EFFECTS OF CALCITONIN GENE-RELATED PEPTIDE ON IRON METABOLISM, IL-6, AND MYELIN PROTEIN ZERO (MYELIN P0) EXPRESSION IN SCHWANN CELLS

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การศึกษาผลของแคลซิโตนินยีนรีเรทเตทเปปไทด์ต่อเมตาบอลิซึมของธาตุเหล็ก IL-6 และการแสดงออกของไมอีลินโปรตีน P0 ในเซลล์ชวานน์

นางสาวหทัยชนก เบญจรงค์รัตน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	EFFECTS OF CALCITONIN GENE-RELATED PEPTIDE ON IRON METABOLISM, IL-6, AND MYELIN PROTEIN ZERO (MYELIN P0) EXPRESSION IN SCHWANN CELLS
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หทัยชนก เบญจรงค์รัตน์ : การศึกษาผลของแคลซิโตนินยีนรีเรทเตทเปปไทด์ต่อเมตาบอลิซึมของธาตุเหล็ก IL-6 และการแสดงออกของไมอีลินโปรตีน P0 ในเซลล์ชวานน์. (EFFECTS OF CALCITONIN GENE-RELATED PEPTIDE ON IRON METABOLISM, IL-6, AND MYELIN PROTEIN ZERO (MYELIN P0) EXPRESSION IN SCHWANN CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.พูลลาภ ชีพสุนทร, 66 หน้า.

แคลซิโตนินยีนรีเรทเตตเปปไทด์ (CGRP) เป็นนิวโรเปปไทด์ที่เพิ่มขึ้นในปมประสาทไทรเจมินัล (trigeminal ganglion; TG) มีความสัมพันธ์กับอาการปวดศรีษะไมเกรนและอาการปวดบริเวณปากและใบหน้า CGRP ออกฤทธิ์โดยการจับกับ CGRP receptor บนเซลล์ประสาทและเซลล์ค้ำจุน งานวิจัยส่วนใหญ่ที่ตีพิมพ์เกี่ยวกับการปรับเปลี่ยนอาการปวดโดยเซลล์ค้ำจุนใน TG ได้มุ่งความสนใจไปที่ satellite cells เนื่องจากความสัมพันธ์ที่ใกล้ชิดระหว่าง satellite cells กับตัวเซลล์ประสาทรับความรู้สึกใน TG ในทางตรงกันข้าม Schwann cells ซึ่งเป็นเซลล์ค้ำจุนอีกชนิดหนึ่งที่ถูกมองว่าทำหน้าที่แค่สร้าง myelin sheath หุ้มรอบเส้น ประสาท แต่จากหลักฐานที่มีอยู่แสดงถึงความเป็นไปได้ที่ Schwann cells จะมีส่วนเกี่ยวข้องกับการปรับเปลี่ยนอาการปวด ดังนั้นการศึกษาครั้งนี้จึงมีวัตถุประสงค์ที่จะศึกษาการตอบสนองของ Schwann cells ต่อการกระตุ้นด้วย CGRP และศึกษากลไกในระดับเซลล์ของการตอบสนองดังกล่าว โมเลกุลเป้าหมายที่ใช้ศึกษาได้แก่ โปรตีน P0 และ IL-6 โดย P0 ถูกใช้เป็นตัวชี้วัดภาวะการถูกกระตุ้นของ Schwann cells การเปลี่ยนแปลงของ IL-6 ถูกใช้เพื่อบ่งชี้ว่าการหลั่ง IL-6 เป็นส่วนหนึ่งของการตอบสนองต่อการอักเสบของ Schwann cells ต่อการถูกกระตุ้นด้วย CGRP หรือไม่ และเนื่องจากธาตุเหล็กมีความสำคัญโดยทำหน้าที่เป็น cofactor ในหลายกระบวนการทำงานพื้นฐานของเซลล์ ได้แก่ การสร้าง ATP การสร้าง myelin sheath และยังเกี่ยวข้องกับการกระตุ้น NF-**K**B ของในการควบคุมการแสดงออกของยีนหลายชนิดในการตอบสนองต่อการอักเสบใน Schwann cells ดังนั้นการศึกษาในครั้งนี้จึงมีวัตถุประสงค์ ในการตรวจสอบความสัมพันธ์ระหว่างเมตาบอลิซึมของธาตุเหล็กกับการตอบสนองของ Schwann cells ต่อการกระตุ้นด้วย CGRP โดย transferrin receptor (TfR) ถูกใช้เป็นตัวชี้วัดระดับธาตุเหล็กในเซลล์ที่ถูกกระตุ้นด้วย CGRP ผลการศึกษาพบว่า (๑) CGRP กระตุ้นการแสดงออก P0 ใน Schwann cells และยังชี้แนะว่า P0 สามารถใช้เป็นตัวชี้วัดที่จำเพาะต่อภาวะการถูกกระตุ้นของ Schwann cells ต่อ CGRP อีกด้วย (๒) การแสดงออกของ P0 ใน Schwann cells ที่ถูกกระตุ้นด้วย CGRP ถูกควบคุมด้วย transcription factor Krox-20 (๓) CGRP สามารถกระตุ้น Schwann cells ให้หลั่ง IL-6 และการแสดงออกของ IL-6 receptor และยังชี้แนะว่า IL-6 ที่หลั่งออกมานอกจากจะมีผลไปกระตุ้นเซลล์อื่น (paracrine) แล้วและยังสามารถมีผลกระตุ้นตัว Schwann cells เอง (autocrine) (๔) CGRP กระตุ้น Schwann cells ให้เกิดการส่งสัญญาณภายในเซลล์ผ่านทาง NF-**K**B และ (๕) CGRP กระตุ้นให้เกิดการเปลี่ยนแปลงเมตาบอลิซึมธาตุเหล็กใน Schwann cells และยังแสดงให้เห็นว่า Schwann cells มีกลไกในการรักษาสมดุลของธาตุเหล็กภายในเซลล์อีกด้วย กล่าวโดยสรุป การศึกษาครั้งนี้ได้บรรยายถึงองค์ความรู้ใหม่เกี่ยวข้องของชีววิทยาของ Schwann cells และบทบาทของธาตุเหล็กและ IL-6 ในการเกิด neurogenic pain

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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HATHAICHANOK BENJARONGRAT: EFFECTS OF CALCITONIN GENE-RELATED PEPTIDE ON IRON METABOLISM, IL-6, AND MYELIN PROTEIN ZERO (MYELIN P0) EXPRESSION IN SCHWANN CELLS. ADVISOR: ASSOC. PROF. POONLARP CHEEPSUNTHORN, Ph.D., 66 pp.

Calcitonin gene related peptide (CGRP), a neuropeptide up-regulated in the trigeminal ganglion (TG), has been associated with migraine pain and other orofacial pain symptoms. CGRP exerts its biological effects by binding to the CGRP receptors expressed on neurons and glial cells. Most of the recently published articles on pain modulation in the TG by glial cells have been focused on the satellite cells. This is due primarily to the intimate contact relationships between the satellite cells and the cell bodies of the primary sensory neurons in the TG. Schwann cells, on the other hand, are thought to play a passive role by producing myelin sheaths wrapping around axons. Emerging evidence suggests that Schwann cells may be participated in pain modulation. Thus, the present study aimed to elucidate the involvement of Schwann cells in responses to CGRP and dissect cellular mechanisms underlying such responses. Key molecular targets to be monitored in this study include P0 and IL-6. P0 was used as an activation marker of Schwann cells. Changes in IL-6 expression were monitored to determine whether Schwann cells could release IL-6 as part of their inflammatory response to CGRP. Since iron is an important cofactor required for a number of basic cellular functions, including ATP production and myelin formation and involved in the activation of NF-**K**B, which plays an essential role in the regulation of genes involved in inflammatory responses in Schwann cells. Therefore, this study also aimed to determine the relationship between iron metabolism and response of Schwann cells to CGRP. The TfR were used to monitor intracellular iron levels following the stimulation of CGRP. Results show (1) CGRP stimulates P0 expression in Schwann cells and further suggest that P0 can be used as a specific marker for CGRP-induced Schwann cell activation, (2) CGRP-upregulated P0 expression in Schwann cells is mediated by the transcription factor Krox-20, (3) CGRP induces IL-6 production and IL-6R expression in Schwann cells and suggest that IL-6 released from Schwann cells should have both a paracrine and an autocrine effects, (4) CGRP-stimulated Schwann cell activation is mediated by NF-**K**B and (5) CGRP stimulates changes in iron metabolism in Schwann cells and Schwann cells possess a regulatory mechanism to maintain cellular iron homeostasis. In summary, the present study provides new evidence involving Schwann cell biology and the essential role of iron and IL-6 in neurogenic pain.

Field of Study: Medical Science Student's Signature .....

Academic Year: 2013 Advisor's Signature .....

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LIST OF ABBREVIATIONS

CGRP	Calcitonin-gene related peptide
GPCR	G-protein coupling receptor
TG	Trigeminal ganglion
TfR	Transferrin receptor
CT	Calcitonin
AMY	Amylin
AM	Adrenomedullin
PBN	Parabrachial nucleus
CeA	Central nucleus of amygda
VGCCs	Voltage gated calcium channels
TNC	Trigeminal nucleus caudalis
RAMP-1	Receptor-activity modifying protein
CLR	Calcitonin-like receptor
RCP	Receptor component protein
ICLs	Intracellular loops
ECLs	Extracellular loops
PKA	Protein kinase A
JNK	Jun N-terminal kinase
PKC	Protein kinase C
PLCβ	Phospholipase C beta
CREB	cAMP response element binding protein
NF-**K**B	The nuclear factor kappa B
NGF	Nerve growth factor
BDNF	Brain-derived nerve growth factor
CNTF	Ciliary neurotrophic factor
TNF-α	Tumor necrosis factor alpha
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
TG	Trigeminal ganglion
TMJ	Temporomandibular joint
PGE2	Prostaglandin
COX-2	Cyclooxygenase-2
SCPs	Schwann cell precursors
MDL	Major dense line
IPL	Intraperiod line

P0	Myelin protein zero
PMP22	Peripheral myelin protein
MBP	Myelin basic protein
P2	Protein 2
p75NTR	The neurotrophin receptor p75
GFAP	Glial acidic fibrillary protein
NCAM	Neural cell adhesion molecule
TLRs	Toll-like receptors
MAPK	Mitogen-activated protein kinase
ERK	Extracellular signal-regulated kinases
PI3K	phosphoinositide 3-kinase
JAK	Janus kinase
STAT	Signal Transducer and Activator of Transcription
IKK	IκB kinase
NIK	**NF-κB** inducing kinase
IRE	Iron responsive element
IRP	Iron responsive protein
UTR	Untranslated region
apo-Tf	Apo-transferin
holo-Tf	Holo-transferrin
LPS	Lipopolysaccharide
FAC	Ferric ammonium citrate
DFO	Deferoxamine

CHAPTER I

INTRODUCTION

1.1 Background and rational

Calcitonin-gene related peptide (CGRP) is a neuropeptide that has been reported to be up-regulated in the trigeminal ganglion (TG) and associated with migraine pain and other orofacial pain symptoms. CGRP exerts its biological effects by binding to the CGRP receptors expressed on neurons and non-neuronal cells. There are two types of non-neuronal cells in the TG: the satellite and the Schwann cells. Both cell types express CGRP receptors. Most of the recently published articles on pain modulation in the TG by non-neuronal cells have been focused on the satellite cells. This is due not only to the intimate contact relationships between the satellite cells and the cell bodies of the primary sensory neurons in the TG, but also to the fact that the satellite cells can promote neuronal sensitization by releasing several pro-inflammatory mediators.

Schwann cells, on the other hand, are thought to play a passive, supporting role by producing myelin sheaths wrapping around axons. Myelin composes of lipids and myelin-associated proteins serving as an axon insulator. The myelin protein zero (P0) accounts for more than half of myelin proteins and, therefore, serve as a specific marker for the identification of myelinating Schwann cells. In pathological scenarios, Schwann cells are activated and release neuroactive substances and pro-inflammatory cytokines including TNF-α and interleukin 1β (IL-1β). These cytokines are known to enhance pain sensitivity. Thus, emerging evidence suggests that Schwann cells may be participated in pain modulation.

Iron is an important cofactor required for a number of basic cellular functions, including ATP production and lipid synthesis. This is also true for Schwann cells, which require iron for myelin formation. Iron is also involved in the activation of the transcription factor of NF-**K**B, which plays an essential role in the regulation of genes involved in inflammatory responses in Schwann cells.

Taken together, the present study aimed to elucidate the involvement of Schwann cells in responses to CGRP and dissect cellular mechanisms underlying such responses. A rat Schwann cell line (RT4-D6P2T) was selected as an alternative *in vitro* model of primary rat Schwann cells. Key molecular targets to be monitored in this study include P0, the transferrin receptor (TfR) and IL-6. P0 was used as an activation marker of Schwann cells. Changes in mRNA expression levels of the TfR were used to

monitor intracellular iron levels following the stimulation of CGRP. IL-6 production was monitored to determine whether Schwann cells could release IL-6 as part of their inflammatory response to CGRP.

1.2 Research question

1.2.1. Does CGRP alter iron metabolism in Schwann cells?

1.2.2. Does CGRP stimulate IL-6 release from Schwann cells?

1.3 Objectives

1.3.1. To determine the effect of CGRP on P0 gene expression in Schwann cells

1.3.2. To determine the effect of CGRP on the release of IL-6 from Schwann cells

1.3.3. To determine the effect of CGRP on TfR gene expression in Schwann cells

1.3.4. To determine the relationship between iron metabolism and response of Schwann cells to CGRP

1.4 Hypothesis

1.4.1. Exposure to CGRP alters iron metabolism in Schwann cells, as evidenced by changes in gene expression of P0 and TfR.

1.4.2. Exposure to CGRP stimulates IL-6 release from Schwann cells.

1.5 Keywords

CGRP, Schwann cells, Myelin protein zero, TfR, IL-6

1.6 Benefits of study

The present study provides new evidence involving Schwann cell biology and the essential role of iron and IL-6 in neurogenic pain.

CHAPTER II

LITERATURE REVIEW

2.1 Calcitonin gene-related protein

Calcitonin gene-related peptide (CGRP) belongs to the calcitonin family of peptides including calcitonin (CT), amylin (AMY), and adrenomedullin (AM). CGRP, CT, and AM are encoded by genes on chromosome 11 (Fig. 1). AMY is the only member that generated from gene located on chromosome 12, which is thought to be an evolutionary duplication of chromosome 11. CGRP have been identified in two isoforms; αCGRP and βCGRP, which encoded by different gene in different cell types. α-CGRP and CT are both encoded by *CALCA* gene and β CGRP encoded from *CALCB* gene on the same chromosome (1) . α CGRP is primarily expressed by sensory neurons, while βCGRP is found mostly in enteric neurons and motor neurons (2-5)

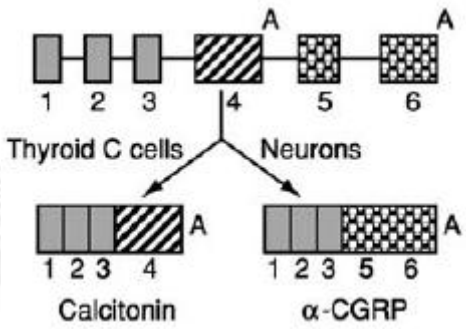

Figure 1 CT and αCGRP splice site.

