การศึกษาโปรตีนจำเพาะในระบบประสาทส่วนกลางของสุนัขที่เป็นโรคพิษสุนัขบ้าในระยะต้นและท้ายของโรค

นายแสงชัย ยิ่งศักดิ์มงคล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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DIFFERENTIAL EXPRESSION OF PROTEINS IN THE CENTRAL NERVOUS SYSTEM OF EARLY AND LATE STAGE RABIES INFECTED DOGS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biomedical Sciences
(Interdisciplinary Program)
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Thesis Title	DIFFERENTIAL EXPRESSION OF PROTEINS IN THE CENTRAL NERVOUS SYSTEM OF EARLY AND LATE STAGE RABIES INFECTED DOGS
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Field of Study	Biomedical Sciences
Thesis Advisor	Professor Thiravat Hemachudha, M.D.

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แสงชัย ยิ่งศักดิ์มงคล : การศึกษาโปรตีนจำเพาะในระบบประสาทส่วนกลางของสุนัขที่เป็นโรคพิษสุนัขบ้าในระยะต้นและท้ายของโรค (DIFFERENTIAL EXPRESSION OF PROTEINS IN THE CENTRAL NERVOUS SYSTEM OF EARLY AND LATE STAGE RABIES INFECTED DOGS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. นพ. ธีระวัฒน์ เหมะจุฑา, 68 หน้า.

โรคพิษสุนัขบ้าเป็นโรคติดต่อได้ในสัตว์เลี้ยงลูกด้วยนม รวมถึงคนและสุนัขที่เป็นตัวแพร่โรคที่สำคัญในประเทศไทยและแถบเอเชีย แอฟริกา และยุโรป อาการในคนและสัตว์ สามารถแยกได้เป็น 2 แบบคือ แบบดุร้ายและ แบบเซื่องซึม มีการศึกษาถึงการเกิดโรคมาเป็นระยะเวลานานแล้ว แต่อย่างไรตามโรคพิษสุนัขบ้าก็ยังไม่ได้หมดไป ในการศึกษาครั้งนี้เป็นการใช้เทคนิคโปรตีโอมิกส์มาศึกษาการแสดงออกของโปรตีนในระยะต้นและท้ายของการเกิดโรค โดยจัดกลุ่มสุนัขที่ออกตามระยะเวลาของการเกิดโรคเป็น 5 กลุ่ม คือ 1)สุนัขที่ป่วยระยะแรกแบบดุร้าย 2)สุนัขที่ป่วยระยะท้ายแบบดุร้าย 3)สุนัขป่วยระยะแรกแบบอัมพาต 4)สุนัขป่วยระยะท้ายแบบอัมพาต และ 5)สุนัขที่ไม่ได้ป่วยจากโรคพิษสุนัขบ้าตามลำดับ แล้วเก็บสมองส่วนฮิปโปแคมปัส สมองกลีบขมับ ก้านสมอง และไขสันหลัง มาแยกโปรตีนด้วยโพลีอคริลาไมด์เจลแบบมิติเดียวร่วมกับวิธีแมสสเปกโตเมทรี และคัดเลือกแยกโปรตีนที่จำเพาะด้วยโปรแกรมทางสถิติและกระบวนการทางชีวสารสนเทศที่เหมาะสม พบโปรตีนที่แสดงออกจำเพาะและมีความต่างกันอย่างมีนัยสำคัญทางสถิติ ในสมองส่วนฮิปโปแคมปัส สมองกลีบขมับ ก้านสมอง และไขสันหลัง จำนวน 26, 7, 35 และ 18 ชนิด ตามลำดับ โปรตีนเหล่านี้มีหน้าที่ เป็นโปรตีนโครงสร้างค้ำจุน เอนไซม์ การตายของเซลล์ ภูมิคุ้มกัน เป็นต้น การศึกษาครั้งนี้ถือเป็นการศึกษาโปรตีโอมในเนื้อเยื่อระบบประสาทส่วนกลางในสุนัขที่ติดเชื้อพิษสุนัขบ้าตามธรรมชาติเป็นครั้งแรกทั้งในระยะแรกและระยะท้าย ช่วยพัฒนาองค์ความรู้ด้านการเกิดพยาธิสภาพในสมองส่วนต่าง ๆ อันเป็นข้อมูลที่สำคัญสำหรับศึกษา กลไกพยาธิกำเนิดในระบบประสาทส่วนกลาง และประยุกต์ไปใช้สำหรับการตรวจคัดกรองและการรักษาโรคพิษสุนัขบ้า

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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SANGCHAI YINGSAKMONGKON: DIFFERENTIAL EXPRESSION OF PROTEINS IN THE CENTRAL NERVOUS SYSTEM OF EARLY AND LATE STAGE RABIES INFECTED DOGS. ADVISOR: PROF. THIRAVAT HEMACHUDHA, M.D., 68 pp.

Rabies is a zoonotic disease. Dogs are an important reservoir host of rabies in Thailand, Asia, Africa and Europe. The clinical manifestations are the same in humans and dogs, and are categorized into 2 forms; furious and paralytic. Although there have been many rabies studies over the years, the pathogenesis of rabies is still unclear. In this study, proteomic analysis was used to investigate changes in host responses in central nervous system (CNS) tissues at early and late stages of disease compared to non-infected dog controls. They were divided in 5 groups; early furious (FE), late furious (FL), early paralytic (DE), late paralytic (DL) and control (N), respectively. Hippocampus, parietal lobes, brainstem and spinal cord tissues from these 5 groups were collected and stored until examination. Proteins were extracted from these tissues and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in combination with liquid chromatography mass spectrometry (LC-MS/MS). The proteins were statistically selected by suitable bioinformatics tools. There were 26, 7, 35 and 18 proteins expressed only in each hippocampus, parietal lobes, brainstem and spinal cord, respectively. They played role in cytoskeleton proteins, enzymes, proteins associated with cell death, inflammation and immunity. This is the first report of changes in proteome data set from rabies-infected CNS tissues in both forms, and in early and late stages of infection. This data will be useful for not only better understanding of the molecular mechanism in many regions of CNS tissues, but also diagnostic and therapeutic of rabies.

Field of Study: Biomedical Sciences Student's Signature .....

Academic Year: 2016 Advisor's Signature .....

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LIST OF ABBREVIATIONS

1-DE	One-dimensional gel electrophoresis
2-DE	Two-dimensional gel electrophoresis
ANOVA	One-way analysis of variance
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
CSF	Cerebrospinal fluid
CVS	Challenge strain virus
DRG	Dorsal root ganglion
G protein	Glycoprotein
HIV-1	Human immunodeficiency virus -1
ICE	Caspase I
IAV	Influenza A virus
Kb	Kilobase
Da	Dalton
L protein	RNA-dependent RNA polymerase or large protein
M protein	Matrix protein
MALDI-MS	Matrix-assisted laser desorption/ionization mass spectrometry
LC MS/MS	Liquid chromatography mass spectrometry
mRNA	Messenger RNA
N protein	Nucleocapsid
P protein	Phosphoprotein
PCR	Polymerase chain reaction
RNP	Ribonucleoprotein complex
RV	Rabies virus
SHBRV	Silver hair bat rabies virus
TUNEL	Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end-labeling

CHAPTER I

INTRODUCTION

Background and Rationale

Rabies is an infectious viral disease of the central nervous system (CNS) that is almost always fatal when symptoms and signs occur. Rabies is a zoonotic disease and dogs are the main source of human rabies deaths, contributing up to 99% of all rabies transmission to humans. Tens of thousands of human deaths are reported every year, mostly in Asia and Africa. Survival cases from rabies have been reported, though recovered patients were mainly infected bat variant rather than dog variant of rabies virus (RV) (Alvarez et al., 1994; de Souza & Madhusudana, 2014; Hattwick, Weis, Stechschulte, Baer, & Gregg, 1972; Porras et al., 1976). Immune functions were more suppressed in dog-related cases than bat-related cases (Hemachudha, Laothamatas, & Rupprecht, 2002). The development of serum antibody is unpredictable as compare to bat-related cases. Serum and cerebrospinal fluid (CSF) rabies antibody appeared during the time of survival or recovery from the rabies (Hemachudha, 1994).

Human rabies can be categorized in atypical, typical or non-classical and classical forms. In classical from, there are two different clinical manifestations; furious and paralytic rabies. These two forms of rabid cases can be classified by the differences of viral dissemination, clinical presentation and host immune response (Hemachudha et al., 1988). The classical furious rabies in human is characterized by fluctuating consciousness, changed mental status, phobic or inspiratory spasms, and autonomic stimulation. Paralytic rabies present flaccid muscle weakness and paralysis, which resembles Guillain-Barré syndrome (Hemachudha et al., 2002). All rabies patients develop coma and death, with an average of 5.7 days for furious rabies, and 11 days in paralytic rabies starting from clinical onset of disease without intensive care support (Hemachudha et al., 2013; Hemachudha et al., 2005). On the other hand, non-classical rabies associated with bat rabies variant exhibit atypical features, such as signs and

symptoms of Horner's syndrome (Hemachudha et al., 2002; Hemachudha & Phuapradit, 1997). The distribution of rabies viral antigen in CNS between furious and paralytic rabies was not different in human cases. Rabies nucleocapsid (N) antigen was found to be confined mainly in midline structures: thalamus, brainstem and basal ganglia during the early clinical phase, and the inflammation was generally mild throughout the neuroaxis (Shuangshoti et al., 2016; Tirawatnpong et al., 1989). In terms of pathogenesis; both forms in dogs share a similar pattern of viral antigen distribution in the CNS regions during the clinical phase. Interestingly, paralytic rabies shows significant inflammation in brainstem, more so than furious form, during the early period. It may be that inflammatory response in this region of paralytic rabies prevents or retards the virus from travelling to the cerebral hemisphere (Shuangshoti et al., 2013; Shuangshoti et al., 2016; Suja, Mahadevan, Madhusudhana, Vijayasarathi, & Shankar, 2009).

Furious rabies shows more viral load in the brain and lower immune response when compared with the paralytic rabies. Moreover, the amount of virus, determined by nucleocapsid (N) gene, showed significantly more manifested distribution in many regions of CNS than paralytic form. The mutation of the rabies nucleoprotein at positions 273 (Glu) and 394 (Gln) may enhance immune evasion and increase pathogenicity (Masatani et al., 2011). The variation of the virus itself could occur as quasispecies during rabies viral infection in neuronal cells in different parts of the brain and spinal cord. In particular, the brainstem is the preferred area of RV manifestation. Neuronal cells in brainstem might be involved in virus replication, dissemination and host immune responses (Shuangshoti et al., 2013). From live cell imaging, the RV bound to p75 neurotrophin receptor (p75NTR) affected the axonal transport process, and resulted in a faster and proficient way to nerve cells (Gluska et al., 2014).

After RV infection into the neuronal cells of the CNS, apoptosis could occur and continue to emit unusual neurotransmitter signals. Pathophysiological changes occur. The furious rabid cases show significantly increased cellular immunity to rabies viral antigen. The levels of cytokine production such as proinflammatory cytokines and nitric oxide are found higher than paralytic rabies. The furious rabies, associated with cell-mediated immune response, has more extensive propagation of RV to the brain than the paralytic (Hemachudha et al., 2013).

Generally, RV propagates through the central nervous system and induces apoptotic processes in the neuronal cells, however the process of cell death are not completely undertaken. There was a report concerning the survival of nerve cells in the different regions of the brain and spinal cord. The results showed some neuronal cells, predominantly in spinal cord and brainstem regions, that had a delayed process in the apoptotic pathway. The evidence of mitochondrial cell death, which can occur from the leakage of cytochrome C in the cytoplasm representing mitochondrial outer membrane permeabilization, is a remarkable mechanism of RV. This allows RV's survival in cells and lower immunological responses than other viruses (Thanomsridetchai et al., 2011). It may suggest that this apoptosis process could be cell protection rather than a pathogenic mechanism.

Rabies virus-host protein interactions have been continuously studies. Protein characterization and functional pathways are being discovered more rapidly than previously, due to modern technologies such as mass spectrometry and compatible computational network programs. The host protein is an important factor that may explain the clinical differences between furious and paralytic rabid cases. The phenomenon could explain the RV's unique mechanism to infect the host cells and present as either of the two clinical manifestations. Some proteins are predominantly expressed during the infection, or when presenting specific clinical signs and symptoms. In Thailand, rabid dogs expressed proteins that are associated with 11 main functional categories such as antioxidant, apoptotic-related protein, cytoskeleton protein, heat shock protein/chaperones and immune regulatory proteins (Thanomsridetchai et al., 2011).

In this study, the four parts of CNS tissues; hippocampus, parietal lobes, brainstem and spinal cord from rabies infected and non-rabies infected dogs were collected. The tissues were prepared, purified and analyzed by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) combined with liquid chromatography mass spectrometry (LC-MS/MS). The proteomic data were analyzed based on reference database in order to determine the protein expressions in different part of CNS tissues of rabid dogs, and explain the correlation between specific expressed proteins in early and late stages of disease. Especially in early stage of rabies

infection, specific expressed proteins were identified for diagnosis or predict the clinical outcomes. The host response, neurophysiology and viral pathogenesis were studied in an appropriate model. The mechanisms of virus-host interaction and specific proteins which lead to the clinical differences in furious and paralytics forms were proposed. Finally, the target proteins important to clinical development of early and late stages rabies infected dogs were found.

2. Research Question

Is there any correlation between specific protein(s) expressed in the central nervous system and clinical rabies?

Are there any difference in protein expression in the central nervous system of early and late stage rabies infected dogs?

