve reinnervation to the plantar surface of thy1-YFP mice over an 8-week period. The drug delivery system is useful for elucidating molecules truly involved in peripheral nerve regeneration *in vivo*. The limit of 4-week administration by a mini-osmotic pump will be overcome by refilling drugs in it. Finally, its further use will provide additional basic knowledge for potential strategies to promote axonal regeneration and reinnervation to target organs in the clinical setting.

【はじめに】

損傷した軸索の再生には、損傷の情報が細胞体に送られる必要がある。MAP キナーゼファミリーがリン酸化され、神経再生の初期に関与することが *in vitro* で報告されているが、*in vivo* での役割についてはよくわかっていない。

損傷した神経の標的臓器から遊離される神経成長因子(NGF)、脳由来神経栄養因子（BDNF）やグリア細胞株由来神経栄養因子（GDNF）などの神経栄養因子が細胞体に逆行性に輸送され、軸索伸長を促進する。しかしどの神経栄養因子が *in vivo* で神経再生に関与するかは明らかになっていない。

本研究は、神経系に特異的に蛍光タンパク（YFP）を発現している thy1-YFP マウスに当講座で確立した坐骨神経切断―再生モデルを適用して、神経再生に関与する分子を明らかにすることを目的とした。

【研究方法】

8-10 週齢の雄 thy1-YFP マウスの右の坐骨神経を切断し、その切断端を 5 mm の間隔をあけてシリコンチューブに結紮し、坐骨神経切断―再生モデルを作製した。薬剤は 4 週間持続注入できるミニ浸透圧ポンプに入れ、シリコンチューブあるいは脊髄髄腔に投与した。

手術前と手術後 1 週ごとに、von Frey フィラメントに対する逃避行動の閾値、thy1-YFP マウスの固有底足趾神経の数、逆行性蛍光マーカーFluro-ruby で標識される後根神経節（DRG）の感覚ニューロンと脊髄前角の運動ニューロンの数を測定した。

神経栄養因子の神経突起伸長に対する効果とその伸長への JNK の関与を検討するために、第 4 から第 6 腰髄の後根神経節からニューロンの初代培養を行った。神経突起が細胞体の 2 倍以上あるものを神経突起があるニューロンとした。

【結果】

モデル作製後 4 週で YFP の蛍光はシリコンチューブを通って遠位端に到達し、術後 6 週で筋肉や皮膚の標的組織に到達した。Fluoro-ruby を遠位端に投与すると、3 週間でかなりのニューロンが感覚ニューロンと運動ニューロンが標識された。これらの形態的な神経再生の結果は行動による機能的回復とよく相関していた。

MAP キナーゼファミリーには ERK、JNK、p38 があり、JNK 阻害薬は機能的回復を 1 週間遅延させたが、ERK あるいは p38 の阻害剤は影響しなかった。JNK 阻害薬で感覚神経の再生も有意に遅れたが、運動神経の再生には影響しなかった。リン酸化 JNK (p-JNK) のシグナルは、6 時間後に坐骨神経の切断の近位端で上昇が見られた。神経切断により、DRG ニューロンの c-Jun がリン酸化されたが、JNK 阻害剤によりそのリン酸化が阻害された。一方、JNK 阻害剤処置により、神経損傷マーカーである ATF3 の発現は 8 週間にわたり増加していた。

NGF、GDNF、BDNF の抗体で神経再生の遅延の効果がこの順番でみられた。これらの神経栄養因子は初代培養 DRG 細胞の神経突起伸長を促進したが、その促進作用は JNK 阻害剤で抑制された。

**【考察】**

1864 年にカウザルギー（複合性局所疼痛症候群）が最初に記載されて以来、この難治性疼痛の治療はできないと考えられてきたが、2005 年に関西医大非常勤講師の稲田有史博士によりコラーゲンチューブを用いた再生医療で治療できることが報告された。この末梢神経再生の機構を明らかにするために、当講座は坐骨神経切断再生モデルを確立した。

本研究では、坐骨神経切断―再生モデルが非浸襲的に機能的回復と末梢神経の足底への組織学的回復が thy1-YFP マウスを用いることにより 8 週間の長期にわたって追跡できること、機能的回復と組織学的回復がよく一致していることを明らかにした。薬剤デリバリー装置は *in vivo* で神経再生に関与する分子の解明に有用である。ミニ浸透圧ポンプは 4 週間持続注入加納であるが、ポンプを取り替えることにより、さらに長期間薬剤を投与することが可能となる。このモデルは軸索の再生と臨床応用に向けた標的臓器への再投射を促進する戦略のために基礎的知識を提供する有用な手段となることが期待できる。