Generation of αCGRP mRNA involved an removing of exon 4 and uses a downstream polyadenylation site after exon 6 to generate an αCGRP mRNA containing exons 1, 2, 3, 5, and 6 (6)

Calcitonin family considerably shares a similarity in their structures (1). The common structural elements of CGRP family including 6 or 7 amino acid ring structures linked by disulfide bridges between conserved cysteine residues and amidated carboxyl-termini (Fig. 2). Calcitonin family members expressed in number of

cell types in peripheral and central tissues with various biological effects (7). Due to the similarity in genetic distribution and structures, calcitonin family considerably has some overlapping biological activity. AMY, AM, αCGRP, and βCGRP all share a vasodilation effects by triggering the vascular smooth muscle relaxation. αCGRP and βCGRP are the most potent vasodilator and the pancreatic hormone. AMY has a minimal vasodilation effect (2). αCGRP has a vasodilation effects 10 times more than AMY and 17 fold more than AM (8, 9).

Both αCGRP and βCGRP have an overlapping biological activities in pain perception and vasodilation, but βCGRP has shown to be less potent than αCGRP (10, 11). βCGRP considerably has less potent in colon smooth muscle relaxation than αCGRP 2.6 times (2). βCGRP has reported the involvement in skin chronic pain conditions, since βCGRP is predominantly expressed in mouse and human keratinocytes (12). αCGRP has impiclated in the development of pathological pain in multiple neurologic conditions such as spinal cord injury and peripheral neuropathy. the upregulation of αCGRP at the injury site initiates axonal regeneration neuronal plasticity along with the development of neuropathic pain (13-16). The released αCGRP from perivascular neurons modulate local blood flow and increase vascular permeability, which further mediate local sterile inflammatory response termed, neurogenic inflammation, which strongly implicated in pathogenesis of migraine pain (17, 18). Thus, evidences have emphasized the important role of αCGRP over βCGRP in pain processing in the nervous system.

2.1.1 Cellular distribution of CGRP and its receptor

αCGRP widely expressed throughout the nociceptive pathway. Small neurons in the dorsal root ganglia (DRG), trigeminal ganglia and vagal ganglia, laminar I of dorsal horn, trigeminal nucleus caudalis (TNC), nucleus of the solitary tract, brainstem, amygdala, hypothalamus, parabrachial nuclear complex and thalamic nuclei can synthesize αCGRP (Fig. 3) (14-16, 19, 20). CGRP plays important roles in neuronal sensitization and pain signal transmission. Recently, it has been reported that plasticity at the CGRP synapse between the lateral Parabrachial nucleus (PBN)

and the central nucleus of amygda (CeA) is associated with central sensitization and pain-related behavior in models of arthritic, visceral, and neuropathic pain (16).

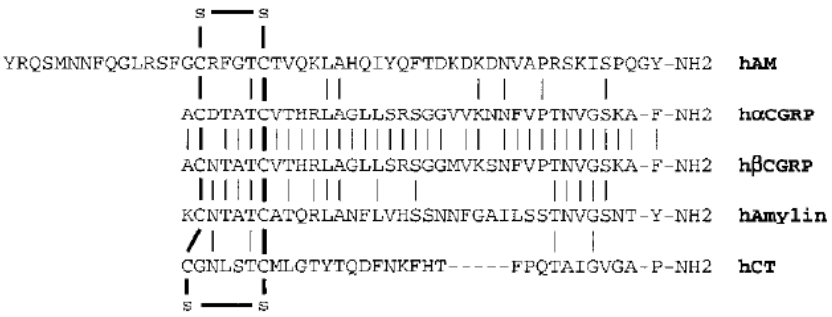

Figure 2 Common structural element of CGRP family

The disulfide bonds between the conserved cysteine residues shared the homology amino acid sequence of the CGRP family peptides (21).

2.1.2 Clinical correlation of CGRP in migraine pain

Elevated CGRP levels in trigeminal pathway relevance to migraine pathophysiology (22). High levels of CGRP in External jugular vein and saliva have been measured during migraine attack (23-25). The significance of CGRP in migraine has been demonstrated by induction of migraine-like headaches following CGRP injection and the reduction of CGRP in blood level using CGRP receptor antagonist olcegepant (BIBN 4096 BS) and telcagepant (MK-0974). Both are drugs that are successfully used for migraine therapy. Moreover, a serotonin type 1 (5-HT1) receptor agonist sumatriptan, which commonly used in the treatment of migraine, has been reported to selectively return CGRP to normal serum levels and alleviate the headache (24, 26-28).

The pathogenesis of migraine headache is not fully understood. The widely accepted migraine vascular theory considers that migraine is caused by a noxious stimulation of trigeminal nociceptive neurons around cerebral arteries, blood vessels

of the dura mater and cranial structures leading to the production of headache-like sensations (3, 29). In animal experiments, the noxious electrical, heat and chemical stimulation such as bradykinins, histamine, serotonin, capsicin or KCl on dura mater stimulate CGRP release and trigeminal nociceptive neuron sensitization (30, 31). Following high neuronal excitation through the high activity of Ca^{2+} channels, K^{+} channels, voltage gated calcium channels (VGCCs), $P2X_3$ receptor, NMDA and AMPAR and the excessive accumulation of active molecules such K^{+}, Ca^{2+}, glutamate, nitric oxide and capsicin, CGRP is found to be released along trigeminal pathway; at either peripheral site, trigeminal ganglion or central site; trigeminal nucleus caudalis, thalamus and amygdala (32-36).

CGRP triggers an intense sterile neurogenic inflammation at peripheral site characterized by edema formation, vasodilation, mast cell degranulation (37). CGRP promotes new protein synthesis including pro-inflammatory cytokines and receptors for pain transmission that further develops peripheral sensitization and reduces the activation threshold of those peripheral nociceptive neurons. The release of CGRP in trigeminal ganglion evokes a cross-activation of neuron-neuron and neuron-glia facilitates inflammation within the TG and prolonged nociceptive pain transmission to central (Fig 4.) (38). CGRP also acts as a neuromodulator at second-order nociceptive neurons in the TNC and at third-order neurons in thalamus including periaqueductal gray and amygdala, where it can induce central sensitization by increase excitatory postsynaptic currents via NMDA receptor (19, 20, 38).

Since peripheral sensitization may well drive central wind-up processes, recent accumulated studies have been focused on the activation mechanism within the first-order neuron of trigeminal pathway, including infllammation within sensory ganglion and glial activation. Recent studies suggest an important role of glial cells in various pain conditions in neuronal activity modulation (39).

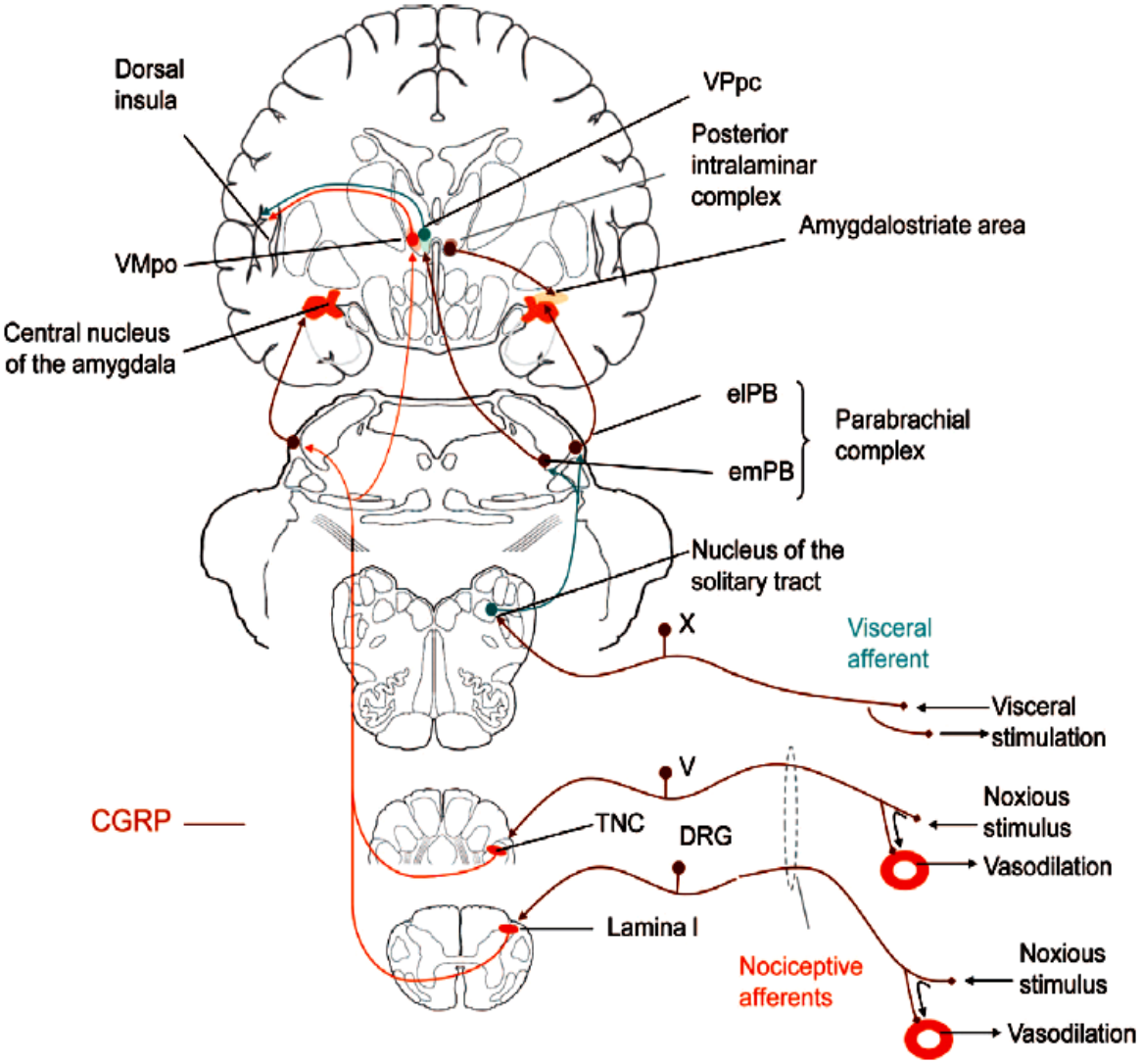

Figure 3 CGRP expression in the sensory pathway

Dorsal root ganglion; DRG, trigeminal (V) and vagal (X) ganglion. Trigeminal nucleus caudalis; TNC, Parabrachial nuclear complex ; the external lateral (elPB) and external medial (emPB) groups. Central nucleus of amygdala; CeA, Ventroposterior parvocellular nucleus of the thalamus; VPpc, Ventral medial posterior nucleus; Vmpo, (16).

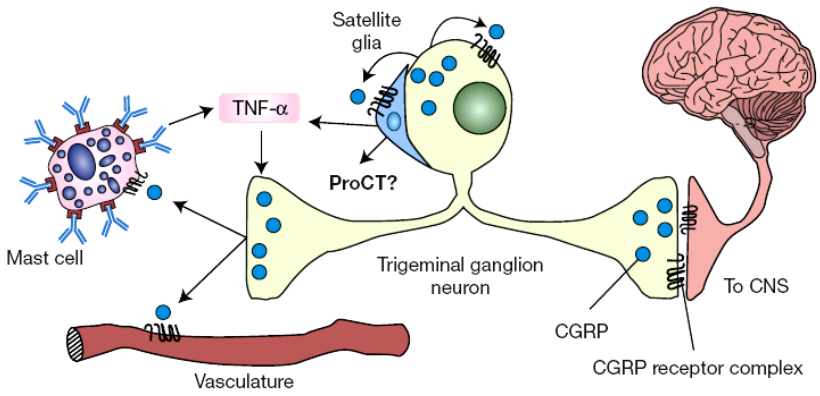

Figure 4 CGRP in pathophysiology of migraine (38)

2.2 CGRP receptor complex

CGRP signals through its CGRP receptor on neuron and non-neuronal cells throughout nociceptive pathway. CGRP exerts its biological activity through CGRP1 receptor. CGRP1 receptor consists of three components: RAMP-1; receptor-activity modifying protein, CLR; calcitonin-like receptor, and RCP; receptor component protein (Fig. 5) (40). RAMP-1 interacts with CLR providing a binding site for CGRP and activates the intracellular signaling through RCP. The structural motifs and amino acid residues in both CLR and RAMP1 are responsible for G-protein coupling receptor (GPCR).

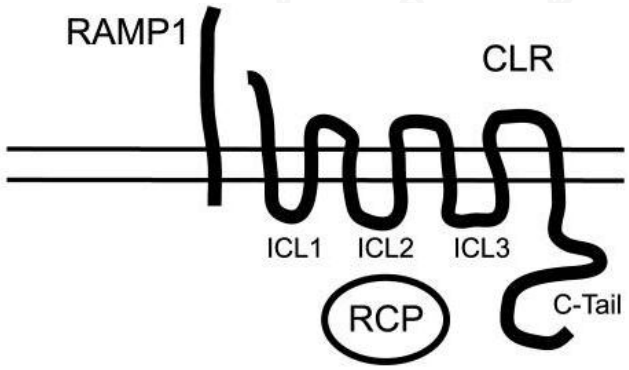

Figure 5 The CGRP receptor complex (40).

RAMP-1 is a single transmembrane protein that plays an important role in CGRP binding and receptor trafficking. It contains a single transmembrane domain α–helix, an extracellular *N*-terminus and a short intracellular *C*-terminus. The *N*-terminus was reported to stimulate a differential glycosylation of CLR at endoplasmic reticulum due to CLR translocation to the cell surface. The short C-terminus has none significant effect on signal transduction when interacts with CLR but potentially contains recognition sequence for intracellular retention in the absence of CLR (41, 42).