3. Objectives

1. To analyze the protein(s) expression in hippocampus, parietal lobes, brainstem, and spinal cord of rabies infected dogs and non-rabies infected dogs

2. To determine specific protein(s) in furious and paralytic rabies-infected dogs

3. To study the difference of specific protein(s) expressed in each stage of infection.

4. Hypothesis

There are differences in specific protein(s) expressed in the central nervous system of early and late stages rabies infected dogs.

5. Key words

Rabies; Proteomics; Furious; Paralytic; Early; Hippocampus

6. Expected benefit and Application

This study is to prove the protein expression in the CNS of early and late stage rabies-infected dogs and explain the conspicuous protein(s) in CNS, and as disease progresses, compare between furious rabies, paralytic rabies and non-rabies infected dogs. The results of this study should explain the host responses to viral infection, which provides insight into rabies disease mechanism.

CHAPTER II

LITERATURE REVIEW

Rabies Virus

Rabies caused by rabies virus (RV) is a unique virus which can travel, via nerve fibers, through the central nervous system (CNS) of humans and other mammals. This virus belongs to the family *Rhabodoviridae* and genus *Lyssavirus*, which is a non-segmented negative-stranded RNA, approximately 12 kb in size. Its genome encodes for five proteins as nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the viral RNA-dependent RNA polymerase (L) (Albertini, Ruigrok, & Blondel, 2011). (Figure 1) There are many variant strains of this virus, each variant has maintained in a particular reservoir host. The preferential sites of RV are motor endplate in the muscle, and entry via the motor route by retrograde transneuronal transfer through the central nervous system tissues, especially in brainstem, thalamus, basal ganglia and spinal cord. It is difficult to diagnose rabies at an early stage based on the clinical signs alone unless local neuropathic pain involving the bitten limb is experienced. Although there is no clinical evidence of brain and spinal cord involvement, there has been a report of magnetic resonance (MR) abnormalities in furious rabies patients (Laothamatas, Sungkarat, & Hemachudha, 2011). The incubation period can vary from weeks to years, but lasts 1-2 months on average. Average survival of furious rabid patient is shorter than paralytic patients (Hemachudha et al., 2013).

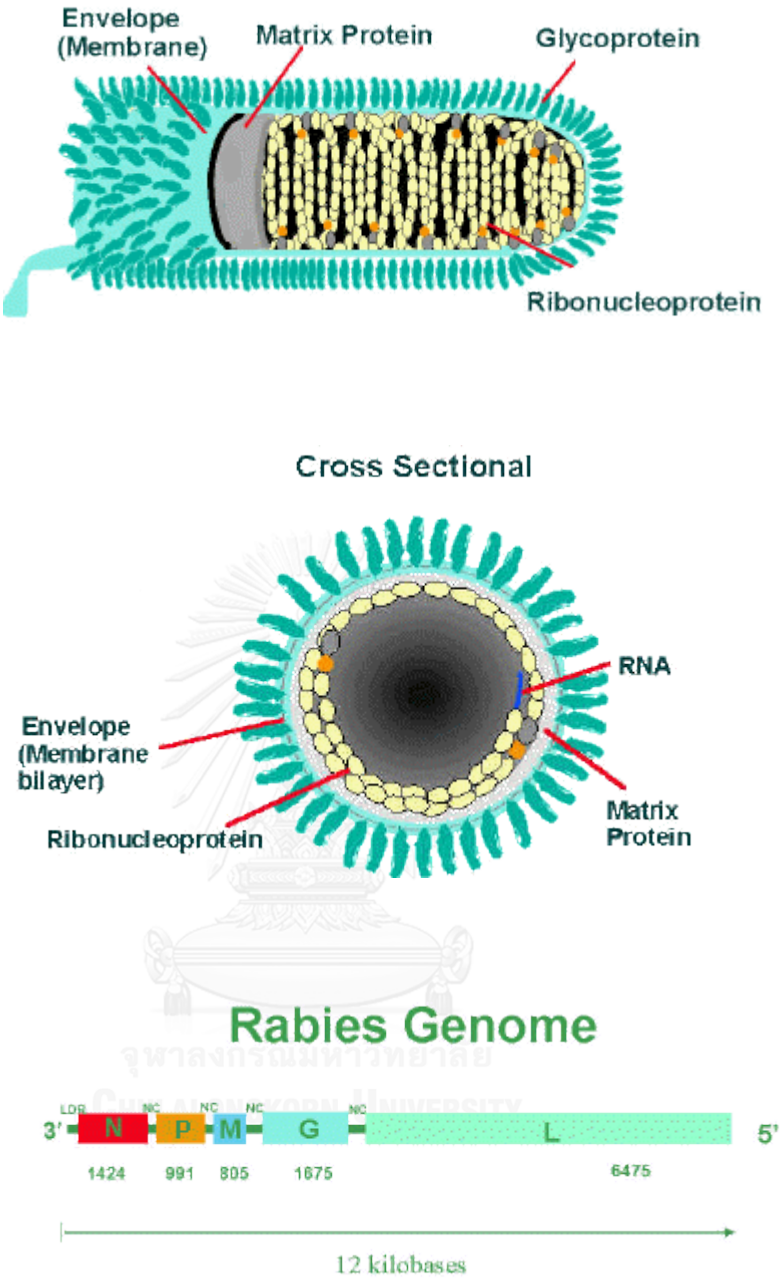

Figure 1 **Structure and genome of rabies virus**

(From *https://www.cdc.gov/rabies/transmission/virus.html*)

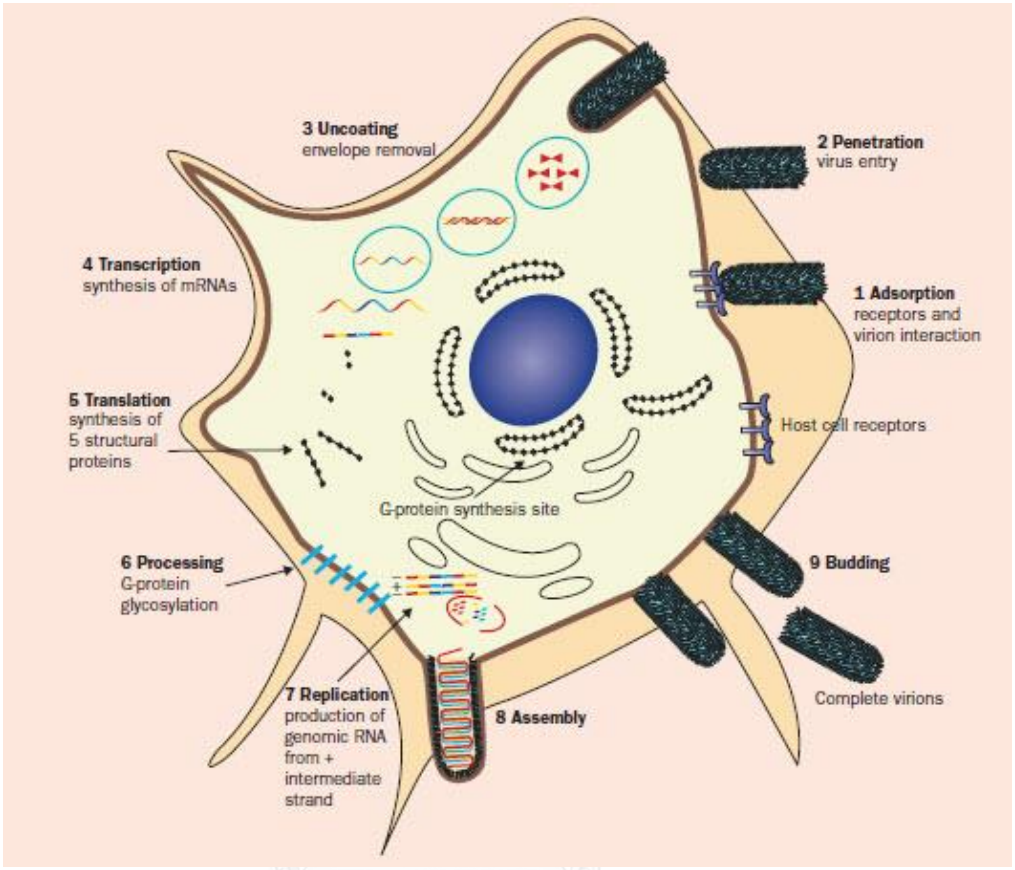

Figure 2 **Concept flow of lyssavirus replication in a host cell. (Rupprecht, Hanlon, & Hemachudha, 2002)**

Clinical manifestations of rabies

RV infection has 2 clinical forms, furious and paralytic rabies, where the survival time, clinical features, viral load, and host immune response are different (Hemachudha et al., 2013). Furious rabies patient presents with hyper-reactivity to external stimuli, constant and persistent fever. Initiation, mentation is normal, but attention period may be shortened. Then three major cardinal signs are: consciousness, phobic spasm and autonomic stimulation signs. In furious rabies, mental status changes to severe agitation and depression with aerophobia and hydrophobia, followed by coma and then death. Paralytic rabies is difficult to diagnose due to the lack of aggression. The major cardinal signs appear late and are not prominent. The sign of muscle weakness of the bitten limb spreads to all limbs, similar to Guillain-Barré syndrome (GBS). The persistent fever from the onset of limb weakness and percussion myoedema, elicited on the chest, deltoid and tight regions, can be used to differentiate rabies from GBS (Hemachudha et al., 2013). The comparison between paralytic and furious rabies form were shown in table 1.

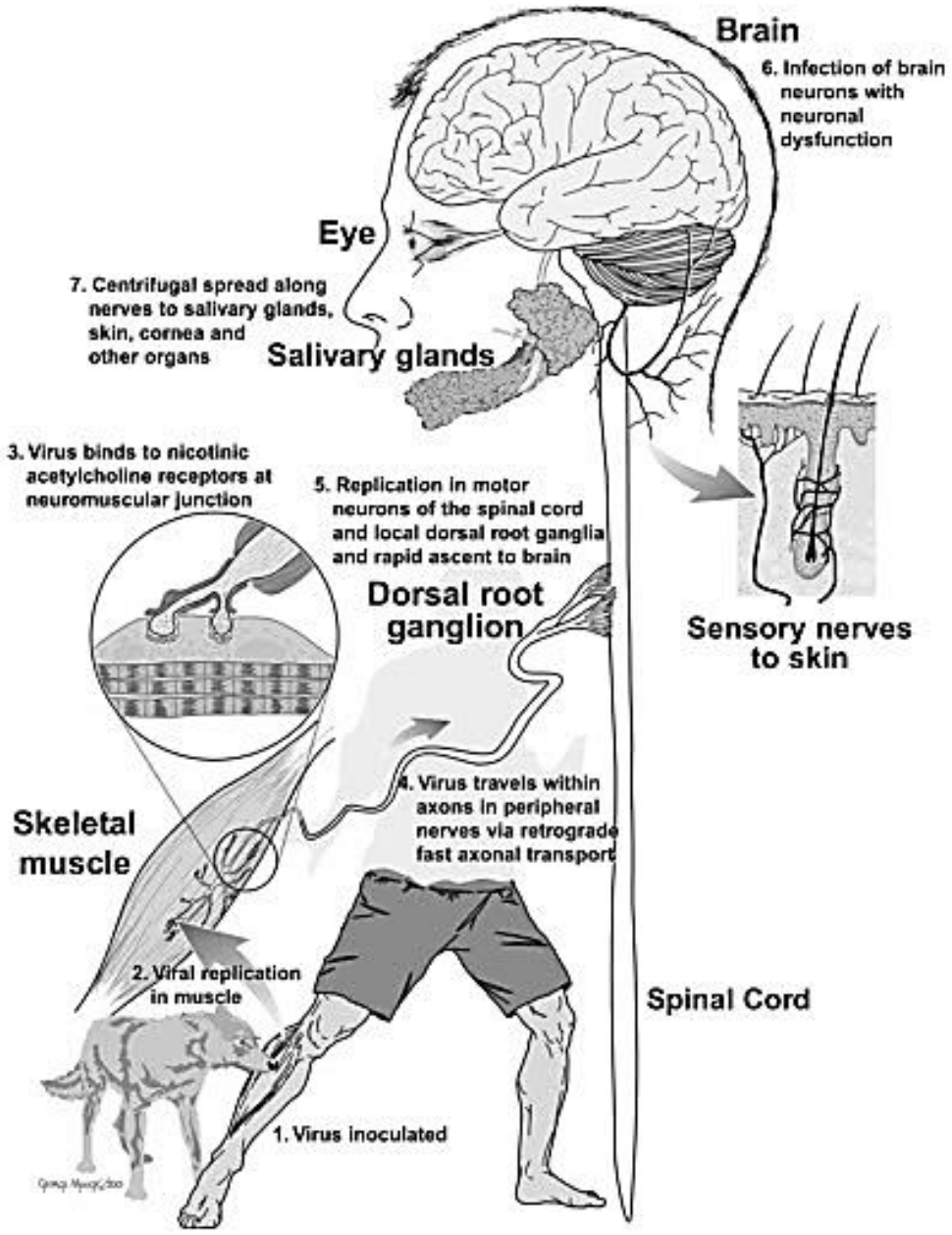

Figure 3 **Schematic diagram showing the steps in the pathogenesis of rabies after an animal bite/peripheral inoculation of rabies virus (Jackson, 2013).**