CLR is a seven transmembrane protein that belongs to the B family of GPCRs known as secretin-receptor family for classical hormone receptor (43). It contains three intracellular loops (ICLs) that are important for G protein interactions. ICL2 plays a prominent role in for signal transduction and cell surface expression (44, 45). The extracellular N-terminus and the extracellular loops (ECLs) are important for CGRP binding and RAMP1 interaction, while the intracellular C-terminus of CLR is required for cell surface expression, receptor activation and internalisation (46-48). The intracellular protein, RCP, is the intracellular protein that binds directly to the CLR/RAMP1 complex at the ICL2 of CLR and activates the cellular signaling pathway. RCP is capable of coupling several subtypes of G-proteins such as $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, $G\beta_\gamma$ that conduct different intracellular signal transductions such as Protein kinase A (PKA), Jun N-terminal kinase (JNK), Protein kinase C (PKC), Phospholipase C beta (PLCβ) (56, 57). Knockdown of RCP expression resulted in decrease of CGRP-mediaed cAMP production. The direct interaction of RCP and CLR suggested that RCP is required for CLR activation (40, 49, 50).

2.3 CGRP receptor signal transductions

To understand CGRP receptor activation following ligand binding, it is essential to appreciate the structure and function of the receptor. The *N*-terminal residues between 2 and 7 disulfide-bonded loop of the 37-amino-acid CGRP ligand has found to be essential for receptor activation, and the removal of the first seven amino acids deletes the sulfide ring to gives an antagonist, $CGRP_{8-37}$ (51, 52). The *C*-terminal phenylalanamide residues 8–18 of CGRP show a strong tendency to form an

α-helix, which are important for ligand binding with the CLR-RAMP1 heterodimer (53). During challenge, *C*-terminal of the CGRP first binds with high affinity to the extracellular N-terminal of both CLR and RAMP1 (Fig. 6B, centre) (54), and the *N*-terminus of ligand interacts with the TM helices and ECLs of CLR (Fig. 6A) (48, 55). RAMP-1 is able to regulate the glycosylation and pharmacology of CLR by induced the conformation changes on its N-terminal (Fig. 6B, left and right) (54). RCP is recruited to the ICL2 of CLR and interacts with G-protein for further intracellular signaling.

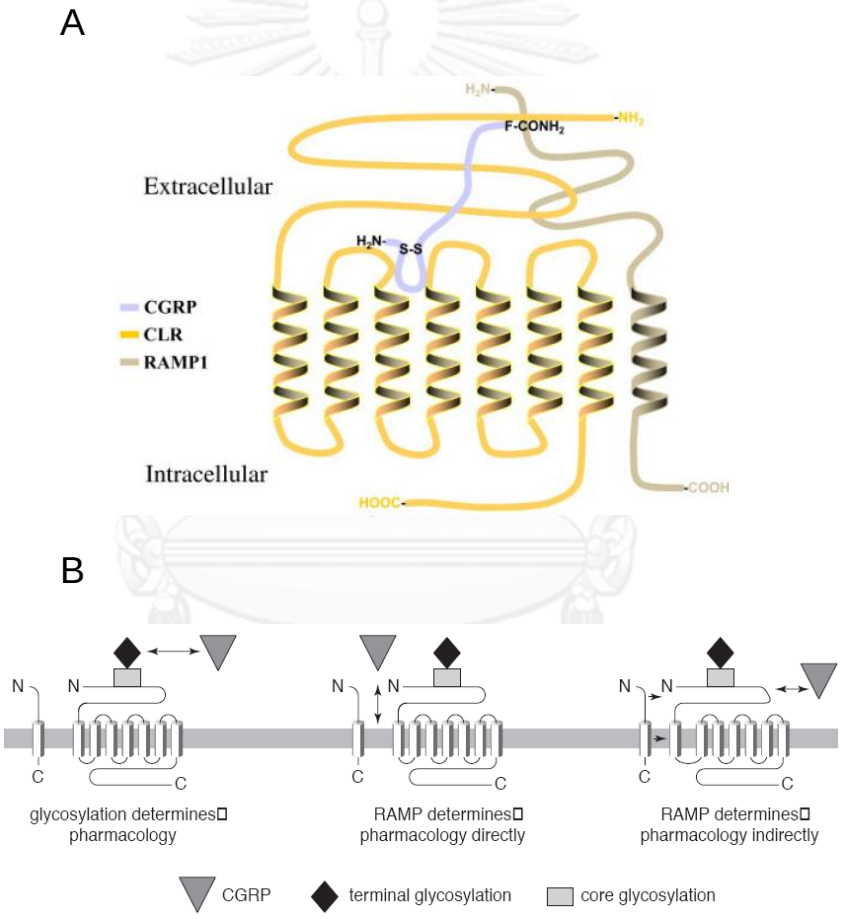

Figure 6 (A) The characteristic of CGRP binding site. (B) Receptor activity modifying proteins (RAMPs) regulate the expression of calcitonin receptor-like receptor (CLR) at the cell surface, its glycosylation, and its pharmacology (48, 54)

Activation of CGRP receptor initiates various intracellular signaling pathways depends on the various subtypes of the coupled G-protein (56, 57). The best understood CGRP-mediated signaling transduction in controlling neuronal function is PKA and PKC. Interaction of $G\alpha_s$ activates cAMP production, protein kinase A activation and cAMP response element binding protein (CREB), which resulted in regulation of various downstream signaling components including K^+ channel, L-type Ca^{2+} channel, NMDA receptor, and $P2X_3$ receptor expression. Interaction of $G\alpha_{q/11}$ activates several signaling downstream including PLCβ, PKC, increases Ca^{2+} transient, IP_3 accumulation and results in various inflammatory molecules production such as NO and pro-inflammatory cytokines TNF-α, IL-1β, IL-6 (50). Furthermore, it has been reported the cross activation of PKA and PKC signaling through NF-κB pathway in inflammatory cytokines production (58-60). Activation of CGRP receptor sensitizes effector proteins, ligands and receptors resulted in long-term modulation of gene expression and neuronal transduction that has been implicated in pain plasticity mechanism (Fig. 7) (16, 61).

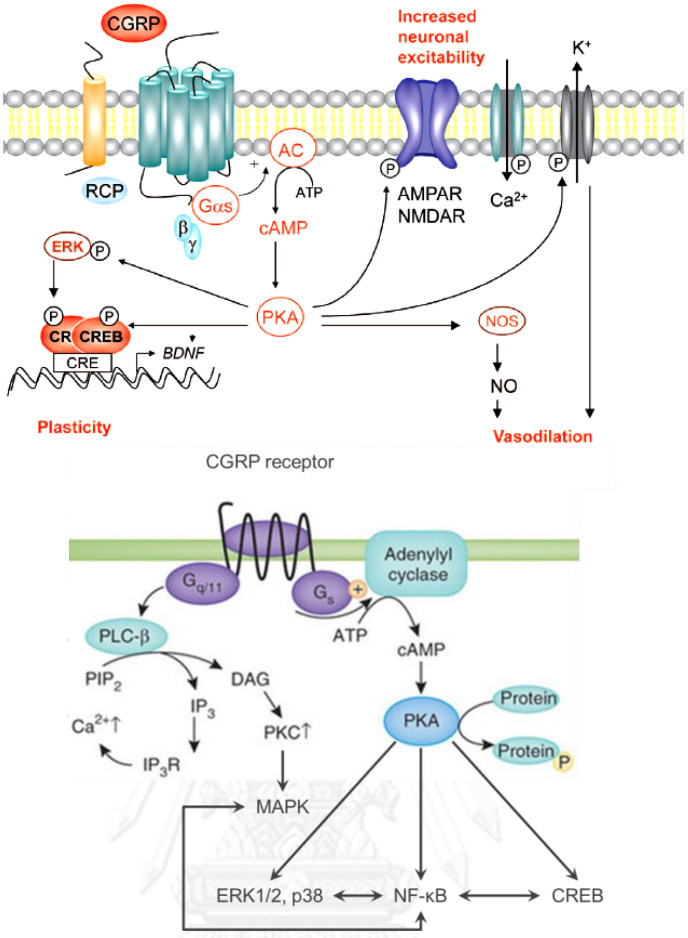

Figure 7 Coupling of intracellular signaling in CGRP receptor activation. (16, 61)

2.4 Role of glial cells in pain signaling modulation

Contribution of neuron-neuron and neuron-glial signaling has been documented during TG neuron activation and in various peripheral pain conditions such as rhinitis, temporomandibular joint (TMJ) inflammation, pulp injury and migraine pain (Fig. 8) (62, 63). The TG comprises of large and small sensory neurons surrounded by two types of glial cells: the satellite cells that wrap around neuronal somata and Schwann cells that ensheath peripheral nerves (Fig 9) (64). 90% of the TG neurons are unmyelinated and 15-50% are CGRP expressed neurons (65, 66). Immunostaining of CGRP receptor has illustrated its expression on Satellite cells and Schwann cells (67-69). Recent studies revealed the potential of glial cells in

participation of neuroinflammation in the TG. Capsicin injection in rat TMJ results in an upregulation of CGRP levels in TG and also found that CGRP stimulation in TG primary glial cell culture increase inflammatory molecules such ciliary neurotrophic factor (CNTF), IL-1β and IL-6 production (70-72).

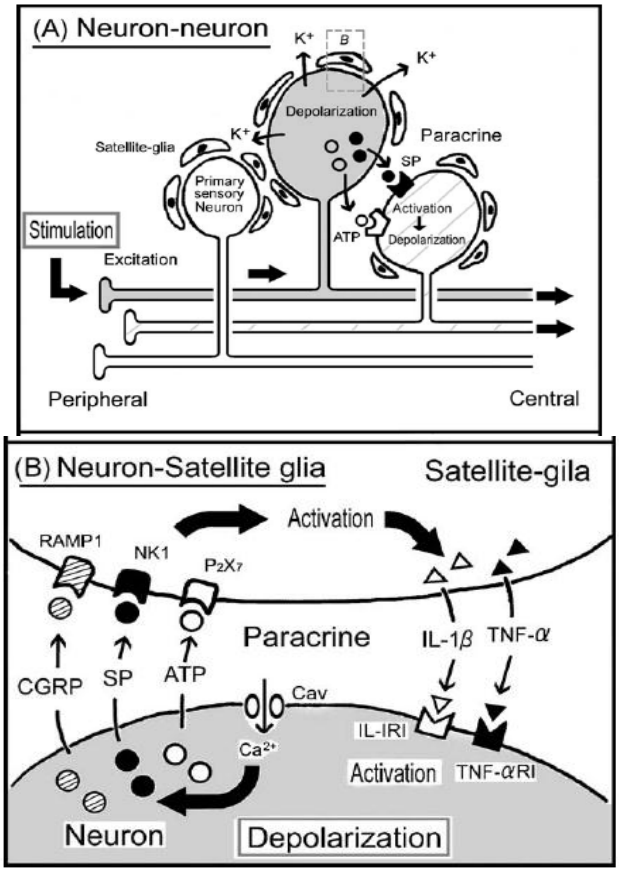

Figure 8 The possibly mechanism for the intraganglion modulation sensory signals (A) Neuron-neuron communication (B) Neuron-glial communication (62).

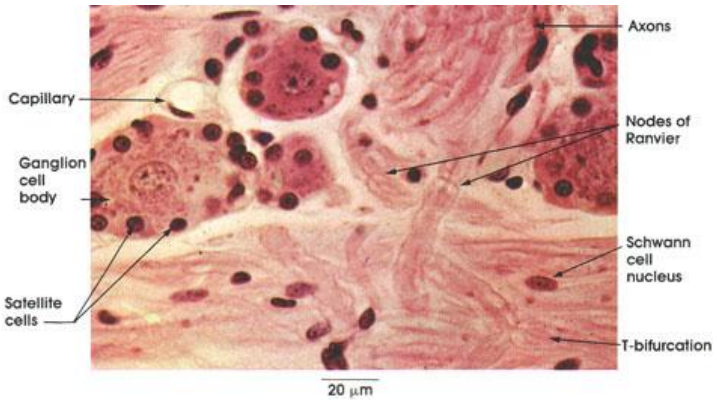

Figure 9 Cell components in sensory ganglion (64)

Previous studies have demonstrated the enhancement of satellite cells in trigeminal neuron excitability during nerve inflammation. Trigeminal satellite cells has found to successfully responded to CGRP by expressing nitric oxide and Prostaglandin (PGE2), cyclooxygenase-2 (COX-2) and the pro-inflammatory cytokines IL-1β (73-75). These findings suggest that satellite cells are responded to the inflammatory phenomenon within TG by induces various inflammatory molecules, which potentially performed a positive feedback that contributes to an intense trigeminal neuronal sensitization. Therefore, role of satellite cells in modulation of chronic pain in sensory ganglion have been explored. Schwann cells have been demonstrated to play a significant role in nerve injury and neuropathy conditions (76, 77). Schwann cells have the capacity to produce inflammatory cytokines and factors that sustain an appropriate environment for neuronal function (78). It has been reported that CGRP-stimulated Schwann cell may be involved in neuronal regeneration in nerve injury model (69). However, role of CGRP-stimulated Schwann cells in aspects of pain hypersensitivity during sensory ganglion inflammation has not yet been explored. Perhaps, Schwann cells in the TG could possibly be another new pain target in TG activation during migraine genesis.

2.4 Schwann cells

Schwann cells are myelinating glia cells in the peripheral nervous system. Schwann cells derive from neural crest cells via two embryonic transitional stages. The first step is the process of gliogenesis, which is characterized by the specification of migrated neural crest cells to generate Schwann cell precursors (SCPs). The second step is the maturation of these cells into immature Schwann cells. The immature Schwann cells differentiate to form mature myelinating and nonmyelinating that found in A-fibers and C-fibers, respectively (79, 80).

Following the promyelinating stage, Schwann cells reduce mitotic activities and create a 1:1 relationship with axons (Fig.11) (81). The myelinating Schwann cells segregate to large axons and lie intervally along the axon. They form basement membranes and begin to extend their plasma membrane around axons, one cell per internode (82). As the myelination begins, the plasma membrane enwraps around the axon and rolls up to form a multiple membrane structure. The junction where inner lip of plasma membrane fused with it first enwrapped roll, called “the inner mesaxon”. The outer roll of plasma membrane fused together with its outer lip called “the outer mesaxon”, which elongates around the axon in a spiral pattern (Fig. 10A) (83). Schwann cell cytoplasm can be seen in each layer of myelin, espectially in the unrolled middle region of myelin tube forming as cytoplasmic clefts, called “Schmidt–Lantermann clefts” and in the lateral region called “paranodal loop or lateral loop” (Fig. 10B) (83). The cytoplasmic surfaces of myelin membrane condense into a compact myelin sheath form the major dense line (MDL), while the two external surfaces form the intraperiod line (IPL) of myelin sheath (Fig. 11) (81).

The repeatedly and tightly enwraps of myelin sheath around axons performed the unique structure of compact myelin which reduces the capacitance of the axon surface and increases the transmembrane resistance (Fig.12) (85). Myelinated segments, referred as, internodes, are interrupted by unmyelinated gaps called the nodes of Ranvier. At these unmyelinated sites provide ion channels and the action potentials are generated for saltatory impulse conduction, resulting in a

10- to 100-fold increase in nerve conduction velocity and in a great reduction of axonal energy consumption. Metabolites, proteins, and ions are thought to transport through the Schwann cell cytoplasm represents uncompacted myelin region such as inner mesaxon, outer mesaxon myelin layer, Schmidt-Lanterman incisures, and paranodal loop or lateral loop in the paranodal region (Fig. 11B) (83, 84).

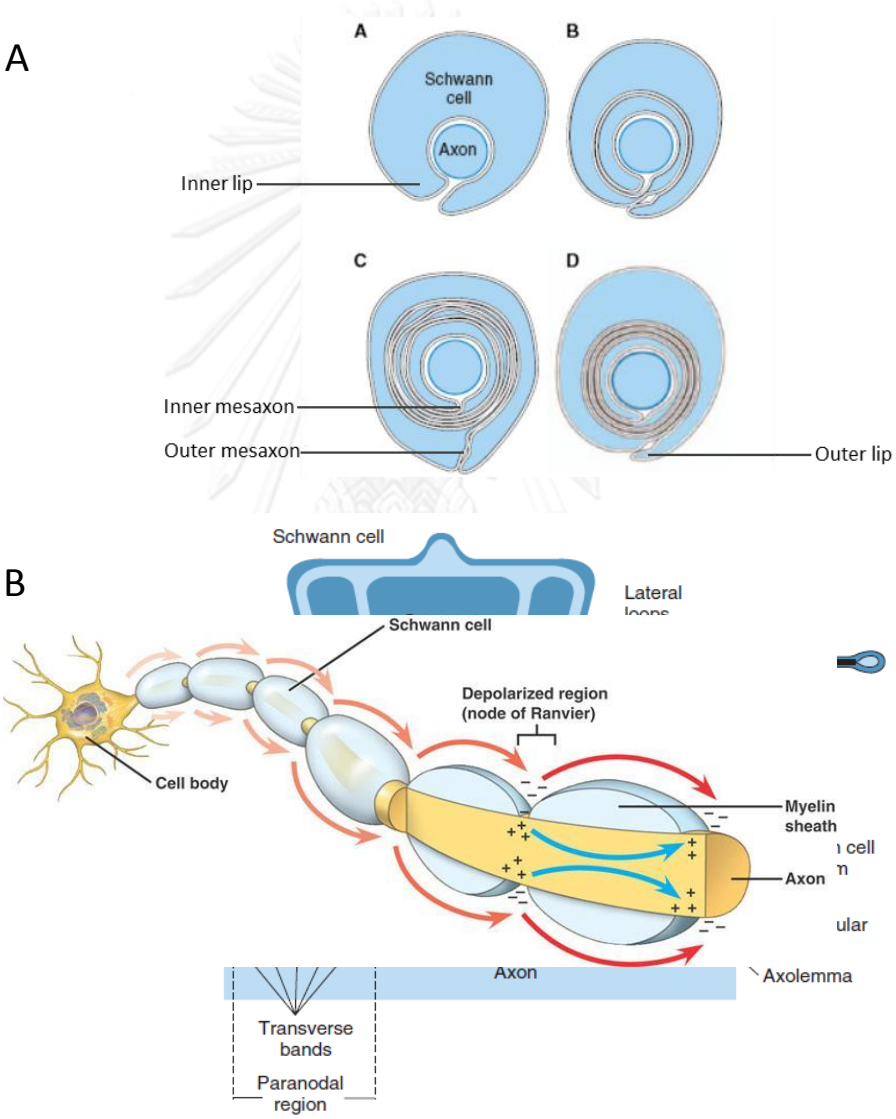

Figure 10 Myelin formation by Schwann cells. (B) The horizontal section of internodal myelin structure in peripheral axon (83).

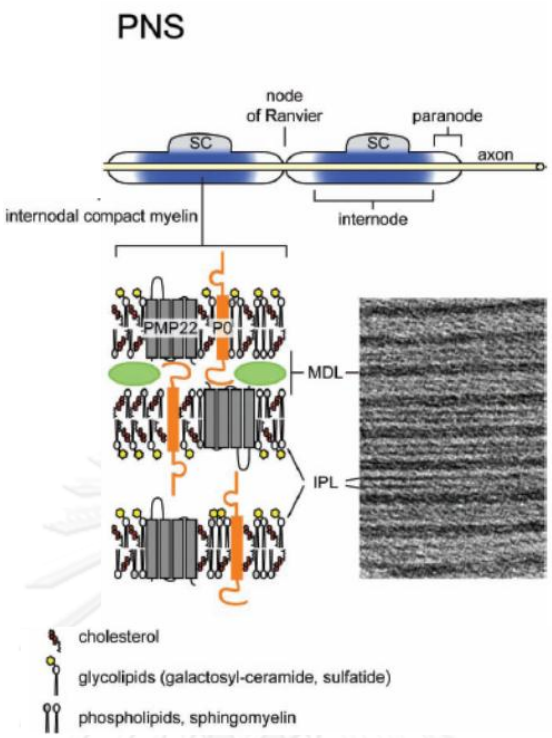

Figure 11 Key stabilization of myelin sheath. IPL, intraperiod lines; MBP, myelin basic protein; MDL, majordense line; P0, myelin protein zero; PLP, proteolipid protein; PMP22, peripheral myelin protein (81)

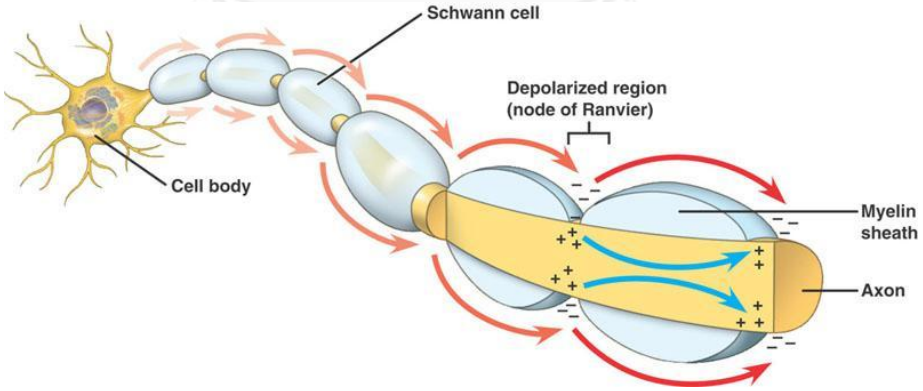

Figure 12 Illustration of Schwann cells with myelinated segments, *internodes*, and unmyelinated segments, nodes of Ranvier, provided ion channels and the action potentials are generated for saltatory impulse conduction (85)

2.6 The myelin Sheath

The myelin membrane contains high content of lipids with the inserted myelin specific proteins (Fig. 11) (81). This unique structure features 70% to 80% lipids constitution of the myelin membrane dry weight. The major lipid classes found in the plasma membrane of myelin include cholesteral, glycolipids, and phospholipids (Table 1) (86). Cholesterol accounts for 20-30% of the total lipids of PNS myelin. It plays a role in myelin membrane compaction and stabilization in corporation with the myelin proteins. Phospholipids account for 50-60% of the total lipids of peripheral myelin with the most abundant ethanolamine phosphoglycerides and sphingomyelin. Compared to CNS, PNS myelin has more sphingomyelin (10–35%), higher content of monogalactosylsphingolipids, with cerebrosides [Gal-C] (14–26%) and sulfatides [SGal-C] (2–7%) and less galactolipid and cholesterol. Galactolipids are almost exclusively located at the extracellular leaflet of the membrane (i.e., facing the intraperiod lines), constituting up to two-thirds of the outer surface of the myelin sheath (81, 83-86). Glycosphingolipids and cholesterol form the key components of the microdomains involved in the cross-communication between Schwann cells and axons frequently referred to as “lipid rafts” in the myelin sheath (84, 86). Inhibition of Cholesterol synthesis enzymes such HMG-CoA (3-hydroxy-3-methyl-glutaryl-CoA) resulted in reduced myelin gene expression and severe hypomyelination. Patients with Cholesterol synthesis disorders such Smith-Lemli-Opitz syndrome (SLOS) reported in absence of myelin and demyelination (87). Previous study has been reported that elevated levels of cholesterol is essential for efficient exiting of the major myelin protein P0 from the endoplasmic reticulum (ER) of Schwann cells, suggested that intracellular cholesterol levels is coupled to the transcription of myelin proteins. This suggested a potential effect of lipid metabolism on myelination and myelinated fiber function (Fig. 13) (81, 87).

Table 1 Lipid composition of the myelin membrane in PNS (86)

Lipids	Bovine	Human	Rabbit	Rat
Cholesterol[a]	24.7	23	27.2	27.2
Total galactolipid[a]	16.6	22.1	27.2	21.5
Gal-C[a]	14.1			
SGal-C[a]	2.5			
Total phospholipids[a]	58.2	54.9	57.6	50.6
Ethanolamine PG[b]	28	35	39	38
Choline PG[b]	21	15	16	19
Serine PG+inositol PG[b]	13	17	14	27
Sphingomyelin	27	34	31	17

[a] Figures are expressed as weight present of total lipids.

[b] Phospholipids are expressed se moles/100 moles total phospholipids. PG. phosphoglycerines.

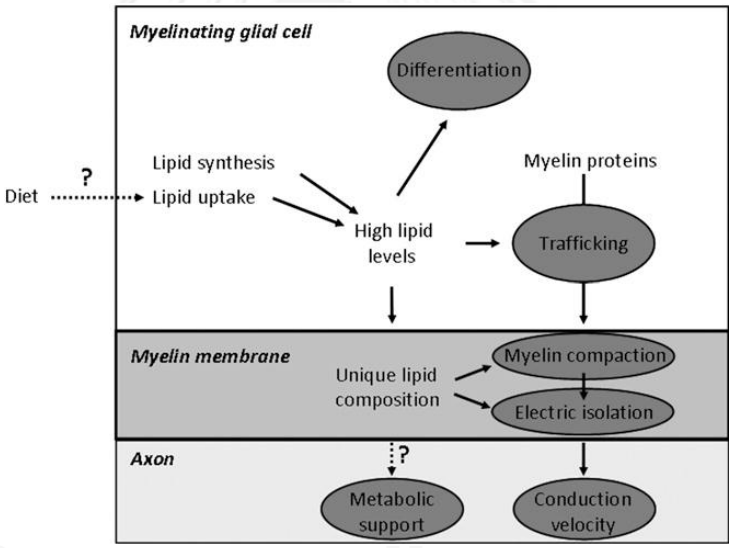

Figure 13 Lipid metabolism influences myelination and myelinated fiber function. High lipid levels from either endogenous synthesis or extracellular uptake are required for synthesis of myelin membrane. Lipids influence myelinating glial cell differentiation and trafficking of myelinproteins to the myelin membrane, also effect myelin compaction, axonal electrostatic isolation, and conduction velocity (87).

Various types of myelin protein expressed in PNS including Myelin protein zero (P0), Peripheral myelin protein (PMP22), myelin basic protein (MBP) and protein 2 (P2). Over half of the myelin protein in compact PNS myelin is P0. The 30-kDa intregral glycoprotein is localized at the base of the extracellular domain near the lipid bilayer of the membrane and extends its intracellular domain to the cytoplasmic surface of membrane. P0 consists of an extracellular N-terminal of a single immunoglobulin variable domain, a transmembrane span and a cytoplasmic *C*-terminal intracellular domain. The extracellular *N*-terminal contains a single Ig-like domain and one site for N-linked glycosylation (Fig. 15) (83, 88). The glycans at the single glycosylation site are sulfate enriched and the terminal of the oligosaccharide oftenly composed of sialic acid or sulfated glucuronic acid, which are a key part of cell-cell adhesion between P0 molecules in the intraperiod line of compact myelin (89). The extracellular domain structure of myelin P0 forms the intraperiod line through homophilic interactions. The cytoplasmic domain of P0 is also important for myelin compaction and adhesion. The mutation at the *C*-terminal has implicated in many demyelinating peripheral neuropathy disease The positive charge at the *C*-terminal induces membrane-membrane binding in the major dense line by interacts to the acidic phospholipids, phosphatidyl serine or other myelin proteins such MBP and P2 (Fig.14) (90). Interaction with phospholipids can be modulated by cytoplasmic domain phosphorylation. The PKC**α** phosphorylation at the *C*-terminal required the receptor for activated C kinase 1 (RACK1) with the NF-**K**B 65-kD adaptor protein (P_{65}) link to P0 and promotes the adhesion (91).

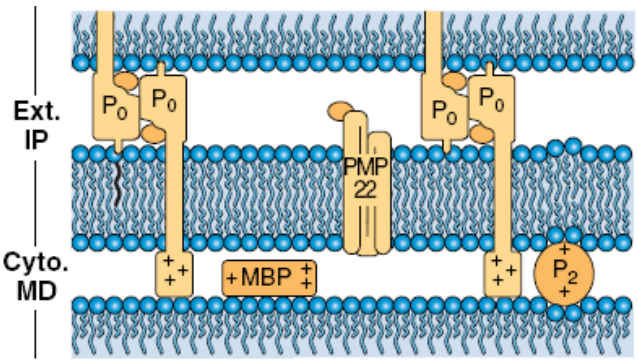

Figure 14 Myelin proteins produced by Schwann cells. The positive charge at the C-terminal induces membrane-membrane binding in the major dense line by interacts to the acidic phospholipids, phosphatidyl serine or other myelin proteins such as MBP and P2. PMP22 also interacts with P0 by interactions at the first and second loop (90)

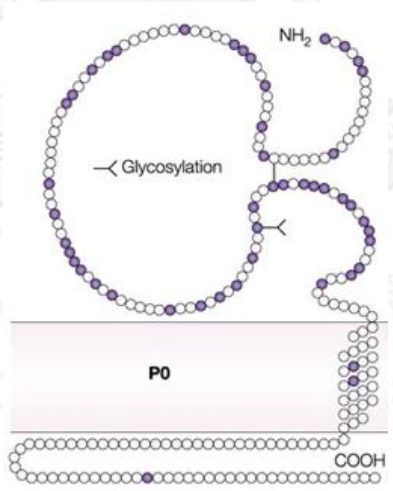

Figure 15 The structural amino acid of P0 (88)

2.7 Cellular markers for Schwann cells

It has been reported that SCPs expresses several phenotypic markers. The neurotrophin receptor p75 (p75NTR) is expressed throughout immature stage of Schwann cells. p75NTR further promotes the expression of the calcium binding protein β (S100-β), which stimulates Schwann cell proliferation and migration (92). They also express glial acidic fibrillary protein (GFAP) and neural cell adhesion molecule (NCAM) throughout the immature phase to pro-myelinating stage and non-myelinated Schwann cells with a complete basal lamina formation. Immature Schwann cells require extrinsic stimuli such as cytokines, axonal membrane bound and extracellular matrix molecules to facilitate the differentiation into either pro-myelinating or non-myelinating Schwann cells (93). Pro-myelinating Schwann cells increase the expression of the exclusive protein of compacted myelin, P0, and a zinc finger transcription factor, Krox20 for initiation of myelination and in order to form a compact myelin structure that facilitates the propagation of axon potentials (Fig. 16) (93, 94).