	Furious	Paralytic
General features in patients infected with dog RABV variants		
Prevalence[2]	2/3 (67%)	1/3 (33%)
Average survival without intensive care support[2]	5·7 days (n=80)	11 days (n=35)
Location of bite and relation to unsuccessful immunisation[2-4]	Anywhere; not related	Anywhere; not related
Prodromal symptoms[2-4]	Non-specific with local neuropathic pain in a third of patients	Non-specific with local neuropathic pain in a third of patients
Rabies characteristics*[2-4]	Present, but might not be seen at all stages	None or minimal, phobic spasms in only half, inspiratory spasms might not be obvious due to weakness of neck muscles and diaphragm; percussion myoedema at deltoids and chest wall (in the absence of hyponatraemia, renal failure, hypothyroidism, and severe cachexia)
Sensory deficits[2-4]	At bitten segment due to ganglionitis; loss of pinprick sensation followed by loss of joint position sense	At bitten segment due to ganglionitis; loss of pinprick sensation followed by loss of joint position sense
Flaccid weakness with areflexia[2-4]	Appears only when comatose	Ascending pure motor weakness, predominantly involving proximal and facial musculature as initial manifestation, while consciousness is fully preserved
Electrophysiological features[2,5]	Subclinical anterior horn cell dysfunction; sensory neuronopathy in patients with local neuropathic symptoms	Evidence of peripheral demyelination or axonopathy; sensory neuronopathy in patients with local neuropathic symptoms
MRI findings in patients infected with dog RABV variants[6,7]		
Prodromal phase	Enhancing hypersignal T2 changes along the brachial plexus and associated spinal nerve roots at levels corresponding with the bitten extremity; non-enhancing ill-defined mild hypersignal T2 changes of the spinal cord, temporal lobe cortices, hippocampal gyri, and cerebral white matter	Enhancing hypersignal T2 changes along the brachial plexus and associated spinal nerve roots at levels corresponding with the bitten extremity; non-enhancing ill-defined mild hypersignal T2 changes of the spinal cord, temporal lobe cortices, hippocampal gyri, and cerebral white matter
Acute neurological (non-comatose) phase	Progression of abnormal hypersignal T2 changes	Progression of abnormal hypersignal T2 changes
Comatose phase	Moderate gadolinium enhancement, especially in limbic structures, thalamus, substantia nigra, tectal plates, brainstem, deep grey matter, cranial nerve nuclei, spinal cord, and cranial and spinal nerve roots	Moderate gadolinium enhancement, especially in limbic structures, thalamus, substantia nigra, tectal plates, brainstem, deep grey matter, cranial nerve nuclei, spinal cord, and cranial and spinal nerve roots
General features of early-stage rabies in naturally infected dogs[7-9]		
Viral load in brain structures[8]	Several times greater than paralytic at all 12 regions examined	Several times lower than furious at all 12 regions examined
Cytokine or chemokine mRNA transcripts[8]	Barely detected; TNFα detectable but at non-significant concentration	TNFα, interferon γ, and interleukin 1β
FLAIR signal abnormality indicative of macrocellular damage revealed by MRI[7,8]	Faint signal in cervical cord, brainstem, temporal lobes, and cerebral hemispheres†	Moderate-to-intense signal in hypothalamus, brainstem, cervical cord, and temporal lobes†
Blood–brain barrier status (examined by presence or absence of contrast-enhanced lesion)[7,8]	Intact; no contrast-enhanced lesion	Intact; no contrast-enhanced lesion
Neuropathology[9]	Caudal–rostral polarity of viral antigen; greater viral antigen reported in many regions, including frontal and occipital cortices and most spinal cord levels; inflammation generally mild throughout the CNS	Prominent inflammation in brainstem, in association with lower extent of viral antigen; caudal–rostral polarity of viral antigen

RABV=Rabies virus, TNFα=tumor necrosis factor α, FLAI=fluid-attenuated inversion recovery. *Change in consciousness, phobic spasms, spontaneous inspiratory spasms, and autonomic dysfunctions.

Table 1 **Feature of furious and paralytic rabies in patients infected with dog RABV variants and it naturally infected dogs. (Hemachudha et al., 2013)**

Pathogenesis of rabies virus

RV is a zoonotic disease that can be transmitted via a bite from a rabid animal. Transmission efficiency depends on severity of bite, site of infection, and amount of virus in the saliva. Non-bite exposure, such as ether transdermal and mucous membrane, are rarely found in this disease (Rupprecht et al., 2002). However, contamination of open wound, a skin abrasion, oral mucous membrane, or the exposure of conjunctiva, or genitalia with the saliva of rabid animal are risks of transmission of rabies. Rarely, RV has been found to spread to humans via aerosol route in bat caves or laboratory accidents with infected aerosolized tissues (Constantine, 1967; Winkler, Fashinell, Leffingwell, Howard, & Conomy, 1973).

In cases where canine RV has successfully inoculated into the wound, the viral glycoprotein binds to the nicotinic acetylcholine receptor on the muscle or other cells at the neuromuscular junction. After viral budding, the virus is taken up into the unmyelinated nerve endings at neuromuscular junctions or at the muscle spindles. Rabies is transported into the CNS by retrograde axoplasmic pathway (Hemachudha et al., 2002). Then the virus infects and replicates in the dorsal root ganglion and anterior-horn cells. At the dorsal root ganglion, viral replication occurs and may be recognized by immune cells, resulting in ganglioneuritis and clinical prodrome of neuropathic pain at the bite site. Rabies can enter directly into nerves without replication in muscle or skin (Shankar, Dietzschold, & Koprowski, 1991). The constant rate of rabies migration into the CNS is 8-20 mm/day, while time taken depends on the inoculum site from the CNS (Tsiang, 1993). The virus can use carbohydrates, phospholipids, gangliosides, neural-cell adhesion molecule (CD56) and low-affinity nerve-growth-factor receptors (p75 neurotrophin receptor) to gain entry into the cells (Thoulouze et al., 1998; Tsiang, 1993; Tuffereau, Benejean, Blondel, Kieffer, & Flamand, 1998). After the virus reaches the CNS, virus localizes, preferably in the brainstem, thalamus, basal ganglion and spinal cord. From the CNS, virus spreads along the neuronal pathway to many organs such as heart, skin and especially saliva glands. The RV sheds from these saliva glands, completing the transmission cycle.

Rabies and host protein interaction

Genomic technology has introduced researchers to a new face of viral-host protein interactions, including virus replication and spread, pathogenic effects on cells, and antiviral responses. Various methods are now available to identify host genes that may be essential for the RV life cycle, antiviral defense or others nonspecifically in RV infection. Each technology is suited for the detection of different classes of genes and different levels of gene expression. In 2001, differential display (DD) cDNA array hybridization, subtraction hybridization (SH) and restriction fragment differential display (RFDD) were used to identify approximately 39 genes from the mouse brains that were injected with RV infection. These included genes involved in regulation of cell metabolism, protein synthesis, synaptic activity, and cell growth and differentiation. By cDNA array hybridization, upregulation of eyes absent homolog 2 (early eye development protein), early growth response protein 3 and signal transducers and activators of transcription 1 (STAT1-transcription factors), major prion protein precursor associated in scrapie-associated fibril protein, programmed cell death 2 (cell death-associated protein), thrombomodulin (anticoagulant), VAMP1 (Exo- and endocytosis) and N-cadherin (cell adhesion) were found and confirmed by using Northern hybridization (Prosniak, Hooper, Dietzschold, & Koprowski, 2001).

RV is transcribed and replicated in neuronal and inflammatory cells of CNS. There were few apoptotic neurons but there was an increase of apoptotic in adaptive immune cells in the rabies infected mice (Fernandes et al., 2011). The experiment used rabies virus that collected from vampire bats. In 2005, 10 rabies patients, 5 furious and 5 paralytic, were post-mortem investigated for evidence of apoptosis and mitochondrial outer membrane permeabilization in brain and spinal cord. The results showed that some neuronal cells, especially in spinal cord and brainstem regions, had a delay in apoptotic processes. It mediated via cytochrome C of the mitochondrial pathway. Downregulation of G protein expression in neuronal cells contributed to pathogenesis by preventing apoptosis and the associated inflammation process. It might be concluded that the apoptosis in brainstem and spinal cord was one of RV's survival method, to stay longer in functional cells. It has also been previously demonstrated that RV-infected cultured purified rat embryonic spinal cord motor neurons resisted cytolysis

and apoptosis. In animal model, similar result was found when RV was injected into rat neonates. Moreover, the RV-infected motoneuronal axons were elongated, indicating that metabolic activity was maintained in these infected cells. On the other hand, hippocampus neurons were apoptotic shortly after infection (Guigoni & Coulon, 2002). These suggested that survival of neuron at different regions is variable, and does not depend on the nature of the virus strain. There are varying explanations on the degree of resistance to apoptosis. Thus, in RV infection, it seems that there are complexities involved in cell death or survival of neurons both *in vitro* and in animal models.

Rabies and proteomic researches

RV infection results in neuronal dysfunction. To prove this theory, proteomics technology was developed to examine the expressed proteins involved in ion homeostasis after RV infection. The results showed that there were upregulation of H^+ATPase and Na^+/K^+ ATPase, while Ca^{2+}ATPase was downregulated. Furthermore, the α-synaptosome-associated protein (SNAP), tripartite motif-containing 9 (TRIM9), syntaxin and pallidin were downregulated after wild type (wt) rabies infection. These proteins are involved in docking and fusion of synaptic vesicles to the presynaptic membrane. Thus downregulation of these proteins results in ion imbalances, leading to neuronal dysfunction (Dhingra, Li, Liu, & Fu, 2007).

In order to extend the knowledge of rabies and host cell responses, BHK-21, neuro-2a (N2a) and Vero cells were used to determine protein expression. In BHK-21 cells after RV infection, the infected cells were prepared and the total protein was run into two dimension gel electrophoresis (2-DE) proteome mapping and followed by liquid chromatography–mass spectrometry (LC-MS/MS) identification. This analysis revealed significant changes in expression of 14 proteins that contain seven protein with known functions: cytoskeleton (vimentin, capping protein), antioxidative stress, regulatory and protein synthesis. Proteomic profiles of mouse N2a cells infected with variant virulence of RVs were identified. There were 35 differentially expressed proteins categorized into cytoskeleton, signal transduction, stress response, and metabolic response groups. The proteomic alterations described here might suggest that the changes in protein expression correlate with the RV adaptability and virulence in N2a cells. Further, the expressed protein after RV infection in Vero cells with 2

different media addition showed similar protein changes. These protein expressions were involved in general cell regulation and in calcium homeostasis. The results from different cell lines were a bit different due to the variants of RV and culture cells. Further experiments to characterize the function of the affected proteins may contribute to the understanding of the mechanism of RV infection and pathogenesis (Kluge et al., 2013; X. Wang et al., 2011; Zandi et al., 2009).

RV successfully invaded the immune systems by evolving to escape or to lower activation of innate immunity and antiviral effects of interferons (IFN). Supporting this sentence, there were previous reports which suggested RV had a different immune response pathway from other lyssaviruses. The results showed that little or no inflammation responses were observed in mice infected with wt RV. The endosomal transmembrane Toll-like receptor 3 (TLR3), cytoplasmic retinoic acid inducible gene-1-like helicase (RIG-I), $IFN_{\alpha/\beta}$, tumor necrotic factor α (TNF_{α}), interleukins were associated with antiviral function (via JAK/STAT signal transduction factor for proinflammatory cytokine) and interferon transcription (via NF-κB) (Hemachudha et al., 2013; Z. W. Wang et al., 2005; Wiltzer et al., 2014). In mouse model, after RV infection, the proteomic profile showed the expressed proteins which play important biological roles such as T and B lymphocyte activation (coronin 1), antiviral activity (peroxiredoxin 1), and cytoskeleton reorganization (cofilin 1). These results strongly hints early divergence on peripheral immune response under influence of viral strain and their pathogenicity (Vaziri, Torkashvand, Eslami, & Fayaz, 2012).

Chaperones and heat shock proteins (HSP) are the host protein family for posttranslational folding. They play a central role in protein homeostasis and protection against proteotoxic stress by preventing protein misfolding and aggregation. The 70-kDa HSP is essential for multiple processes within cells, and presents in both nucleocapsid and purified virions. Previous study in BSR cells (cloned BHK-21 cells) showed that the HSP70 interacts with the N protein of CVS strain of RV. The downregulation of HSP70 resulted in a significant decrease of the amount of viral mRNAs, viral proteins and viral particles (Lahaye, Vidy, Fouquet, & Blondel, 2012). And the eukaryotic cytosolic chaperonin TRiC/CCT complex (chaperone CCTγ) also acts as a host factor that facilitates intracellular RV replication in N2a cells. Knockdown

CTTγ by lentivirus-mediated RNA interference led to RV replication (J. Zhang et al., 2013). Thus it may be concluded that cellular chaperonins and HSP70 are important to RV, and further viruses utilize these as well as compartmentalization for their own benefit.

Clinical presentation and host protein association

The host proteins associated with rabies with different clinical forms has been discussed in many reports. In 2011, Thanomsridetchai and her colleagues reported the proteomic results from natural rabies infected dogs. They found that furious rabies had more viral load in the brain with lower immune response than the paralytic rabies. The furious and paralytic rabid dogs had significantly more expressed proteins when compared with non-rabid dogs. The hippocampus, brainstem and spinal cord samples were prepared for protein separation using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then electrospray ionization (ESI) quadrupole time-of-flight (ESI Q-ToF) mass spectrometry (MS) was used for protein identification. The results showed 11 main functional categories of significantly altered proteins affected by rabies infection. There were associations in previous reports between pathogenesis and expression of gene related to host immune response, cell susceptibility and the expression of protein such as: apoptosis-related proteins, cytoskeletal proteins, heat shock proteins/chaperones, and immune regulatory protein. In summary, they reported large data set of protein changes in furious and paralytic rabies in the hippocampus, brainstem and spinal cord regions (Thanomsridetchai et al., 2011). However the results explained only some expressed protein groups in rabid dogs.