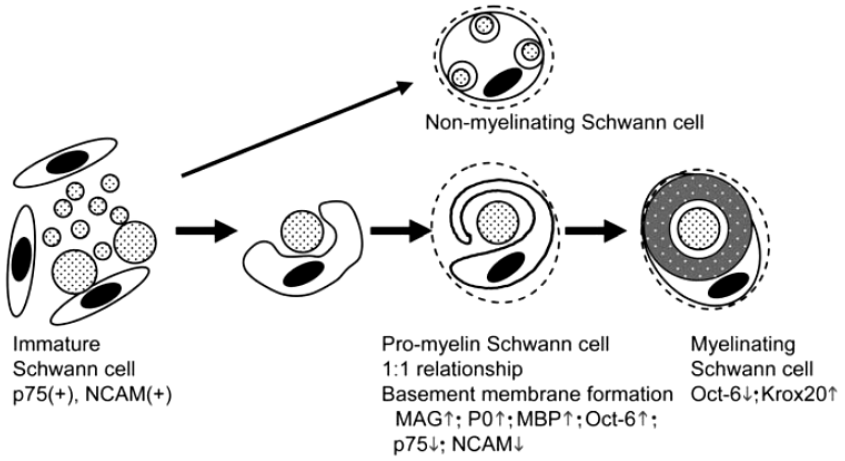

Figure 16 Schwann cells development stages and their markers (93)

Krox-20 *is* zinc-finger transcription factors, a member of the early growth response factor (Egr) gene family. Krox-20 has been implicated as the earliest specific myelination marker, required for the progression of Schwann cells through the pro-myelin stage of differentiation. The *Krox-20* gene has been shown to be responsible

for Schwann cell cycle transition. The mutation of *Krox-20* in the mouse blocks Schwann cells at the pro-myelinating stage. *Krox-20* null mice, although myelin differentiation starts (95), myelin sheaths do not form and Schwann cells continue to proliferate and remain susceptible to death (96, 97). Krox-20 is needed for myelin presenting and long-term maintenance of myelin (98). Krox-20 has a capacity to bind and transcribe genes encoding P0, and also regulate the HMG CoA reductase gene that involved the myelin lipid synthesis (99, 100). *Krox-20* mutations are associated with Charcot-Marie-Tooth, Dejerine-Sottas, and hereditary sensory and motor neuropathies, underlying the pivotal role of this protein in myelin formation (101). Krox-20 is able to modulate Schwann cell responsiveness independent of several axonally derived and extracellular signaling by arresting cells from the cell cycle, downregulating the proliferative molecules (93).

2.8 Schwann cells and inflammation

As a supporting glia that closely interacts with axon, Schwann cells provide trophic molecules to support and maintain the environment for axon. Following peripheral nerve injury conditions, activated Schwann cells immediately release pro-inflammatory mediators such as cytokines (IL-1β, IL-6 and TNF-α), the complement system and arachidonic acid metabolites within the first 24h. These bioactive molecules induce T-cells, neutrophils and macrophages infiltrate the site of an injury within two days (102, 103). Signaling through C-Jun and ERK drive Schwann cells into dedifferentiation phase associated with rises in GFAP and p75NTR mRNAs, decrease amount of myelin proteins including P0 and also initiate myelin phagocytosis with macrophages (104-106). Local paracrine signals and molecular products synthesized by both macrophages and activated Schwann cells such cytokines and inflammatory molecules can directly modulate phagocytic activity. The pro-inflammatory cytokine IL-6 as a potent pain-related cytokines has been reported in amplification of neuronal hypersensity and leading to the maintenance of neuropathic pain (107).

2.8.1 Interleukin 6 (IL-6) and interleukin 6 receptor (IL-6R)

IL-6 is a pleiotrophic cytokine that has been reported to be upregulated within first 24h following nerve injury correlating with the development of

hyperalgesia and allodynia (108). IL-6 is one member of the glycoprotein 130 family that play multiple effects in inflammatory responses, neuroprotective effects and promote regeneration (109, 110). Transcription of IL-6 mRNA has been reported in high neuronal activity following L-type voltage-dependent Ca2+ channels, NMDA-mediated glutamatergic depolarization and incubation of ATP. The IL-6 promoter can directly be stimulated by the transcription factors CREB and NF-κB (107, 111-113). IL-6 exerts its effects through type I transmembrane glycoprotein termed IL-6R dimerized with the two molecules of glycoprotein 130 as a IL-6 receptor complex and signals through JAK/STAT, P38/MAPK, ERK/MAPK, and PI3K pathway (Fig. 17) (113).

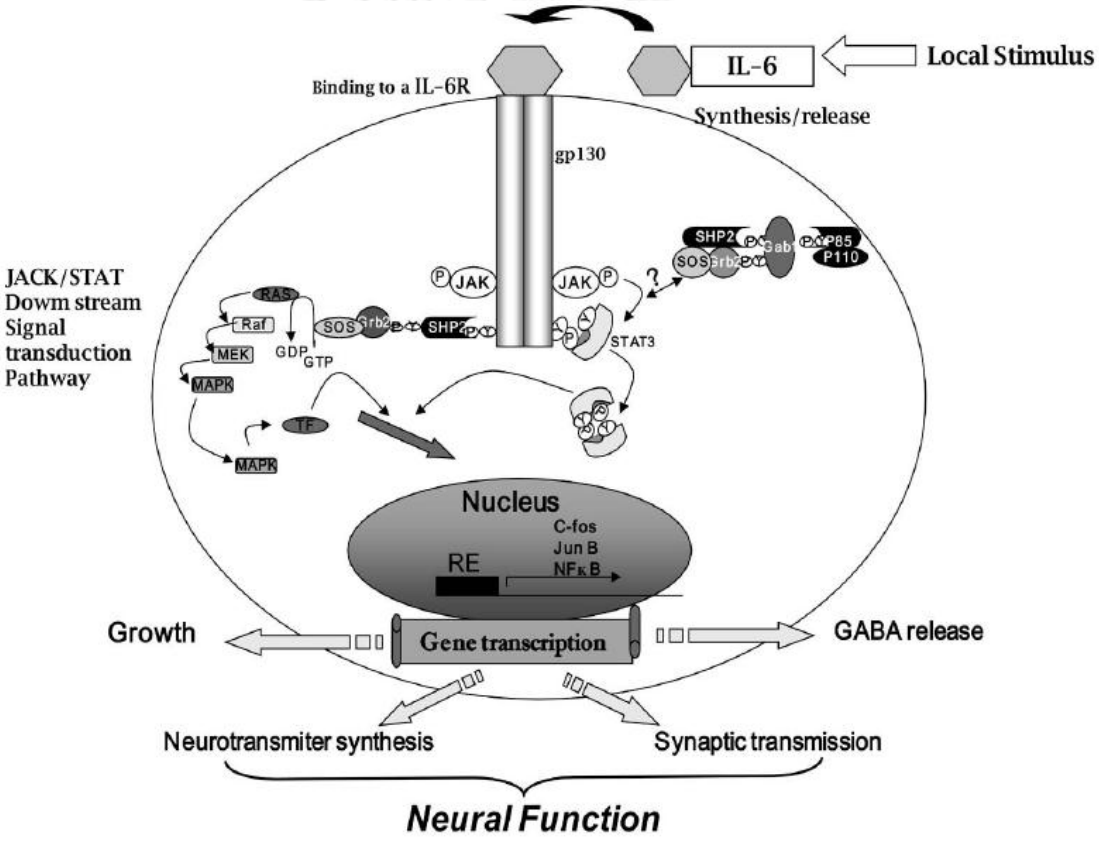

Figure 17 The intracellular signaling and function of the IL-6/IL-6 receptor complex (113).

The nuclear factor NF-κB pathway has long been described in a broad production of inflammatory cytokines including IL-6. NF-κB can signal via two separate pathways. The “canonical” pathway occurs through the activation of IL-1 receptor family such as IL-1 and TNF receptor families, the Toll-like receptors (TLRs) during challenge with microbial products and proinflammatory cytokines that leading an activation of p65/RelA orp65/c-Rel complexes. The other is an “alternative” pathway, which is activated by T cell/B cell activation factors and receptor activator of NF-κB ligand (RANKL and TNFSF11), resulting in activation of RelB/p52 complexes (114, 115). The activity of these two pathways is tightly regulated by interaction of inhibitory IκB protein with the NF- κB complexes. Activation of NF-κB resulted in IκB kinase (IKK) or NF-κB-inducing kinase (NIK) mediated IκB phosphorylation and degradation by ubiquitinal process in proteosome. The degradation of IκB frees the NF- κB complex to enter the nucleus and activate target gene expression (Fig. 18) (116).

The nuclear factor kappa B (NF-κB) is another transcription factor that considered as a pro-myelinating transcription factor (117, 118) NF-κB was highly upregulated in pre-myelinating Schwann cells, and then its expression progressively declined until it was nearly absent in adults. NF-κB activation is required for regulating axonal size and myelination level for proper myelin formation (118, 119). NF-κB has shown the protective effect in nerve growth factor (NGF) signaling induced anti-apoptotic death during 7days of sciatic nerve injury (120). Laminin activates NF-κB in Schwann cells to enhance neurite outgrowth. Schwann cell proliferation is unaffected by NF-κB inhibition (121). The transcriptional activity of NF-κB is significantly enhanced by treatment with forskolin. In nerve crush injury model, it has been that shown the novel function of NF-κB to initiates axonal regeneration and remyelination (114, 122).

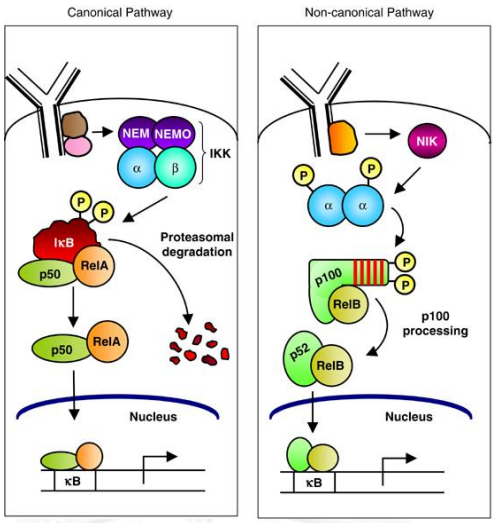

Figure 18 NF-KB signaling pathway (116).

2.8.2 Schwann cells and IL-6

In normal conditions, IL-6R expression is highly found in non-myelinating Schwann cells. In myelinating Schwann cells IL-6R presents at the perinuclear region, in membrane domains of the myelin sheath and the nodes of ranvier. Immunochemistry study reveals an upregulation of IL-6R expression in denervated Schwann cells between the myelin ovoids during Schwann cell proliferation period after nerve crush injury (123). During acute inflammation, IL-6 recruits chemokine expression initially for neutrophils and subsequently for monocytes T cells infiltration to the inflammatory site after 24–48 h to prevent increased tissue damage from the accumulation of neutrophil-secreted proteases and reactive oxygen species. IL-6 is also reported in the induction of pro-inflammatory genes that potentiated myelin debris clearance during demyelination process (124). IL-6 can evoke NMDA and other glutamate receptor function, increase in intracellular calcium concentrations and release of nitric oxide and substance P which exert long-term inflammatory action that further prolongs pain transmission (125, 126). Increase IL-6 has been shown to facilitate neurite outgrowth and myelin formation via activation of JAK/STAT3 and phosphatidylinositide 3-kinase/protein kinase B (PI3K/Akt) pathway. An incubation of IL-6 in cultured Schwann cells resulted in the upregulation of the transcription factor Krox-20 and myelin protein MBP, PMP-22 and P0 via JAK2/STAT3 pathway (127, 128).

The P0 and MBP promoters contain the sequences that are required for IL-6/IL6R activation. This suggests the multiple roles of IL-6 in pathophysiology of neuroinflammation, neuropathic pain and neural repair (129).

2.9 Iron

Iron is a basic element for living organism and cellular activity. Iron is a key component for cellular oxidative metabolism, DNA synthesis and repair. Iron also participates redox cycling in free radicals generation and detoxification. The essential role of iron has been reported in oligodendrocytes and Schwann cells biology and development (130, 131). These myelinating glia required high levels of cellular metabolism during myelination process. Iron is required as a cofactor of various basic enzymes for energy metabolism, hence, glucose synthesis via pentose-6-phosphate pathway (glucose-6-phosphate dehydrogenase, dioxygenase, succinic dehydrogenase and NADH dehydrogenase), lipid synthesis (HMG-CoA reductase, squalene epoxidase, Lipid saturase and desaturase), cytochrome oxidase system including cytochromes a, b, and c and the Fe-sulfur complexes of the oxidative chain for ATP production, as well as Ribonucleotide reductasefor total regulation rate of DNA synthesis and repair by formation of deoxyribonucleotides from ribonucleotides during cell division and DNA repair. Since iron had been evidenced in both CNS and PNS myelinating glia biology, high levels of these enzyme activities are implicated during myelination (132, 133). Iron deficiency has been reported to impair metabolic activity, fatty acid composition and cytochrome oxidase activities with a decrease of the myelin protein, MBP, concentration in early postnatal rats (130). Treatment of iron promotes Schwann cell differentiation by increase P0 and MBP expression together with the decrease of Schwann cell proliferative marker, p75NTR (134). The dysregulation of iron processing, storage, or availability affect both the quantity and the quality of myelin (131).

2.9.1 Iron uptake and intracellular iron pool

The uptake iron requires apo-transferrin (apo-Tf), which binds to two molecules of Fe^{3+} present in the blood serum. Iron-bound holo-transferrin (holo-Tf) binds with high affinity to TfR1 on the cell surface and the complex undergoes

endocytosis via clathrin-coated pits into the endosomes. Changes of endosome acidification triggering the release of Fe^{3+} from holo-Tf and reduced Fe^{3+} to Fe^{2+} before exporting into the cytoplasm as an intracellular free iron. Cytoplasmic iron presented in two forms, free low molecular weight iron and ferritin-stored iron (Fig 20) (134).

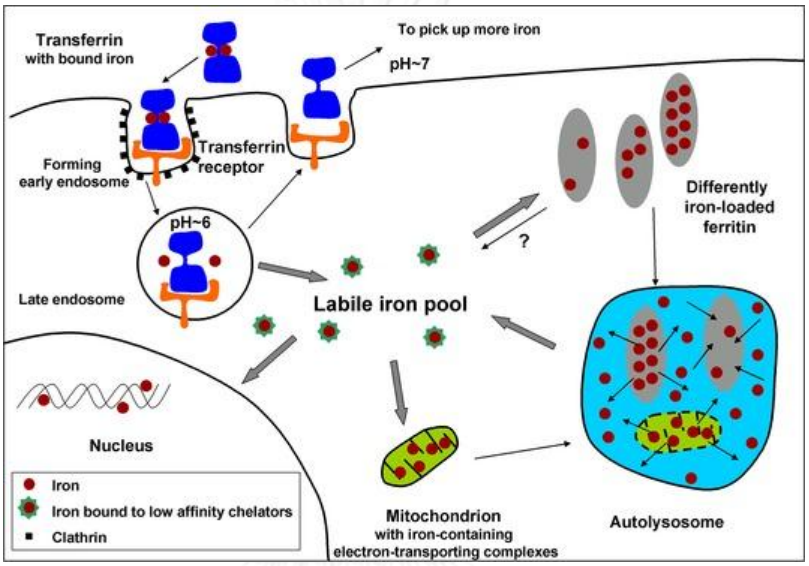

Figure 19 Iron transprot and cell iron homeostasis (134)

Two forms of free iron are available in cellular living organism, Fe^{2+} and Fe^{3+}. The generation of free radicals through Fenton reaction mechanism was initiated by hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet-}$). The reaction between H_2O_2 and Fe^{2+} produce hydroxide (OH^-) and the highly reactive hydroxyl radical ($OH^{\bullet}$), and the reaction between Fe^{3+} and $O_2^{\bullet-}$ generate O_2. The impaired homeostasis of free iron leads to oxidative damage resulting from an excess of cellular iron and an accumulation of reactive oxygen species. The intracellular iron sequester protein such ferritin play a protective role against iron toxicity. As an iron storage protein, ferritin capable to stores 4,500 molecules of Fe^{3+} per molecule. Ferritin is divided into two subtypes, which are H- and L-ferritin. H-ferritin has a ferroxidase activity. It plays a role in rapid iron detoxification by convert Fe^{2+} to Fe^{3+}, while L-ferritin stores iron for long-term usage and associates with iron nucleation and mineralization. Ferritin

expression levels are regulated by the cytoplasmic low molecular weight iron levels, in order to control cellular iron in homeostasis (135).

2.9.2 Cellular iron homeostasis

Key regulation of cellular iron metabolism involved a post-transcriptionally control of Tf and ferritin expression level via IRE/IRP system. Intracellular iron controls the interactions between the cytosolic trans-acting mRNA protein; iron regulatory protein (IRP) and the structural element; iron responsive element (IREs). IREs structure is located in the 3'-untranslated region (UTR) of TfR mRNA and in the 5'UTRs of ferritin mRNA. The bifunctional protein, IRP1, has a prominent role in cellular iron regulation; its functional switch depends on the assembly of an iron-sulphur (4Fe–4S) structure (135, 136). In iron repletion, the assembly of iron in the 4Fe–4S structure limits IRE interaction but allows the binding of citrate to perform an isomerization of citrate to isocitrate as an aconitase enzyme. In iron depletion, IRP1 acts as a RNA-binding protein that allows the interaction with IRE in the 3'-UTR of TfR mRNA and triggers TfR synthesis and protect the mRNA degradation, where as, binding of IRP1 and IRE in the 5'-UTR inhibits ferritin synthesis and leads to ferritin mRNA degradation within lysosome to maintain the proper LIP level (Fig. 20) (134, 137, 138). An alteration of TfR and ferritin expression level is indicated as a parameter for cellular iron status.

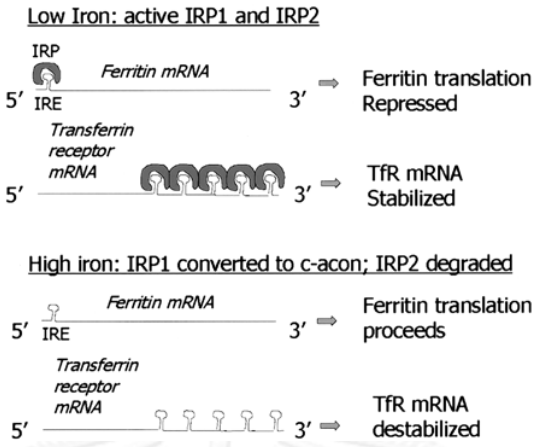

Figure 20 Post-trancriptional regulation of transferrin receptor and ferritin genes. (Transferrin receptor; TfR, IRP1, iron regulatory protein 1; IRP2, iron regulatory protein 2; IRE, iron responsive element) (138)

2.9.3 Iron and Schwann cells

Tf and TfR have been reported to be expressed by Schwann cells during myelination. The immunohistochemistry and immunoblot revealed the cytoplasmic accumulation of **Tf** in Schwann cells in the sciatic nerve (139). Tf has shown to promote Schwann cell differentiation during late embryonic and potentiate nerve regeneration following nerve crush scenario (140). TfR expression is upregulated in Schwann cells within an early day following sciatic nerve injury coincides with the high upregulated in endoneural iron uptake (141). An abnormal accumulation of iron frequently found in the early stages of demyelination diseases (142). Treatment of iron chelator such as deferoxamine successfully reduces inflammatory molecules production via inhibition of NF-κB activation, prevents DNA damage and cell apoptosis in nerve injury and demyelinating neuropathy models, suggesting iron may have closely relationship in triggering cellular inflammation (143, 144).

CHAPTER III

MATERIAL AND METHODS

3.1 Chemicals

Human calcitonin gene-related peptide (CGRP), human CGRP fragment 8-37 ($CGRP_{8-37}$), Lipopolysacharride (LPS), ferric ammonium citrate (FAC), deferoxamine (DFO) and NF-κB inhibitor (Cardamonin) were obtained from Sigma Aldrich (St.Louis, MO, USA). Dulbecco's Modified Eagle'sMedium (DMEM), pennicillin-streptomycin and trypsin-EDTA were purchased from Hyclone (South logan, UT, USA). Fetal bovine serum (FBS) was obtained from Gibco (Paisley, UK). Calcein acetoxymethylester (Calcein AM) and TRIzol® reagent were purchased from Invitrogen (Carlsbad, CA, USA). GoScript RT reverse transcription kits were obtained from Promega (Madison, WI, USA). Power SYBR® green mastermix was purchased from Applied Biosystems (Warrington, UK). ELISA kit for IL-6 detection was purchased from Thermosciencetific (Rockford, IL, USA). All cell culture vessels were purchased from Corning (Tewksbury, MA, USA).

3.2 Cell culture and treatments

A Rat schwann cell line, RT4-D6P2T, was purchased from ATCC (Manassas, VA, USA) and maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C in DMEM containing 10% FBS. After 48h prior to experiment, cells were treated. Cells were treated with FAC (500 µg/ml), LPS (1 µg/ml), (DFO (1mM), CGRP (0.01, 1 µM), CGRP (1 µM) combined with either DFO or $CGRP_{8-37}$. The treatment times were indicated in each experiment.

3.3 Measurement of intracellular iron levels

Calcein-AM is a membrane permeable ester, that reacts with cytosolic non-specific esterases resulting in the production of membrane impermeable and fluorescent calcein molecules. It has been reported that calcein fluorescence is quenched following chelation of the intracellular free iron (Tenopoulou M et al., 2007). Thus, the degree of quenching gives an estimate of the amount of chelatable iron. Briefly, cells were plated at a density of $2x10^4$ cells/well in 96-well

plates. In each experiment as indicated in results, 1 μM of calcein AM was added into the cultured media for 30 min prior to time point examined. Cells were washed twice with PBS and calcein fluorescence was monitored at excitation/emission wavelengths of 488 nm/518 nm using a Synergy HT microplate reader (Biotek, USA). Untreated cells and cells that were treated with FAC and DFO were served as controls for this assay.

3.4 RNA extraction and qPCR

Cells were plated at a density of $3x10^5$ cells/well in 6-well plates. After the treatment, total RNA was extracted using Trizol reagent and quantified using Nanodrop spectrophotometer (Thermoscientific, USA). A 1μg of the total RNA was reverse transcriped with random primers using GoScript RT kit in a 20 μl reaction volume. qPCR was performed with a Step one plus Real-Time PCR System (Applied biosciences, USA) with a total volume of Power SYBR® green mastermix 20 μl. Each reaction mixture contains 5 μl of 2X SYBR® green mastermix, 0.4 μl of 10uM primer, 1 μl of 25ng cDNA, and 13.2 μl of ddH_2O. The PCR conditions are 94°C for 15 sec, 60°Cfor 1 min, 40 cycles. The relative quantification was performed by the comparative cycle threshold method using glyceraldehydes- 3-phosphate dehydrogenase (GAPDH) as an internal control (Schmittgen TD and Livak KJ., 2008) Primer sequences for all targets were as followed: Krox-20; F 5'-CAGTACCCTGGTGCCAGCTG-3', R 5'-CTGTGGTTG AAGCTGGTGG-3'; P0 ;F 5'-GCCCTGC TCTTCTCTTCTTT 3' , R 5'-CCAACACCACCCCATACCTA-3; IL-6;F 5'-CCAGTTGCCTTCTTG GGACT-3', R 5'-CCTCTGTGAAGTCTCCTCTCC-3'; IL-6R; F 5'-TCACAGAGCAGAGAATGGACT-3', R 5'-GTATGGCTGATA CCACAAGGT-3'; GAPDH; F 5'-GGC AAATTCAACGGCACAGT-3', R 5'-AGATGGTGATGGGCT TCCC-3'.

3.5 ELISA assay

Following each treatment, cell-free supernatants were collected. The amount of secreted IL-6 was determined using IL-6 ELISA kits. The cultured medium was diluted 1:5 with the standard diluents and the experiment was performed according to the manufacturer's instructions. Absorbance was measured immediately at excitation/emission wavelengths of 450 nm/550 nm after stop reaction using a

Synergy HT microplate reader (Biotek, USA). The cytokines production levels were calculated using standard curve.

3.6 Statistical analysis

All statistical test were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). One-way ANOVA followed by LSD post hoc testing was used for multiple comparisons of data. Data are presented as mean ± standard error of the mean (SEM) from three independent experiments in triplicate. Differences were considered statistically significant at $p<0.05$

CHAPTER IV

RESULTS

4.1 CGRP upregulates P0 expression in Schwann cells.

The myelin protein zero (P0) accounts for more than half of myelin proteins produced by Schwann cells and serves as a specific marker for the identification of Schwann cells in addition to GFAP and S100 protein. However, it is not known whether P0 could be used as an activation marker for Schwann cells. Therefore, the first aim of my study was to explore such possibility. CGRP and LPS were used to stimulate cultured Schwann cells for different periods of times (3, 6 and 24 h) and expression of P0 at each time point was examined by qRT-PCR. CGRP was selected because it has been reported to be up-regulated in the TG and expected to play an important role in pain transmission associated with migraine headache. LPS was used as a positive control for CGRP stimulation. Cultured Schwann cells were stimulated with CGRP alone (1 μM) or combined with $CGRP_{8-37}$ (1 μM). These concentrations were selected according to a recent study on neurogenic inflammation of rat TG (144). Results showed that CGRP significantly upregulated P0 expression in a time-dependent manner beginning at 6 h, compared to the unstimulated control cultures. The ability of CGRP to induce P0 expression was completely inhibited in the presence of $CGRP_{8-37}$ (Fig. 21A). In contrast to CGRP, LPS upregulated P0 expression in cultured Schwann cells only at 3 h post-stimulation. At 6 and 24 h post-stimulation, P0 expression was observed at the baseline levels. These results indicate that CGRP stimulates P0 expression in Schwann cells and further suggest that P0 can be used as a specific marker for CGRP-induced Schwann cell activation.

The ability of CGRP to induce P0 expression in Schwann cells was further confirmed by monitoring expression of Krox-20, the transcription factor known to regulate P0 expression. Results demonstrated that CGRP significantly upregulated Krox-20 expression in a time-dependent manner beginning at 6 h. This pattern coincides with that of P0. The presence of $CGRP_{8-37}$ inhibited Krox-20 expression induced by CGRP (Fig. 21B). These results suggest that CGRP-upregulated P0 expression in Schwann cells is mediated by Krox-20. Since P0 plays an important role

in myelin adhesion and compaction, it is hypothesized that upregulation of P0 by CGRP could stabilize myelin structure, thereby facilatating pain transmission.

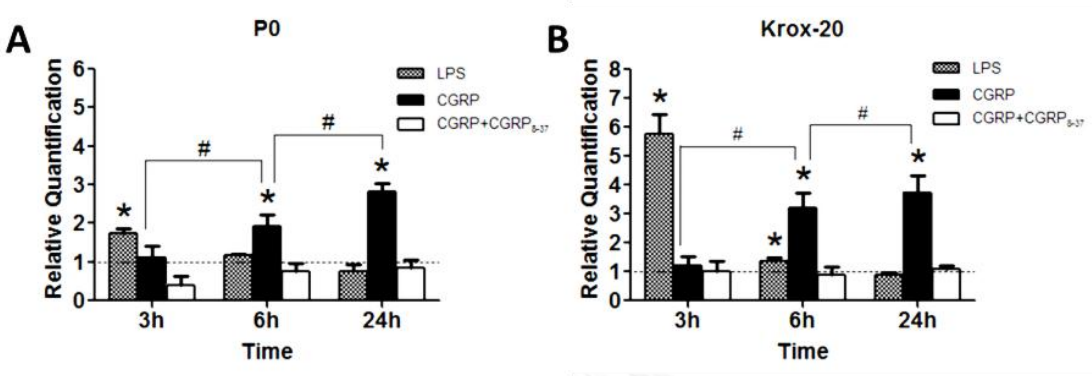

Figure 21 CGRP upregulates expression P0 and Krox-20 in Schwann cells.

Relative quantification of **(A)** P0 and **(B)** Krox-20 mRNA levels at 3, 6, and 24h post-CGRP stimulation. Dashed line represents baseline expression in the unstimulated control cultures. Data are presented as the means ± SEM of three independent experiments performed in triplicate. (* $P<0.05$; compared with the unstimulated control at each time point).