In general, the clinical signs and symptoms present after the cellular, organic or systemic dysfunction or failure of body homeostasis. The host protein is one of the essential determinants which changes spontaneously with a stimuli such as viral infection. There were proteomic studies in early stages of viral infections such as porcine reproductive and respiratory syndrome (PRRS). Diagnosis PRRS in early stages using upregulated levels of protein(s), which acted as a biomarker, allowed for rapid identification despite aviremia (Genini et al., 2012). In 2013, Venugopal and his team reported the novel expressed proteins in furious and paralytic rabies in human

brain tissue. They found the overexpression of karyopherin alpha 4 (KPNA4) and upregulation of calcium calmodulin dependent kinase 2 alpha (CAMK2A) in paralytic rabies. Glutamate-ammonia ligase (GLUL) were overexpressed in both paralytic and furious forms (Venugopal et al., 2013). It might be possible that these proteins could be used as diagnostic biomarkers in rabies infected humans. However there has been no report, to date, exhibiting the expressed proteins in early stage of rabies in dogs. Further studies to identify the different expressed protein(s) at different stages of infection are necessary to establish biomarkers of rabies for early detection.

The advantages of mass spectrometry over the 2-DE proteomic analysis are purity of sample, higher sensitivity, and MS requires small number of samples per run. Moreover, there are large amounts of information of identified protein obtained from the MS. The error data will be decreased because of high purity of sample and MS machine's ability. The artifacts from MS are also lower than 2-DE assay. The opportunity of protein matching from LC MS/MS machine is high reliable because protein database is constantly updating. In this study we used 1D-PAGE LC-MS/MS as a tool to analyze the express protein(s) at different clinical stages of RV infections.

CHAPTER III

MATERIALS AND METHODS

The schematic diagram in the figure below shows the experimental design to analyze the expression of proteins in hippocampus, parietal lobes, brainstem and spinal cord of rabies infected and non-rabies infected dogs.

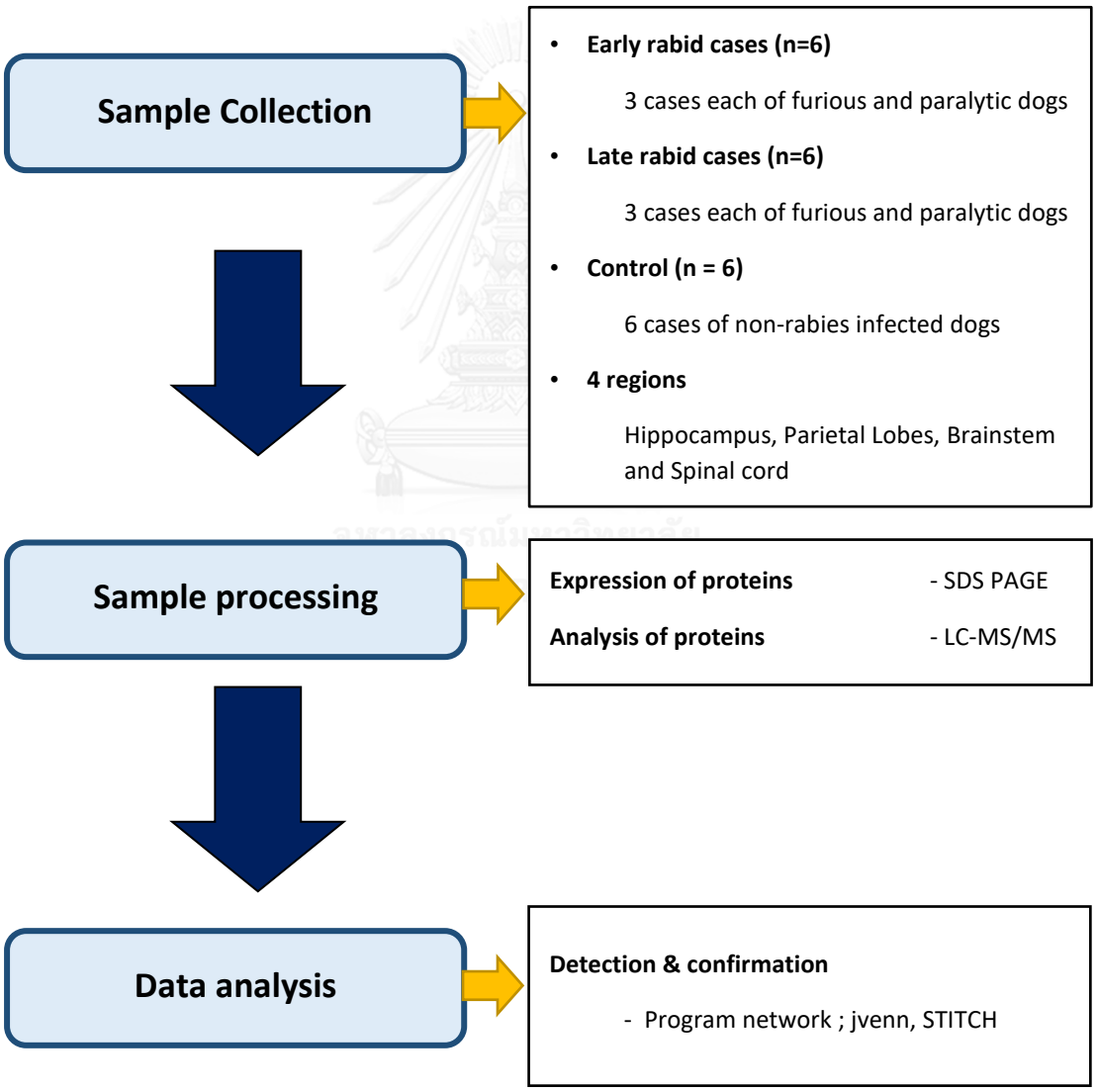

Figure 4 **Flow chart of the experimental design**

Sample collection

Samples in this study were categorized into 5 groups. They were early furious rabies dogs, early paralytic rabies dogs, late furious rabies dogs, late paralytic rabies dogs and non-rabies infected dogs. Dogs were observed and diagnosed at Quarantine and Rabies Diagnostic Unit of the Queen Saovabha Memorial Institute (QSMI). As previously described, the dogs were classified and categorized using the same criteria as Dr. Laothamathas's report in 2008 (Laothamatas et al., 2008). Infectious stages were clinically defined as early or late, based on whether the dog remained fully conscious (early) or had lapsed into coma (late). Paralytic rabies infected dogs were clinically found with paralyzed hind limb, with minimal or none of the furious symptoms. The dogs were quarantined and died naturally without any supportive treatment. Tissues from the central nervous system of the furious and paralytic dogs were collected during early and late stage of rabies infection. The hippocampus, parietal lobes, brainstem (midbrain, pons and medulla) and cervical part of spinal cord were taken and stored at -70^{o}C until examination.

Sample preparation for proteomics analysis

The sample tissues, hippocampus, brainstem, parietal lobes, and spinal cord were ground to powder with liquid nitrogen using pre-chilled mortar and pestle. Powdered tissues were homogenized in 0.1% SDS solution using a microhomogenizer (IKA®-WERKE, Germany). Sample buffer (5x) (0.25 M Tris pH 6.8, 10% glycerol, 0.01% bromophenol blue, 10 mM dithiothreitol, 2% SDS and 2% β-mercaptoethanol: all from Sigma) were added. Samples were boiled and protein concentration were determined using the Lowry protein assay (Lowry, Rosebrough, Farr, & Randall, 1951). This technique is a biuret reaction in which the peptide bonds of proteins react with copper under alkaline conditions to produce Cu^{+}. Briefly, solution A (ratio 1:1:2:4 of 0.2% $CuSO_4$ with 0.4% Tartaric acid, 20% Na_2CO_3, 0.8N NaOH, and 5% $NaC_{12}H_{25}SO_4$, respectively) was mixed with sample and incubated at 37^{o}C for 30 mins, and then solution B (1:5 of Folin's phenol and water) was added, mixed and incubated at 37^{o}C for another 30 mins, for enhanced color development. Blue color could be measured at

750 nm. The protein concentration of sample tissues were determined and calculated with standard serum albumin. The samples were then stored at -20ºC until further use.

Protein fraction by SDS-PAGE

SDS-PAGE is a technique widely used to separate protein according to their electrophoretic mobility. Sodium dodecyl sulfate (SDS) is a detergent that can bind to proteins with fairly constant charge to mass ratios. This binding confers a negative charge to the polypeptide and destroys high order protein structures. The electrophoretic migration rate through a gel is therefore determined by the size of the complexes. Molecular weights are determined by simultaneously running marker proteins of known molecular weight. Preparation method was modified from Laemmli's method (Laemmli, 1970). All samples were prepared and mixed with loading dye, and run into 12.5% acrylamide gel.

Acrylamide gels were prepared in 12.5 % concentration and used as separating gel. Briefly, the reagents; 40% acrylamide/bis, 1.5 M Tris-buffer, pH 8.8, 10% SDS and 10% Ammonium persulfate (APS) were mixed together, and TEMED was then added as the last substance. These solutions were mixed and poured into a degassed cassette and allowed to polymerize for 45-60 min. When the gel has polymerized, the line between the stacking gel and separating gel was seen. Then, stacking gel at 5% acrylamide concentration were prepared and poured above the separating gel. Briefly, area above the separating gel were dried and the comb was placed on the cassette. The reagents; 40% acrylamide/bis, 0.5 M Tris-buffer, pH 6.8 10% SDS and 20% APS, were mixed together and TEMED were added into the last substance. The stacking gel was poured to stacking cassette and left to solidify. For electrophoresis, running buffer (pH 8.3) was a composition of 0.1% SDS, 0.19 M glycine, and 25 mM Tris-based dilute with distilled water. The comb was removed from the gel and the electrophoresis cell was assembled (Bio-rad Laboratories, Inc). The inner and outer chambers were filled with running buffer. Each sample was mixed with dye and buffer, and then heated at 90-100ºC for 5 mins. Protein samples were loaded onto the gel and electrophoresis cell was connected to power supply, at 50 and 60 V for approximately 2 hrs or until the dyes

reach almost to the end of the gel. The power supply was then stopped, the gel was silver stained to view the protein bands.

Silver staining

Silver staining is available for detecting proteins separated by gel electrophoresis. The most important advantage of silver staining gels is the increased sensitivity obtained over other staining methods. Basically, protein detection depends on the binding of silver ions to the amino acid side chains, primarily the sulfhydryl and carboxyl groups of proteins, followed by reduction to free metallic silver. Protein bands are imaged in the gel due to differences in oxidation/reduction potentials between sites in gels occupied by protein and adjacent sites not occupied by protein. Briefly, the gel was fixed and gently stirred for about 30 mins with fixative reagent composed of 50% methyl alcohol, 12% acetic acid and 0.018% formaldehyde. The gel was washed 2 times with 35% ethyl alcohol and was sensitized 2 times with 0.02% sodium thiosulfate ($Na_2S_2O_3$). Then in the developing step, the gel was soaked in 6% sodium carbonate, 0.0004% $Na_2S_2O_3$ and 18.5% formaldehyde and was stopped reaction with 1.46% sodium EDTA. A photo of the gel was taken using Gene Genius Bio Imaging System machine (SynGene), and was stored in 0.1% acetic acid to continue protein digestion.

Protein Digestion for LC-MS/MS

Fifteen gel pieces per sample containing the interested protein were cut out from the stained gel. They were cut into small pieces (approximately 1 mm^2) and transferred to 96-well plates. Gel pieces were de-stained and washed twice by nanopure water. Gel pieces were then dehydrated by adding 50% acetonitrile (ACN) in 25 mM NH_4HCO_3 buffer at room temperature for 15 mins and then washed once with 100% ACN. Dithiothreitol (DTT) solution was then added to cover the gel pieces, waited for 1 hour, and incubated in dark area with 25 mM iodoacetamide in 10 mM NH_4HCO_3 buffer for 1 hour at room temperature. This step was for alkyl group adding and to destroy disulfide bonding and prevent re-bonding, a reaction called carbamidomethyl (C) reaction. After two more washes, the solvent was removed and the gel was dried by a SpeedVac concentrator (Savant; Holbrook, NY). Next step was to rehydrate gel particles in 1% (w/v) trypsin solution (Promega) in 25 mM NH_4HCO_3 buffer (pH 8.0).

After rehydration, the gel pieces were incubated at 37°C for at least 16 hours. Then, extraction was performed by 50% ACN/0.1% trifluoroacetic acid (TFA) solution.

Liquid Chromatography-Mass spectrometry (LC-MS)

LC-MS/MS analysis of digested peptide mixtures was performed using a Waters SYNAPT™ HDMS™ system. The 1D-nanoLC was carried out with a Waters nanoACQUITY UPLC system. Four microlitre of digests was injected onto the RP analytical column (20 cm x 75 μm) packed with a 1.7 μm Bridged Ethyl Hybrid (BEH) C18 material (Waters). Peptides were eluted with a linear gradient from 2% to 40% acetonitrile developed over 60 minutes at a flow rate of 350 nl/min. This was followed by a 15 min period of 80% acetonitrile to clean the column before returning to 2% acetonitrile for the next sample. The effluent samples were electrosprayed into a mass spectrometer (Synapt HDMS) for MS/MS analysis of peptides. Argon gas was used in the collision cell to obtain MS/MS data. MS/MS spectra thus obtained were processed using Max Ent 3, a deconvolution software for peptides (Ensemble 1, Iterations 50, auto peak width determination) within MassLynx 4.0.

Proteins quantitation and identification

For proteins quantitation, DeCyder MS Differential Analaysis software (DeCyderMS, GE Healthcare (Johansson et al., 2006; Thorsell, Portelius, Blennow, & Westman-Brinkmalm, 2007) was used. Acquired LC-MS raw data were converted and the PepDetect module was used for automated peptide detection, charge state assignments, and quantitation based on the peptide ions signal intensities in MS mode. The analyzed MS/MS data from DeCyderMS were submitted to database search using the Mascot software (Matrix Science, London, UK, (Perkins, Pullen, & Thompson, 1999). The data was searched against the NCBI database for protein identification. Database interrogation was; taxonomy (Viridiplantae); enzyme (trypsin); variable modifications (carbamidomethyl, oxidation of methionine residues); mass values (monoisotopic); protein mass (unrestricted); peptide mass tolerance (1 Da); fragment mass tolerance (±0.4 Da), peptide charge state (1+, 2+ and 3+), max missed cleavages (1) and instrument = ESI-TRAP. Proteins considered as identified proteins had at least one peptides with an individual mascot score corresponding to $p<0.05$.

Data normalization and quantification of the changes in protein abundance between the control and treated samples were performed and visualized. Briefly, peptide intensities from the LC-MS analyses were transformed and normalized using a mean central tendency procedure. They performed statistical tests of variance of differences (ANOVA) for these data sets that statistically significant proteins ($p < 0.05$). Protein functional network programs such as VENN (Bardou, Mariette, Escudie, Djemiel, & Klopp, 2014), and STITCH (Szklarczyk et al., 2016) were used to compare and identify the expressed proteins and find the relationship between proteins and functions.

CHAPTER IV

RESULTS

Protein Identification

1. SDS PAGE analysis

Proteins were extracted from 4 different parts of CNS; hippocampus, parietal lobes, brainstem and spinal cord, and were analyzed by SDS-PAGE. There were eighteen samples from different parts of the CNS; six were non-rabies infected (N), three samples each from early stage of furious form (FE), late stage of furious form (FL), early stage of paralytic form (DE) and late stage of paralytic form (DL) of rabies, respectively. SDS-PAGE, illustrating the differentially expressed proteins in hippocampus (Figure 5), parietal (Figure 6), brainstem (Figure 7) and spinal cord (Figure 8), respectively from clinical and control groups are shown.

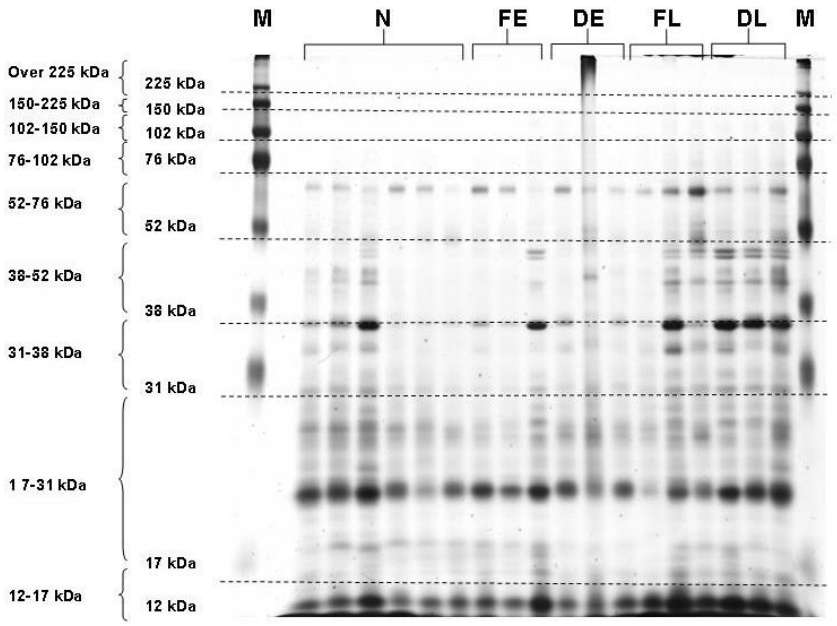

Figure 5 **SDS-PAGE of differential expressed proteins in hippocampus of dogs; N, FE, DE, FL and DL groups. M is ladder or marker for protein measurement.**

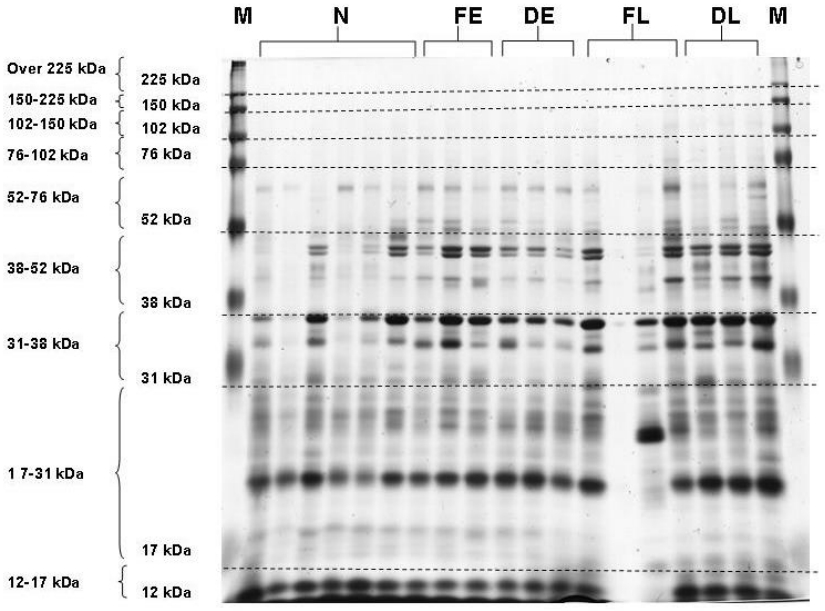

Figure 6 **SDS-PAGE of differential expressed proteins in parietal lobe of dogs; N, FE, DE, FL and DL groups. M is ladder or marker for protein measurement.**

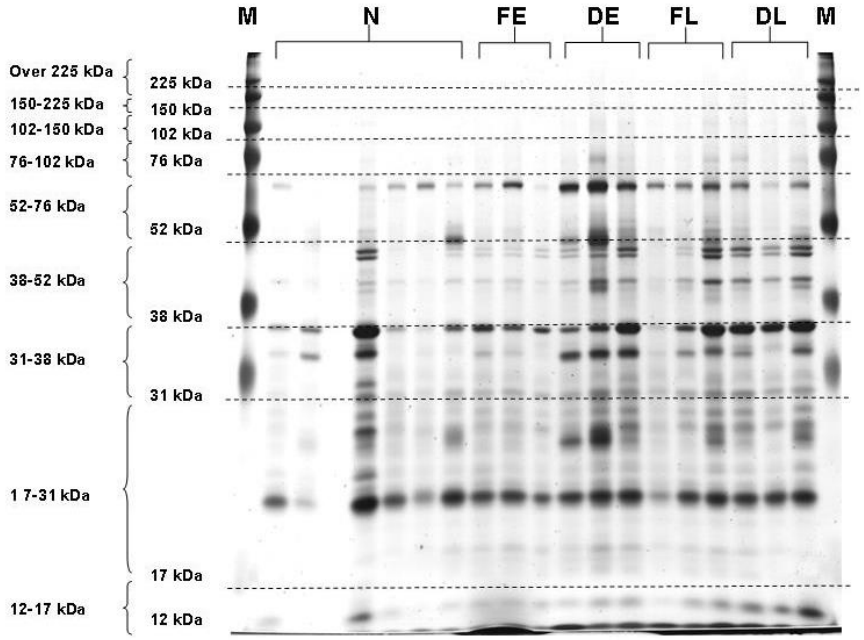

Figure 7 **SDS-PAGE of differential expressed proteins in brainstem of dogs; N, FE, DE, FL and DL groups. M is ladder or marker for protein measurement**.

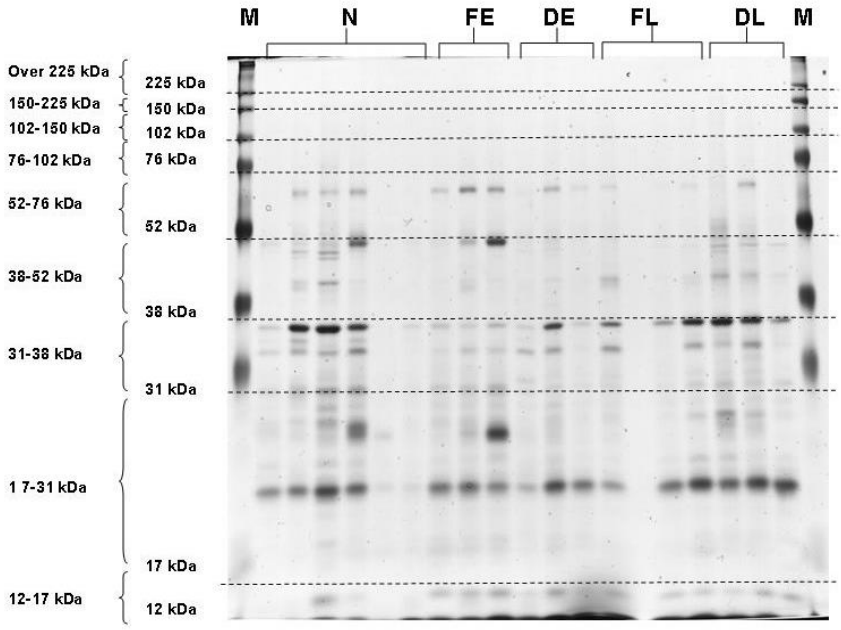

Figure 8 **SDS-PAGE of differential expressed proteins in spinal cord of dogs; N, FE, DE, FL and DL groups. M is ladder or marker for protein measurement**.

Protein quantitation and identification by LC-ESI-Q-TOF MS/MS analyses

Subsequent analyses of these differentially expressed proteins were done by LC-ESI-Q-TOF. The criteria for the significant changes in protein abundance were defined as p value must be < 0.05 and mean values of the five groups using multiple comparisons must have p value < 0.05. Summary proteins of all five canine groups in different CNS tissues are shown in Table 1

Table 2 **Summary of expressed proteins in spinal cord, brainstem, parietal lobes and hippocampus found in 5 groups of dogs**

CNS regions	Expressed proteins found in					
	Total	**Early furious (FE)**	**Early paralytic (DE)**	**Late furious (FL)**	**Late paralytic (DL)**	**Non-Rabies (N)**
Spinal Cord	2278	2235	2109	2093	2099	2205
Brainstem	2776	2426	2541	2541	2503	2673
Parietal lobe	2115	1917	2000	2002	1953	2057
Hippocampus	2874	2669	2764	2697	2564	2760

Expressed proteins in different parts of the brains

The expressed proteins were detected in spinal cord, brainstem, parietal lobe and hippocampus tissues, were compared using Venn diagram produced by jvenn web application (http://jvenn.toulouse.inra.fr/app/example.html). The results are shown in Figure 9 and dominant proteins are summarized and displayed in Table 2.

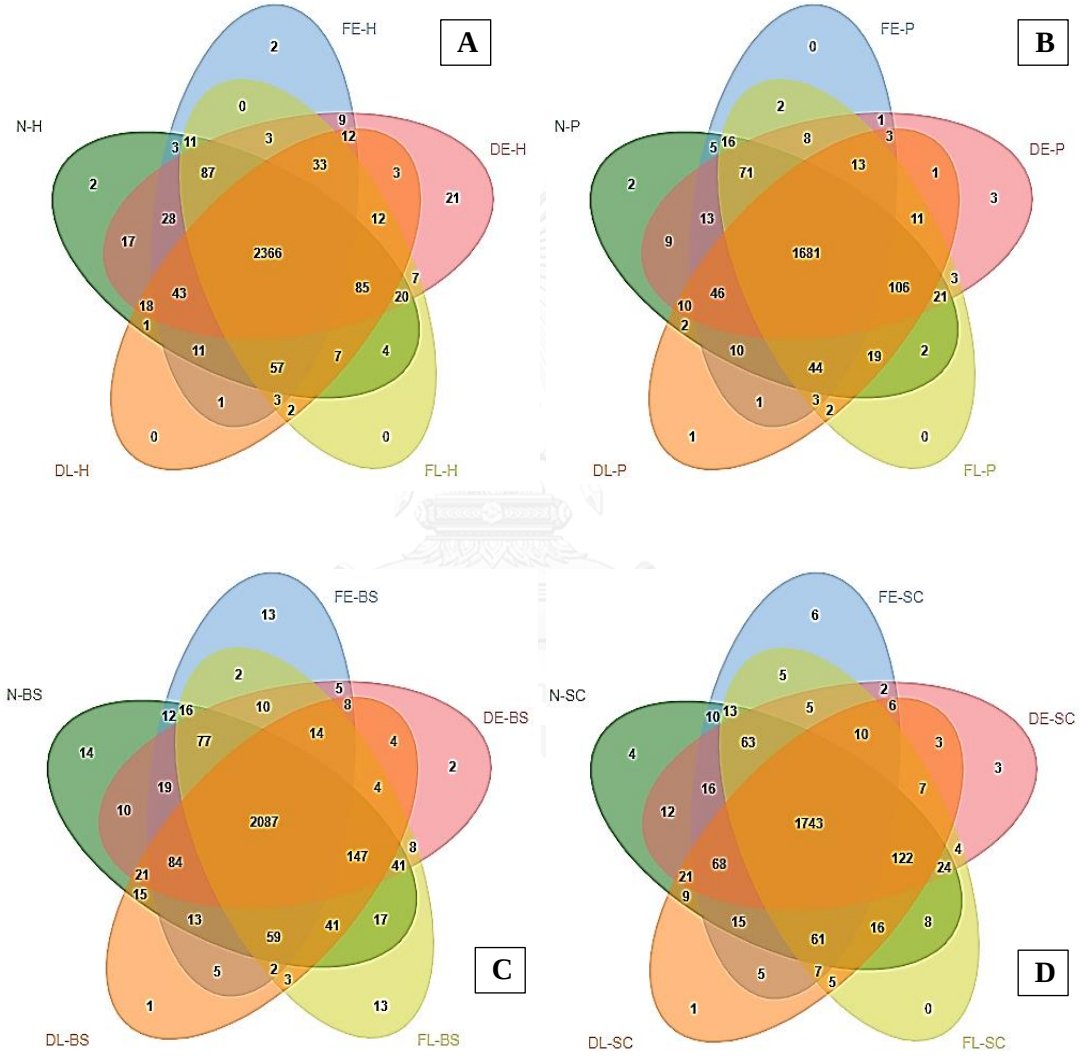

Figure 9 **Venn diagrams of expressed proteins found in hippocampus (A), parietal lobe (B), brainstem (C) and spinal cord (D) compared with Non-rabid, FE, DE, FL and DL groups**.