4.2 CGRP simulates expression of IL-6 and IL-6R in Schwann cells.

In pathological setting, Schwann cells are activated and release neuroactive substances including pro-inflammatory cytokines TNF-α and IL-1β. These cytokines are known to enhance pain sensitivity. IL-6 is a pleiotropic cytokine acting through IL-6R. Upregulated IL-6 levels following nerve injury correlates well with the development of thermal hyperalgesia and allodynia (9, 10 in proceeding). Emerging evidence suggests that IL-6 may play a role in pain and hypersensitivity associated with inflammation. To investigate whether Schwann cells could release IL-6 as part of their activation in response to CGRP, IL-6 expression and release was examined using qRT-PCR and ELISA, respectively. Cultured Schwann cells were treated with LPS (1 µg/ml), CGRP (1 µM) alone or CGRP (1 µM) in the presence of $CGRP_{8-37}$ (1 µM) for 3, 6 and 24 h. Results demonstrated that LPS induced Schwann cell expression of IL-6. IL-6 expression reaches its highest point at 3 h and reduce to basal levels at 24h. In contrast, CGRP induced Schwann cell expression of IL-6 in a time-dependent manner. The presence of $CGRP_{8-37}$ completely inhibited CGRP-induced IL-6 expression in Schwann cells (Fig. 22A). ELISA assay was used to determine the amount of IL-6 released from Schwann cells. Results demonstrated that CGRP induced IL-6 release deom Schwann cells in a time-dependent manner. The significant levels of IL-6 were found at 6 h and reached its highest levels at 24 h (Fig. 22B). In addition to paracrine effect, it is hypothesized that IL-6 released from Schwann cells could also have an autocrine effect. Therefore, mRNA expression of IL-6R in cultured Schwann cells was wxamined using qRT-PCR. LPS transiently induced Schwann cell expression of IL-6R at 3 h post-stimulation and then declined to basal levels by 24 h. In contrast to LPS, CGRP induced a significant increase in IL-6R observed at 24 h post-stimulation. The presence of $CGRP_{8-37}$ completely inhibited CGRP-induced IL-6R expression (Fig. 22C). These results indicate that Schwann cells produce IL-6 in response to CGRP and also suggest that IL-6 released from Schwann cells should have an autocrine and a paracrine effects.

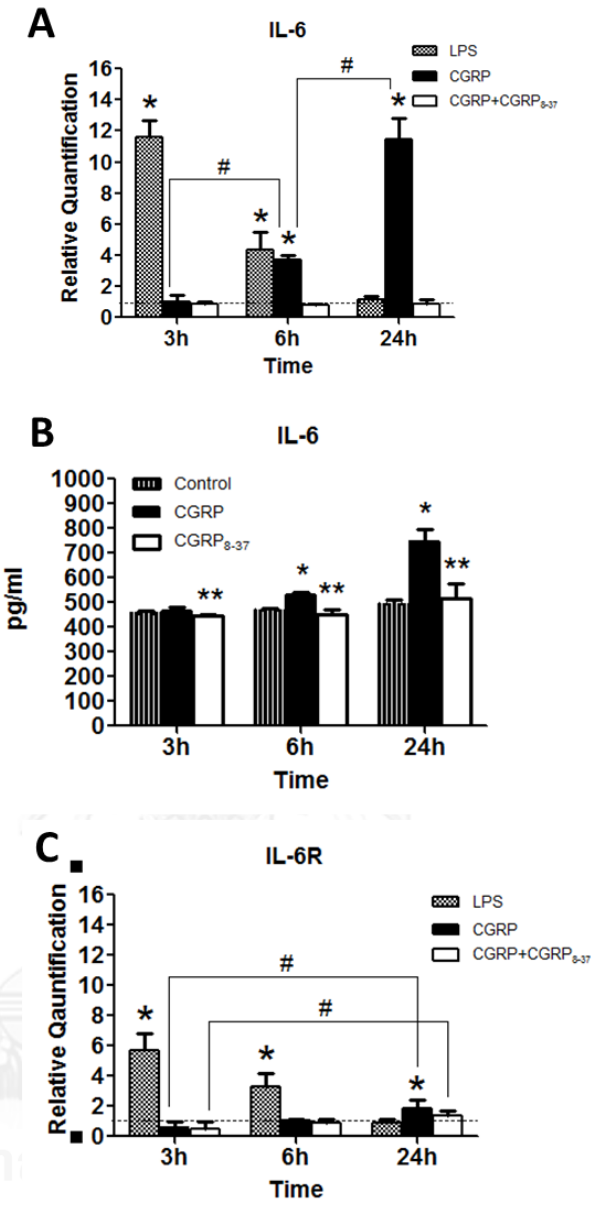

Figure 22 CGRP induces IL-6 and IL-6R expressions in Schwann cells.

(A) IL-6 mRNA expression levels. (B) IL-6 protein levels in the cell cultured media. (C) IL-6R mRNA expression levels. Dashed line represents baseline expression in the unstimulated control cultures. Data are presented as the means ± SEM of three independent experiments performed in triplicate. (* $p<0.05$ statistically significant compared with control, (** $p<0.05$ statistically significant compared with CGRP, # $p<0.05$ statistically significant compared between time point).

4.3 CGRP-stimulated Schwann cell activation is mediated by NF-κB.

In this set of experiments, cardamonin, which is an inhibitor of NF-κB activaton, was used to investigate the involvement of NF-κB signaling in Schwann cell activation by CGRP. Results demonstrated that cardamonin significantly abolished CGRP-induced mRNA expression of P0, Krox-20, IL-6 and IL-6R and the production of IL-6 in a concentration-dependent manner (Fig. 23). These results indicate that NF-κB signaling is essential for CGRP-stimulated cellular activation of Schwann cells.

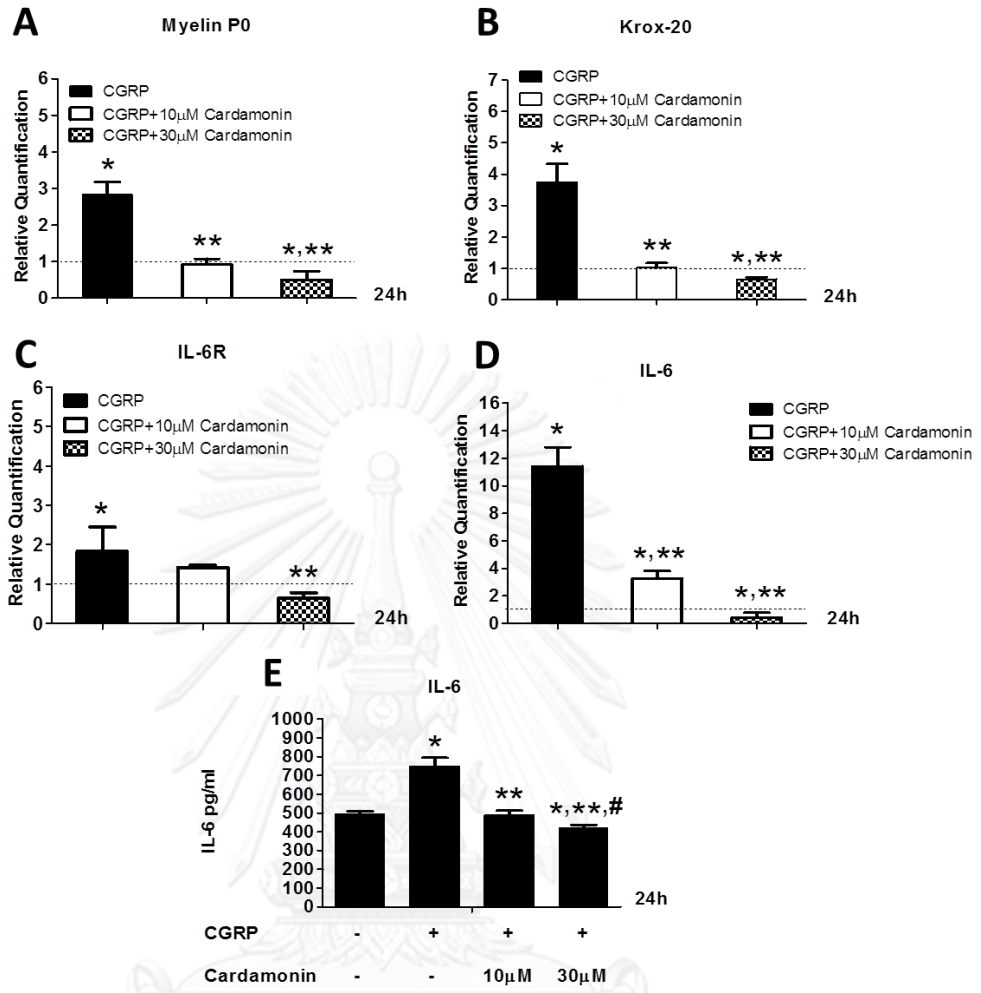

Figure 23 NF-κB signaling is essential for CGRP-induced Schwann cell activation.

mRNA expression levels of **(A)** P0, **(B)** Krox-20, **(C)** IL-6R and **(D)** IL-6 were determined using qRT-PCR. Dashed line represents baseline expression levels in the unstimulated control cultures. Data are presented as the means ± SEM of three independent experiments performed in triplicate. (* $p<0.05$ statistically significant compared with control; ** $p<0.05$ statistically significant compared with CGRP; # $p<0.05$ statistically significant compared with cardamonin (10 μM). **(E)** IL-6 protein levels were determined by ELISA. Data are presented as the means ± SEM of three independent experiments performed in triplicate. (* $p<0.05$ statistically significant compared with control; ** $p<0.05$ statistically significant compared with CGRP; # $p<0.05$ statistically significant compared with cardamonin (10 μM).

4.4 Effect of CGRP on iron metabolism in Schwann cells.

It is established that mRNA expression of the TfR is regulated by cellular iron status. Furthermore, upregulation of TfR mRNA in Schwann cells has been reported following nerve injury (140, 145-147). To explore whether cellular activation of Schwann cells by CGRP is linked to cellular iron status, TfR expression in Schwann cells following CGRP stimulation was examined at different periods of times using qRT-PCR. Cultured Schwann cells were stimulated with LPS or CGRP in the presence or absence of $CGRP_{8-37}$ at the same concentrations described in previous experiment. Results showed that both CGRP and LPS did not cause a significant change in TfR mRNA expression in cultured Schwann cells compared to that of the unstimulated control cultures at all time points examined (Fig. 24). This finding suggests that activation of Schwann cells by CGRP may not be linked to cellular iron metabolism. However, it is also possible that CGRP could alter cellular iron metabolism in Schwann cells, which, in this case, cannot be monitored through changes in TfR mRNA expression.

The effect of CGRP on iron metabolism in Schwann cells was further examined using an iron-sensitive fluorescence dye calcein (CA). It has been shown that CA fluorescence is quenched following chelation of the intracellular free iron (148). Thus, the degree of quenching gives an estimate of the amount of chelatable iron. In this set of experiments, cultured Schwann cell were incubated with FAC (500µg/ml), DFO (1mM) or CGRP (1 µM) in the presence or absence of $CGRP_{8-37}$ (1 µM) for 3, 6 and 24 h. Incubation with FAC and DFO served as control for this experiment. Results demonstrated that incubation with FAC increased intracellular iron levels in Schwann cells in a time-dependent manner, as reflected by a continuous decrease in CA fluorescence intensity. In contrast, incubation with DFO decreased intracellular iron levels in Schwann cells as reflected by an increase in CA fluorescence intensity reaching the plateau at 3 h. After 6 h, CA fluorescence intensity began to drop toward baseline levels. By 12 h, CA fluorescence intensity was observed below baseline values. CGRP caused a significant decrease in CA fluorescence intensity during the first 30 min of incubation. After that CA fluorescence intensity was increased by peaking at 3 h post-incubation before returning to the baseline values

(Fig. 25A). A low concentration of CGRP (0.01 μM) also gave a similar pattern of changes in CA fluorescence intensity compared to high concentration (1 μM CGRP), but in a lower amplitude, thus suggesting a dose-dependent effect of CGRP on intracellular iron levels in Schwann cells (Fig. 25B). Incubation with $CGRP_{8-37}$, the CGRP receptor antagonist, reduced the effects of CGRP on CA fluorescence intensity in a concentration dependent manner (Fig. 25C). Together, results demonstrate that CGRP stimulates changes in iron metabolism in Schwann cells. These results also emphasize that Schwann cells possess a regulatory mechanism to maintain cellular iron homeostasis. It is speculated that changes in intracellular iron levels following CGRP stimulation could represent stage of Schwann cell activation.

To further examine whether TfR is included in a regulatory mechanism, by which Schwann cells use to maintain cellular iron homeostasis, TfR mRNA expression levels in Schwann cells that were incubated with DFO was examined using qRT-PCR. Results showed that incubation with DFO increased TfR mRNA expression in a time-dependent manner, beginning at 6 h (Fig. 25D). This finding indicates that Schwann cells increase iron uptake by upregulating TfR expression in response to cellular iron depletion by DFO, thus raising the possibility that Schwann cell activation by CGRP is linked to iron metabolism.

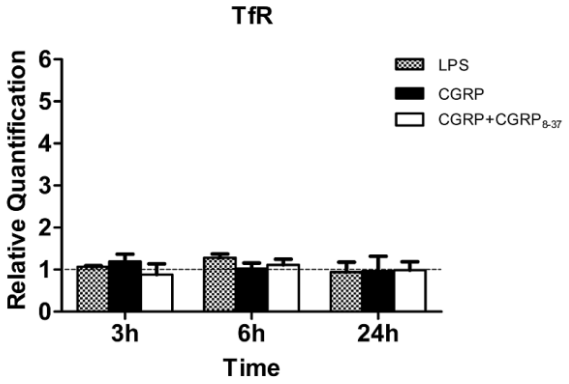

Figure 24 Effect of CGRP on TfR mRNA expression in Schwann cells

Relative quantification of TfR mRNA levels in Schwann cells stimulated by CGRP. Dashed line represents baseline expression of TfR in the unstimulated control cultures. Data are presented as the means ± SEM of three independent experiments performed in triplicate.(* P<0.05; compared with the unstimulated control at each time point).