Table 3 **Summary of proteins uniquely expressed only in FE, DE, FL, DL and N groups in hippocampus (H), parietal (P), brainstem (HS) and spinal cord (SC).**

Group	Protein name	NCBI ID	Gene code	Uniprot ID	MASCOT scores (MS/MS)	MW (Da)	ANOVA *p* value
H-FE	Interleukin-1 receptor accessory protein	gi|34222652	IL1RAP	Q9NPH3	8.60	5868.0	<0.01
	protocadherin-10	gi|533144494	PCDH10	Q9P2E7	11.42	735.39	<0.01
H-DE	Cytoskeleton-associated protein 2	gi|172047279	CKAP2	A5D7U0	11.64	2627.27	<0.01
	POTE ankyrin domain family member F	gi|215273934	POTEF	A5A3E0	75.87	1790.95	<0.01
	Selenocysteine-specific elongation factor	gi|259016384	EEFSEC	P57772	13.47	3486.58	<0.01
	Solute carrier family 35 member F2	gi|395861430	SLC35F6	Q8N357	12.42	7350.40	<0.01
	Transmembrane protein 115	gi|507929861	TMEM115	Q12893	24.44	1634.85	<0.01
	Thyrotropin-releasing hormone receptor-like	gi|532003197	H671_21711	A0A061HUH4	14.36	1864.01	<0.01
	Uncharacterized protein C3orf20 homolog	gi|532030773	NA	Q8CDN1	12.38	1573.90	<0.01
	Inositol hexakisphosphate and diphosphoinositol-pentakisphosphate kinase 1	gi|545549684	PPIP5K1	Q6PFW1	12.01	8159.95	<0.01
	Hormone-sensitive lipase	gi|558192237	Lipe	P15304	13.53	5622.77	<0.01
	Probable RNA-binding protein 19	gi|562845642	Rbm19	Q8R3C6	9.48	1655.80	<0.01
	Cytochrome P450 1A1	gi|821134768	Cyp1a1	P00185	11.64	5223.43	<0.01
	Neurofilament heavy polypeptide	gi|83305012	Nefh	P16884	9.44	1240.63	<0.01
	DnaJ homolog subfamily B member 7	gi|836707936	DNAJB1	P25685	4.38	840.70	<0.01
	Histone-lysine N-methyltransferase 2D	gi|852756208	KMT2D	O14686	13.88	1283.62	<0.01
	Protein cordon-bleu	gi|884871056	Cobl	Q5NBX1	24.82	1059.51	<0.01
	Calponin homology domain-containing protein 2	gi|928185789	LRCH2	Q5VUJ6	20.80	1718.825	<0.01
	Tetratricopeptide repeat protein 6	gi|942074158	TTC6	Q86TZ1	6.53	530.26	<0.01
	Ubiquitin carboxyl-terminal hydrolase 35	gi|942092280	USP35	Q9P2H5	16.25	1718.82	<0.01
	RNA polymerase II subunit A C-terminal domain phosphatase	gi|946651111	CTDP1	CTDP1	14.52	3364.13	<0.01
	MHC class II transactivator	gi|946775822	CIITA	P33076	8.14	827.39	<0.01
	Collagen alpha-1(XXIV) chain	gi|946782823	COL24A1	Q17RW2	16.90	2116.16	<0.01

NCBI = National Center for Biotechnology Information; NA = Not applicable

Table 3 **Summary of proteins uniquely expressed only in FE, DE, FL, DL and N groups in hippocampus (H), parietal (P), brainstem (HS) and spinal cord (SC).(continue)**

Group	Protein name	NCBI ID	Gene code	Uniprot ID	MASCOT scores (MS/MS)	MW (Da)	ANOVA *p* value
P-DE	RNA-binding protein PNO1	gi|75045936	PNO1	Q7YRD0	12.3	767.58	< 0.01
	Protein shisa-4	gi|81899557	Shisa4	Q8CA71	15.22	593.60	0.04
	Transport and Golgi organization protein 6 homolog	gi|836698949	TANGO6	NA	10.25	898.10	0.04
P-DL	Ras-responsive element-binding protein 1	gi|884911295	RREB1	Q92766	13.13	2549.65	< 0.01
BS-FE	Matrix metalloproteinase-25	gi|123796969	Mmp25	Q3U435	14.31	970.63	0.04
	Junction plakoglobin	gi|205371866	JUP	P14923	35.27	1935.00	<0.01
	Keratin, type II cytoskeletal 6B	gi|238054404	KRT6, K6B,KRTL1	P04259	82.76	1407.64	<0.01
	Growth/differentiation factor 2	gi|341940736	Gdf2	Q9WV56	5.13	6818.49	<0.01
	Vacuolar protein sorting-associated protein 13C	gi|471368315	VPS13C	Q709C8	11.43	2281.26	<0.01
	Rab-like protein 6	gi|504167661	RABL6	Q3YEC7	26.93	2103.97	<0.01
	Dynein heavy chain 9, axonemal	gi|507953616	DNAH9	Q9NYC9	19.78	1272.58	<0.01
	Uncharacterized protein C4orf50 homolog	gi|533192170	NA	NA	11.40	2231.02	<0.01
	ATP-binding cassette sub-family A member 12	gi|562858086	ABCA1	O95477	8.510	4772.91	<0.01
	Cytochrome b	gi|729262	MT-CYB	P41303	17.42	2759.61	<0.01
	Septin-7-like	gi|821140214	NA	K9K3C6	13.32	1695.77	<0.01
	Voltage-dependent T-type calcium channel subunit alpha-1I	gi|852773982	CACNA1I	Q9P0X4	11.30	3578.50	<0.01
	AP-3 complex subunit delta-1	gi|940779969	AP3D1	O14617	7.11	5731.32	<0.01
BS-DE	Pre-rRNA-processing protein TSR2 homolog	gi|395860967	TSR2	H0WMU0	8.59	1192.67	0.03
	Solute carrier family 22 member 6	gi|75061925	SLC22A6	Q5RCH6	8.89	1276.41	<0.01

NCBI = National Center for Biotechnology Information; NA = Not applicable

Table 3 **Summary of proteins uniquely expressed only in FE, DE, FL, DL and N groups in hippocampus (H), parietal (P), brainstem (HS) and spinal cord (SC).(continue)**

Group	Protein name	NCBI ID	Gene code	Uniprot ID	MASCOT scores (MS/MS)	MW (Da)	ANOVA *p* value
BS-FL	Keratin, type II cytoskeletal 1	gi|238054406	**KRT1**	P04264	108.17	1657.75	<0.01
	Keratin, type I cytoskeletal 10	gi|269849769	**KRT10**	P13645	117.39	2342.93	<0.01
	Histone-lysine N-methyltransferase 2B	gi|341940998	**Kmt2b**	O08550	12.78	3513.90	<0.01
	Adhesion G protein-coupled receptor B3	gi|408359980	**Adgrb3**	Q80ZF8	10.82	2210.36	<0.01
	Protein-glutamine gamma-glutamyltransferase 2	gi|504146883	**TGM2**	P21980	16.06	2268.17	<0.01
	ADP-ribosylation factor-like protein 2	gi|504174384	ARL2	P36404	4.83	1371.09	<0.01
	Leucine-rich repeat-containing protein 66	gi|511890606	LRRC66	Q68CR7	7.99	929.55	<0.01
	Zinc finger protein 883-like	gi|823422072	AAES_23497	A0A0Q3TZE4	21.8	827.41	<0.01
	Treacle protein	gi|826333277	TCOF1	Q13428	14.06	2696.34	<0.01
	HBS1-like protein	gi|831226722	HBS1L	Q9Y450	12.68	1718.80	<0.01
	Interferon-induced very large GTPase 1-like	gi|850327144	H671_3g10074	A0A061IE60	6.22	1224.82	0.02
	Coiled-coil domain-containing protein 148	gi|918612290	CCDC148	Q8NFR7	4.22	719.33	<0.01
	Zinc finger protein 211-like	gi|942094541	ZNF211	Q13398	2.03	1342.02	<0.01
BS-DL	Histone acetyltransferase p300	gi|821489148	EP300	Q09472	6.91	5265.25	<0.01
SC-FE	APC membrane recruitment protein 1	gi|488508853	Q5JTC6	AMER1	20.09	970.42	<0.01
	Homeobox protein Hox-A4	gi|507931222	Q00056	HOXA4	16.71	1585.81	<0.01
	Uncharacterized protein C12orf50 homolog	gi|514472568	NA	NA	10.68	2592.35	<0.01
	Guanylate-binding protein 7-like	gi|821479492	P32455	GBP1	8.39	2057.92	<0.01
	PH domain leucine-rich repeat-containing protein phosphatase 2	gi|847015497	Q6ZVD8	PHLPP2	8.25	5099.12	<0.01
	Roundabout homolog 2	gi|946643500	Q9HCK4	ROBO2	10.5	4309.92	<0.01
SC-DE	Protein S100-A8	gi|115442	P05109	S100A8	60.79	1421.70	<0.01
	Forkhead-associated domain-containing protein 1	gi|507936952	B1AJZ9	FHAD1	3.59	1219.65	0.025
	NXPE family member 3-like	gi|823404538	Q969Y0	NXPE3	6.92	772.44	0.042
SC-DL	T-cell differentiation antigen CD6	gi|504146195	P30203	CD6	7.78	1298.01	0.03

NCBI = National Center for Biotechnology Information; NA = Not applicable

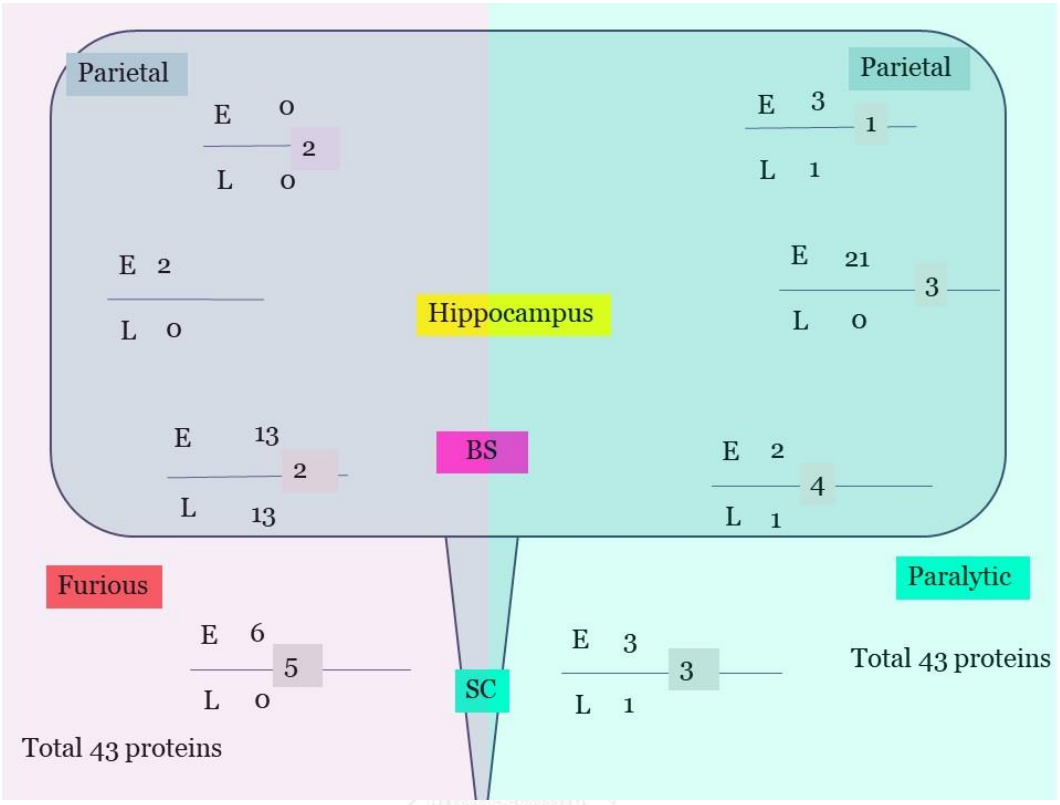

Figure 10 Summary depiction of expressed proteins in hippocampus (H), parietal lobe (P), Brainstem (BS) and spinal cord (SC) regions. The left part was expressed proteins found in furious form; early (FE) and late (FL) stage and the right part was expressed proteins found in paralytic form; early (DE) and late (DL) stage.

Characterization of the differentially expressed proteins

From the MASCOT search engine and NCBI database, the identified proteins were compared and classified into 13 main categories in furious form; namely, apoptotic process, cell adhesion, cell cycle, cell development, cell growth, cytoskeleton, inflammation, immune response, metabolism, signal transduction, transcription, translation and transportation, as shown in Figure 11A. Whereas in paralytic form, there were 14 categories, namely apoptotic process, cell cycle, cell division, chaperone, cytoskeleton, DNA repair, homeostasis, inflammation, immune response, metabolism, signal transduction, transcription, translation and transportation, as shown in Figure 11 B.

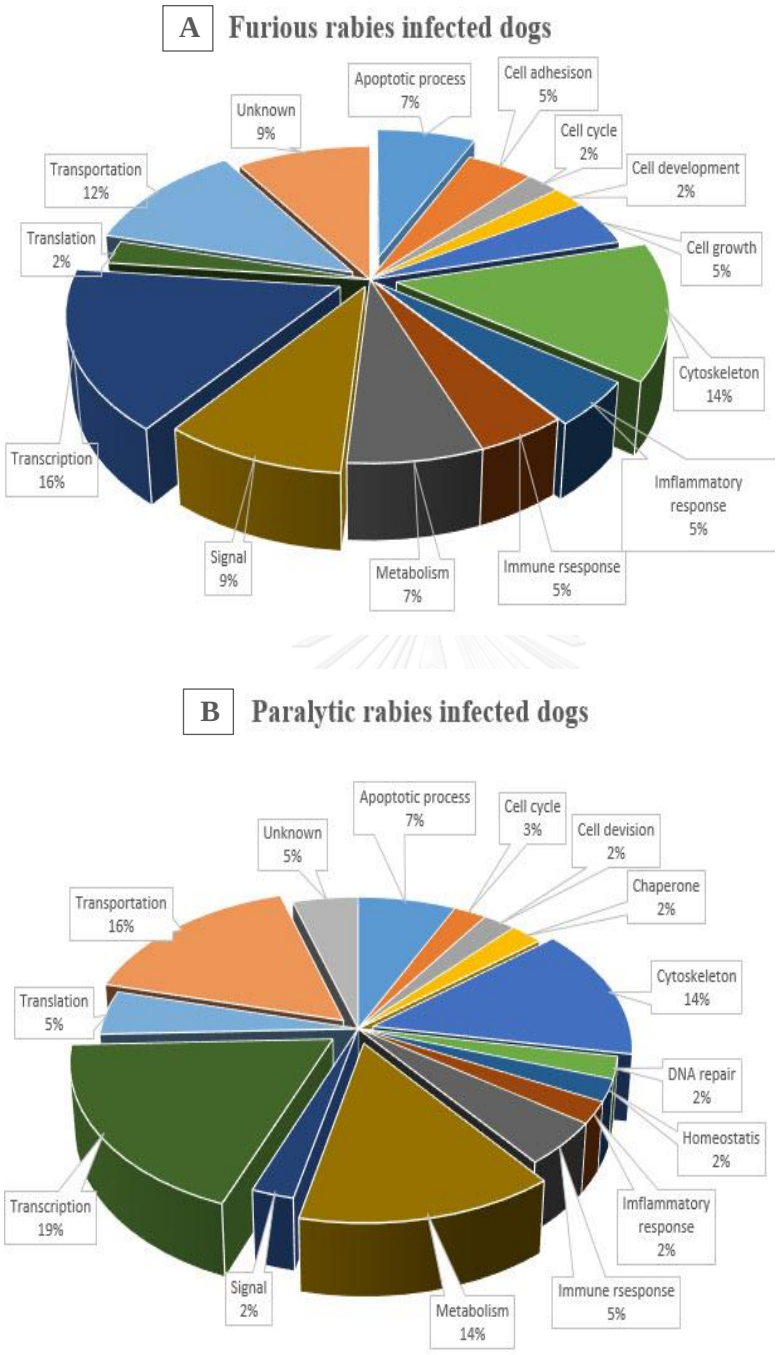

Figure 11 Summary of proteins expressed in hippocampus, brainstem and spinal cord of dogs naturally infected with rabies. These significantly different proteins were classified based on their molecular function.

Protein-chemical inhibitor relationship

The proteins expressed in early and late stages of furious and paralytic rabid dogs in 4 CNS parts were analyzed by STITCH. The proteins were expressed specifically in each stage of RV infection were delineated as a protein and chemical interactive network with huge database, texts and prediction method. The chemical inhibitors and drugs were used to analyze protein-chemical interactions in different stage of rabies infections.

The nine chemical inhibitors were caprofen (non-steroid anti-inflammatory drug), prednisolone cortisone (steroid anti-inflammatory drug), hydrogen peroxide (associated with ER stress), 3-methyladenine, chloroquine, wortmannin (autophagy inhibitor), ribavirin (antiviral drug) and cisplatin (apoptotic inhibitor). It is known that in rabies pathogenesis and disease progression, most of the RV moves from the site of infection or neuromuscular junction by retrograde axonal transportation and into the dorsal-root ganglion, through to the anterior horn cell of the spinal cord ascending to the brainstem, and then spreads throughout the brain (Hemachudha et al., 2013). The STITCH version 5.0 was used with the inhibitors to find the protein binding affinity, results of which are shown in Figures 12-15 and table 4.

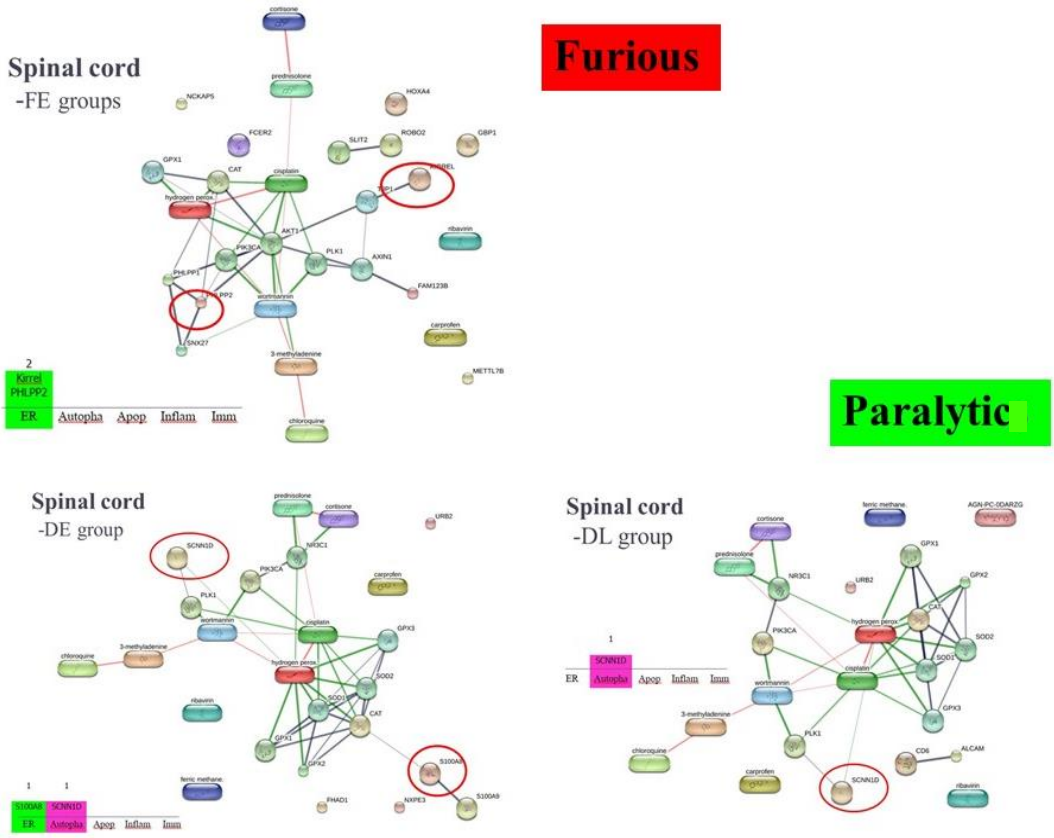

Figure 12 **The interaction network between inhibitors and proteins expressed only in spinal cord was predicted by STITCH. Red circle was to label expressed proteins that interacted with inhibitor and reference proteins from database.**

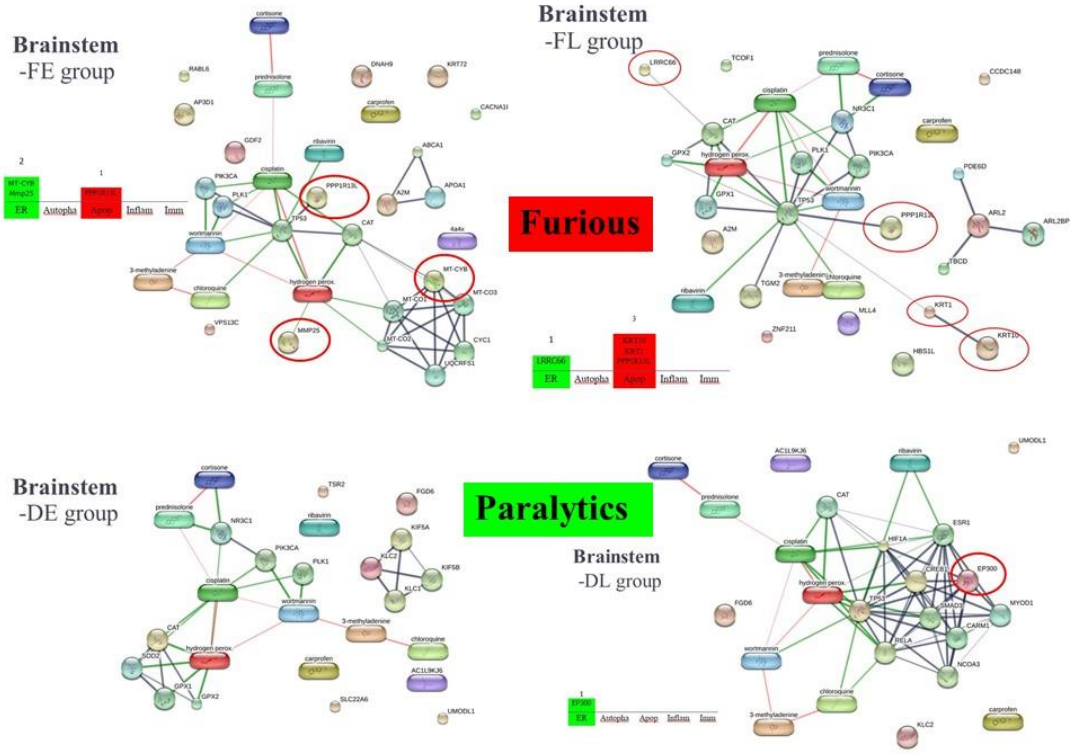

Figure 13 **The interaction network between inhibitors and proteins expressed only in brainstem was predicted by STITCH. Red circle was to label expressed proteins that interacted with inhibitor and reference proteins from database.**

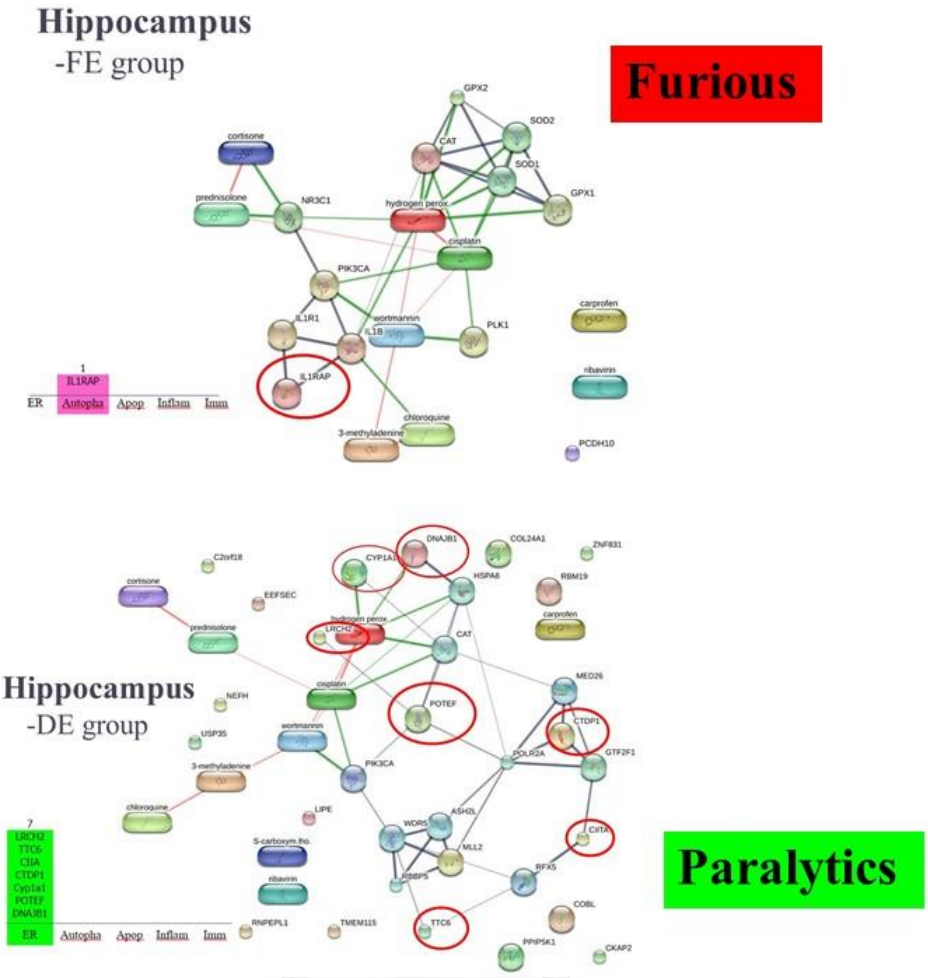

Figure 14 **The interaction network between inhibitors and proteins expressed only in hippocampus was predicted by STITCH. Red circle was to label expressed proteins that interacted with inhibitor and reference proteins from database.**