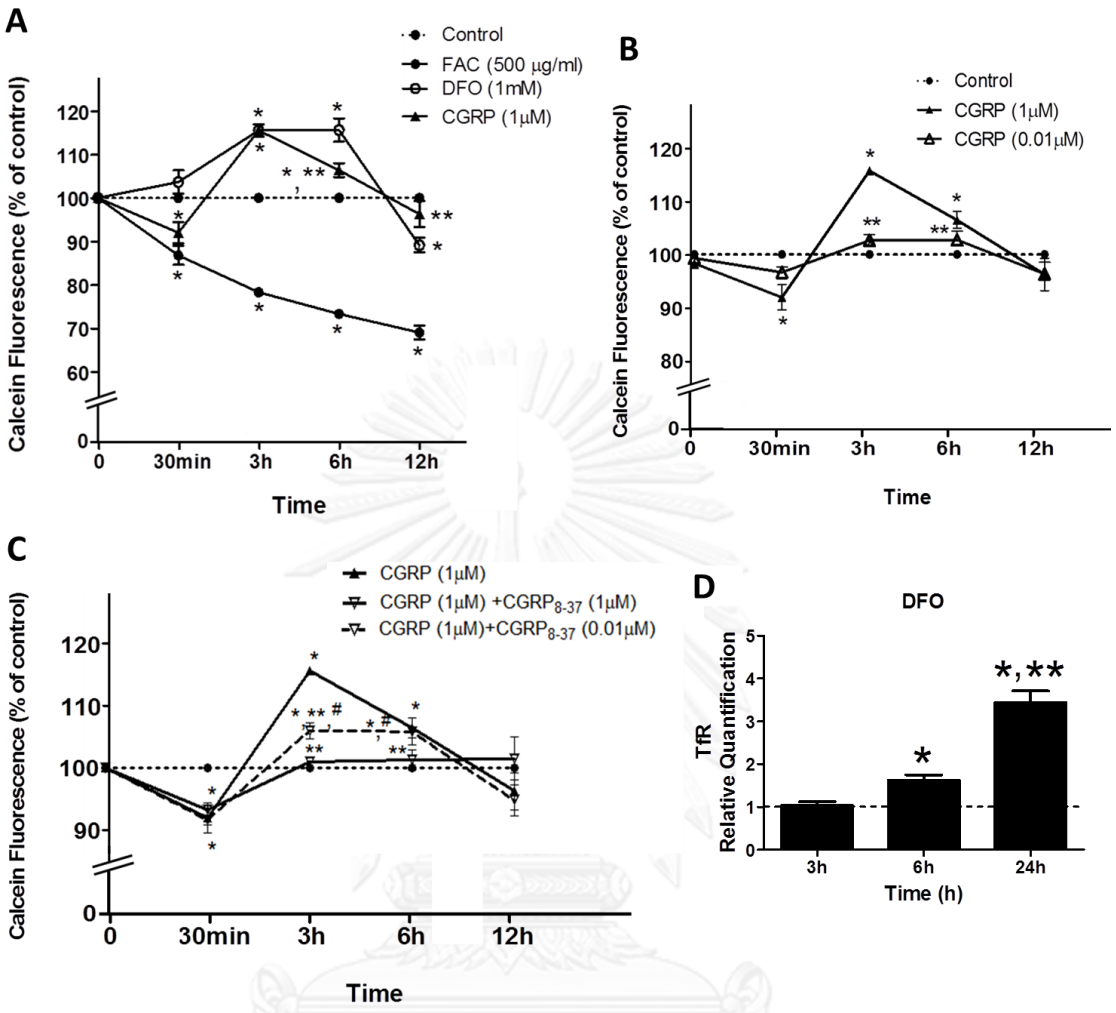

Figure 25 CA fluorescence assay and qRT-PCR for TfR.

(A) Effects of CGRP on intracellular iron levels in Schwann cells, compared to FAC and DFO. **(B)** Concentration-dependent effects of CGRP on intracellular iron levels in Schwann cells. **(C)** Concentration-dependent effects of CGRP antagonist ($CGRP_{8-37}$) on intracellular iron levels in Schwann cells. (* $p<0.05$ compared with the control, ** $p<0.05$ compared with 500 µg/ml FAC (A) or compared with 1 µM CGRP (B, C) or compared with 6 h (D), # $p<0.05$ compared with 1 µM CGRP+1 µM $CGRP_{8-37}$) (C)), **(D)** DFO-induced TfR mRNA expression. Dashed line represents baseline expression in the unstimulated control cultures. Data are presented as the means ± SEM of three independent experiments performed in triplicate (* $p<0.05$ statistically significant).

4.5 Relationship between intracellular iron and CGRP-stimulated Schwann cell activation.

In this set of experiments, iron chelator DFO was used to determine whether intracellular iron status is linked to the cellular activation of Schwann cells by CGRP. DFO (1 mM) was co-incubated with CGRP to deplete the availability of intracellular iron during cellular activation of Schwann cells by CGRP. CGRP caused a significant increase in CA fluorescence intensity during the first 30 min of incubation. After that CA fluorescence intensity was increased by peaking at 3 h post-incubation before returning to the baseline values. In contrast, the presence of DFO during cellular activation by CGRP caused an increase in CA fluorescence intensity peaking at 3 h. After that CA fluorescence intensity continued to decrease. By 12 h which was the last time point examined, CA fluorescence intensity was significantly dropped below baseline values (Fig. 26A). These results suggest that cellular activation of Schwann cells during iron depletion (by DFO) leads to an accumulation of free intracellular iron.

At 3 h compared to CGRP stimulation alone, the presence of DFO during stimulation induced a significant increase in P0 mRNA levels, which subsequently was declined below the baseline values at 6 and 24 h, respectively. This pattern is in contrast to that of Schwann cells that were stimulated by CGRP alone (Fig. 26B). It was also observed that the presence of DFO during CGRP stimulation enhanced the production of IL-6 from Schwann cells (Fig. 26C). Taken together, these results suggest that intracellular iron status is linked to the cellular activation of Schwann cells by CGRP.

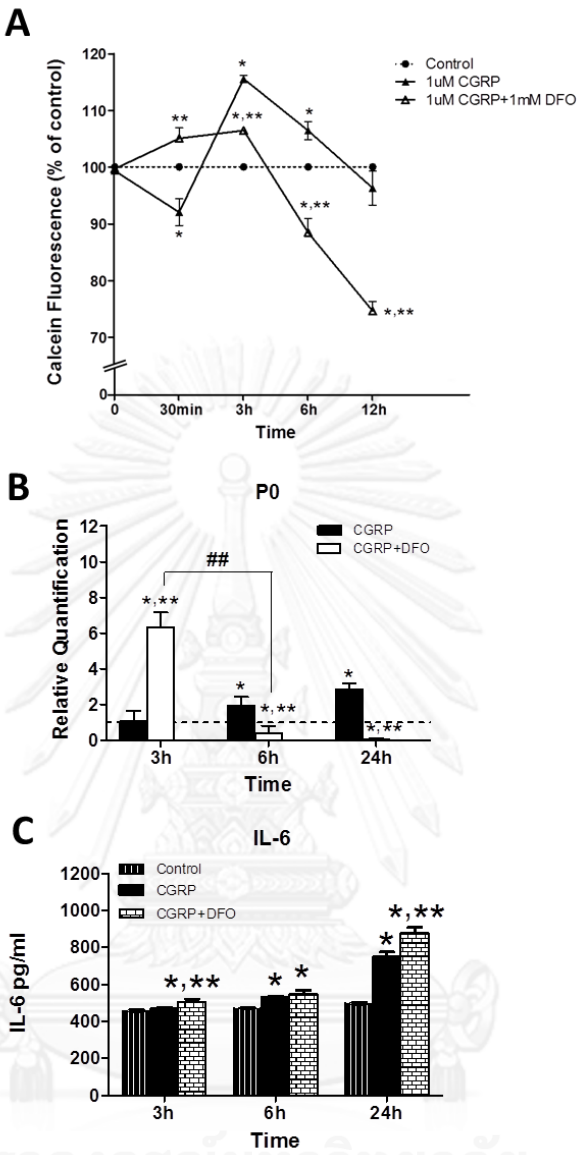

Figure 26 Effects of Iron chelator DFO in CGRP-stimulated Schwann cell activation.

CA fluorescence intensity of cultured Schwann cells following stimulation with CGRP or CGRP combined with DFO. **(B)** Relative quantification of P0 mRNA levels. **(C)** IL-6 protein levels in the cell culture media determined by ELISA. Data are presented as the means ± SEM of three independent experiments performed in triplicate. (* $p<0.05$ statistically significant compared with control; ** $p<0.05$ statistically significant compared with CGRP).

CHAPTER V

DISCUSSION

The present study describes cellular activation of Schwann cells by CGRP, which is a neuropeptide that has been reported to be up-regulated in the TG and associated with migraine pain. The results demonstrate that CGRP induces Schwann cell activation as indicated by up-regulation of myelin P0 and Krox-20, a key transcription factor regulating the expression of P0 and the expression of IL-6 and its receptor. It is also demonstrated that NF-κB signaling is essential for CGRP-stimulated cellular activation of Schwann cells. Moreover, the results suggest that cellular activation of Schwann cell by CGRP is linked to intracellular iron status.

It is established that P0 plays roles in myelin adhesion and compaction and is exclusively expressed by Schwann cells of the peripheral system. The finding that CGRP up-regulates P0 mRNA expression in Schwann cells prompts us to propose that P0 can be used as a specific marker for Schwann cell activation stimulated by CGRP. However, further study is required to elucidate whether P0 will be as useful as the calcium binding protein β (S100β) and glial acidic fibrillary protein (GFAP) in monitoring stages of Schwann cell activation. To my knowledge, this is a novel finding that may provide important new insight into role of Schwann cells in pain facilitation. This is because up-regulation of P0 and Krox-20 by CGRP may enhance myelin compaction and thereby increasing nerve conduction velocity. In addition, previous studies have reported an increase in CGRP expression in central neurons, which coincides with a transient increase in dendritic spine motility indicating the plasticity of the nociceptive circuit underlying the development of neuropathic (149-151). Moreover, it has been reported that repetitive nerve stimulation to injured peripheral nerve promotes nerve transduction and neo-innervation by increase axonal diameter, myelin thickness and neurite sprouting co-exhibited with pain and increased CGRP expression as well as several neurotrophic factors for neuronal plasticity, such as NGF and BDNF (152-154).

The important of CGRP in the inflammatory component of migraine pathophysiology is documented (74, 75). In the second set of experiments, it was found that CGRP stimulated Schwann cells to produce IL-6, which was accompanied by an increase in expression of IL-6R. These findings are in agreement with previous studies showing that pro-inflammatory cytokine IL-6 is elevated in the TG following CGRP administration. However, these studies did not identify cellular source of IL-6.

Thus, the results presented herein complement previously described reports (70, 71). Increase in IL-6 expression in sensory ganglion has been reported to be involved in neuropathic pain development by prolonging neuronal activity via mobilization of presynaptic vesicles and postsynaptic glutamate receptors (108, 126) The finding that up-regulation of IL-6 in Schwann cells accompanied by an increase in expression of IL-6R coincides with a previous report demonstrating that IL-6R expression is regulated by IL-6 (155). Taken together, it suggests that IL-6 derived from Schwann cells could exert both a paracrine and an autocrine effects.

The results from the third set of experiments demonstrate that NF-**κ**B signaling is essential for CGRP-stimulated cellular activation of Schwann cells. Inhibition of NF-**κ**B by cardamonin suggests that NF-**κ**B regulates Krox-20 expression, which in turn controls P0 mRNA levels. This is in agreement with results from previous study showing a reduction in Krox-20 expression in mice expressing a transgenic suppressor of NF-**κ**B (122, 156). The NF-**κ**B subunit p65 has been reported to enhance histone deacetylation and activates activity of P0 promoter. Overexpression of p65 mutants exhibits an increase in *P0* promoter activity, thus suggesting that NF-**κ**B regulates myelination phenotypes of Schwann cells at transcription level (157). The results also indicate that NF-**κ**B is required for CGRP-induced IL-6 expression and production in Schwann cells. Thus, it appears that NF-**κ**B signal is essential for CGRP-induced Schwann cell myelination and inflammation.

Increase in TfR expression in Schwann cells has been consistently reported following nerve injury (140, 147). This suggests that TfR may be a functional marker associated with Schwann cell activation and response to injury. It also suggests that Schwann cell activation could be linked to cellular iron status. The results from this study indicate that both LPS and CGRP alter intracellular iron levels in Schwann cells without changing in expression of TfR. Based on results from CA fluorescence assay, which reveals a brief increase in intracellular iron levels following CGRP stimulation followed by a long reduction period and subsequently a period of returning to the baseline level, these findings can be interpreted that cellular activation of Schwann cells associates with mobilization of intracellular iron, most likely, between iron in the free pool and iron stored in the ferritin to restore intracellular iron homeostasis during the activation. Thus, in this case there is no need for Schwann cells to uptake more iron from extracellular environment. On the other hand, changes in TfR expression in Schwann cells described in the nerve injury models could reflect a bigger demand of Schwann cells for iron, for example during proliferation phase to

duplicate DNA and during remyelination to synthesize lipid and myelin proteins. This speculation is in agreement with the fact that iron is an essential cofactor of many key enzymes involved in such processes (130).

The relationship between intracellular iron status and CGRP-stimulated Schwann cell activation was described in the last set of experiments using iron chelator DFO. It is speculated that intracellular iron depletion by DFO could decrease expression of P0 and IL-6 expression induced by CGRP. This hypothesis was simply based on the assumption that cellular activation of Schwann cells depends on intracellular iron status as suggested by the afore-mentioned results. As expected, it was found that co-incubation between CGRP and DFO led to the inhibition of P0. However, the primary cause of this effect is unlikely to be the result of intracellular iron depletion. On the opposite, it results from an intracellular accumulation of iron as indicated by a continuous decrease in CA fluorescence intensity over time (Fig. 6A). Subsequently, free iron enhances the generation of intracellular ROS through the Fenton reaction. Supportively, accumulation of intracellular ROS has been shown to down-regulate several myelin proteins including P0 and PMP22 (158). The finding that DFO increases expression of IL-6 is opposite to what would be expected. Increase in IL-6 expression coincides with intracellular accumulation of iron suggesting that IL-6 expression in Schwann cells may be linked to cellular iron status. Indeed, it has been reported that activation of NF-**κ**B is required iron as cofactor (159). Furthermore, DNA binding activity of NF-**κ**B is enhanced by ROS (160)

In summary, the present study provides new evidence involving Schwann cell biology and the essential role of iron and IL-6 in neurogenic pain by showing (1) CGRP stimulates P0 expression in Schwann cells and further suggest that P0 can be used as a specific marker for CGRP-induced Schwann cell activation, (2) CGRP-upregulated P0 expression in Schwann cells is mediated by the transcription factor Krox-20, (3) CGRP induces IL-6 production and IL-6R expression in Schwann cells and suggest that IL-6 released from Schwann cells should have both a paracrine and an autocrine effects, (4) CGRP-stimulated Schwann cell activation is mediated by NF-**κ**B and (5) CGRP stimulates changes in iron metabolism in Schwann cells and Schwann cells possess a regulatory mechanism to maintain cellular iron homeostasis.

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APPENDIX

APPENDIX

SPECIFIC BUFFER AND REAGENTS

1. Dulbecco's Modified Eagle's medium (DMEM) stock medium	1 liter
Sodium Pyruvate powder	110 mg
Penicillin-Streptomycin solution	10 ml
HEPES, free acid	10 ml
Sterilized by filtering through a 0.45 μm membrane filter	
Store at 4°C	
2. 1X Phosphate Buffered Saline (PBS)	1 liter
Phosphate Buffered Saline dry powder in foil punch 1 packet	
Add ddH_2O up to 1 liter and sterilized by autoclaving	
3. 10X Phosphate Buffered Saline (PBS)	100 ml
Phosphate Buffered Saline dry powder in foil pouch 1 packet	
Add ddH_2O up to 1oo ml and sterilized by autoclaving	
4. 70% Ethanol	1 liter
Ethanol	700 ml
ddH_2O	300 ml
Store at -20°C	
5. Phenol-Chloroform (25: 24: 1)	500 ml
Phenol	250 ml
Chloroform	240 ml

Isoamyl alcohol (2-propanol) 10 ml

Store at 4°C

6. LPS

Stock 1 mg/ml

Final Conc. 1 μg/ml of LPS

VITA

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