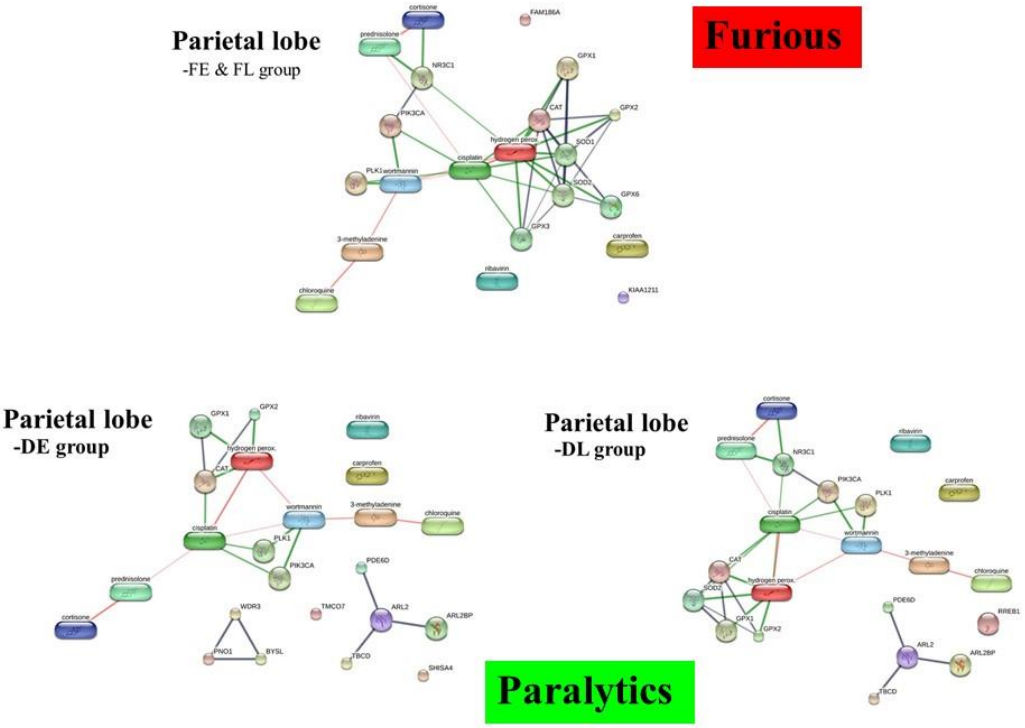

Figure 15 **The interaction network between inhibitors and proteins expressed only in parietal lobe was predicted by STITCH. Red circle was to label expressed proteins that interacted with inhibitor and reference proteins from database.**

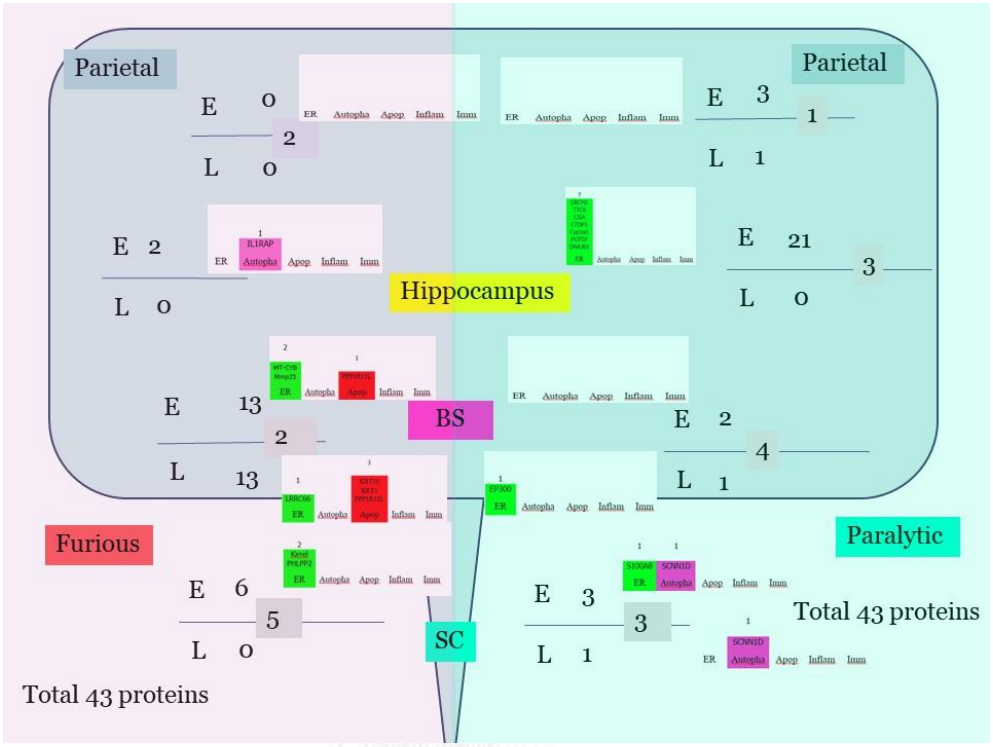

Figure 16 **Summary of the dominant proteins expressed in hippocampus, parietal lobe, brainstem and spinal cord, respectively. The protein names in green were found from STITCH network and are related to endoplasmic reticulum stress, protein names in pink were found from STITCH network, related to autophagy, protein names in red were found in apoptotic process.**

Table 4 **Information of interested proteins expressed only in FE, DE, FL, DL and N groups in hippocampus (H), parietal (P), brainstem (HS) and spinal cord (SC).**

Gene symbol	Region/ RV stage	Protein information and function
PHLPP2	**SC-FE**	**PH domain leucine-rich repeat-containing protein phosphatase 2** Protein phosphatase that mediates dephosphorylation of 'Ser-473' of AKT1, 'Ser-660' of PRKCB isoform beta-II and 'Ser- 657' of PRKCA. AKT1 regulates the balance between cell survival and apoptosis through a cascade that primarily alters the function of transcription factors that regulate pro- and **antiapoptotic** genes. Dephosphorylation of 'Ser-473' of AKT1 triggers apoptosis and decreases cell proliferation. Also controls the phosphorylation of AKT3. Dephosphorylation of PRKCA and PRKCB leads to their destabilization and degradation
KIRREL	**SC-FE>FL**	**Kin of IRRE-like protein 1** Plays a significant role in the normal development and function of the glomerular permeability. Signaling protein that needs the presence of TEC kinases to fully trans-activate the transcription factor AP-1 (By similarity)
S100A8	**SC-DE**	**Protein S100-A8** S100A8 is a calcium- and zinc-binding protein which plays a prominent role in the regulation of **inflammatory processes and immune response**. It can induce neutrophil chemotaxis and adhesion. Predominantly found as calprotectin (S100A8/A9) which has a wide plethora of intra- and extracellular functions. The intracellular functions include: facilitating leukocyte arachidonic acid trafficking and metabolism, modulation of the tubulin-dependent cytoskeleton during migration of phagocytes and activation of the neutrophilic NADPH-oxidase. Activates NADPH- oxidation.
SCNN1D	**SC-DE<DL**	**Amiloride-sensitive sodium channel subunit delta** Sodium channel, non-voltage-gated 1, delta subunit; Sodium permeable non-voltage-sensitive ion channel inhibited by the diuretic amiloride. Mediates the electrodiffusion of the luminal sodium (and water, which follows osmotically) through the apical membrane of epithelial cells. Controls the reabsorption of sodium in kidney, colon, lung and sweat glands. Also plays a role in taste perception

Table 4 **Information of interested proteins expressed only in FE, DE, FL, DL and N groups in hippocampus (H), parietal (P), brainstem (HS) and spinal cord (SC). (continue)**

Gene symbol	Region/ RV stage	Protein information and function
MMP25	**BS-FE**	**Matrix metallopeptidase 25**; May activate progelatinase A inflammatory response
MT-CYB	**BS-FE**	Mitochondrially Encoded Cytochrome B; Component of the ubiquinol-cytochrome-c reductase complex (complex III or cytochrome b-c1 complex), which is a respiratory chain that generates an electrochemical potential coupled to ATP synthesis (By similarity)
PPP1R13L	**BS-FE>FL**	**RelA-associated inhibitor** Protein phosphatase 1, regulatory subunit 13-like; Regulator that plays a central role in regulation of apoptosis and transcription via its interaction with NF-kappa-B and p53/TP53 proteins. Blocks transcription of HIV-1 virus by inhibiting the action of both NF-kappa-B and SP1. Also inhibits p53/TP53 function, possibly by preventing the association between p53/TP53 and ASPP1 or ASPP2, and therefore suppressing the subsequent activation of apoptosis**.**
KRT1	**BS-FL**	**Keratin, type II cytoskeletal 1** Keratin 1; May regulate the activity of kinases such as PKC and SRC via binding to integrin beta-1 (ITB1) and the receptor of activated protein kinase C (RACK1/GNB2L1). In complex with C1QBP is a high affinity receptor for kininogen-1/HMWK
KRT 10	**BS-FL**	**Keratin, type I cytoskeletal 10**
LRRC66	**BS-FL**	**Leucine-rich repeat-containing protein 66**

Table 4 **Information of interested proteins expressed only in FE, DE, FL, DL and N groups in hippocampus (H), parietal (P), brainstem (HS) and spinal cord (SC). (continue)**

Gene symbol	Region/ RV stage	Protein information and function
EP300	**BS-DL**	**Histone acetyltransferase p300** E1A binding protein p300; Functions as histone acetyltransferase and regulates transcription via chromatin remodeling. Acetylates all four core histones in nucleosomes. Histone acetylation gives an epigenetic tag for transcriptional activation. Mediates cAMP-gene regulation by binding specifically to phosphorylated CREB protein. Also functions as acetyltransferase for nonhistone targets. Acetylates 'Lys-131' of ALX1 and acts as its coactivator in the presence of CREBBP. Acetylates SIRT2 and is proposed to indirectly increase the transcriptional activity of TP53 through acetylation **Uniprot (KW-0945)-**viral immune evasion Viral or cellular protein involved in a host-virus interaction. Viruses interact with many cellular pathways to achieve their replication cycle. Entry into the host cell, transport to the viral replication sites or viral exit from the host cell are all steps that require specific interactions between the virus and its host. Additionally, the evasion from the host immune response requires a lot of viral proteins to associate with and inhibit cellular proteins with antiviral functions.
IL1RAP	**H-FE**	**Interleukin 1 receptor accessory protein;** Co-receptor with IL1R1. Associates with IL1R1 bound to IL1B to form the high affinity interleukin-1 receptor complex which mediates interleukin-1-dependent activation of NF-kappa-B and other pathways. Signaling involves the recruitment of adapter molecules such as TOLLIP, MYD88, and IRAK1 or IRAK2 via the respective TIR domains of the receptor/co-receptor subunits. Recruits TOLLIP to the signaling complex. Does not bind to interleukin-1 alone; binding of IL1RN to IL1R1, prevents its association with IL1R1 to form a signaling complex. **GO:0004908**: Combining with interleukin-1 to initiate a change in cell activity. Interleukin-1 is produced mainly by activated macrophages and is involved in the inflammatory response.
POTEF	**H-DE**	**POTE ankyrin domain family, member F**

Table 4 **Information of interested proteins expressed only in FE, DE, FL, DL and N groups in hippocampus (H), parietal (P), brainstem (HS) and spinal cord (SC). (continue)**

Gene symbol	Region/ RV stage	Protein information and function
CYP1A1	**H-DE**	**Cytochrome P450, family 1, subfamily A, polypeptide 1;** Cytochromes P450 are a group of heme-thiolate monooxygenases. In liver microsomes, this enzyme is involved in an NADPH-dependent electron transport pathway. It oxidizes a variety of structurally unrelated compounds, including steroids, fatty acids, and xenobiotics **GO:0009615**: response to virus Any process that results in a change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of a stimulus from a virus.
DNAJB1	**H-DE**	DnaJ (Hsp40) homolog, subfamily B, member 1; Interacts with HSP70 and can stimulate its ATPase activity. Stimulates the association between HSC70 and HIP. UniProtKB - P25685 – GO: 00900084; negative regulation of inclusion body assembly Any process that decreases the rate, frequency, or extent of inclusion body assembly. Inclusion body assembly is the aggregation, arrangement and bonding together of a set of components to form an inclusion body. GO: Hsp70 protein binding Interacting selectively and non-covalently with Hsp70 proteins, any of a group of heat shock proteins around 70kDa in size.
LRCH2	**H-DE**	**Leucine-rich repeats and calponin homology (CH) domain containing 2**
TTC6	**H-DE**	**Tetratricopeptide repeat domain 6**

Table 4 **Information of interested proteins expressed only in FE, DE, FL, DL and N groups in hippocampus (H), parietal (P), brainstem (HS) and spinal cord (SC). (continue)**

Gene symbol	Region/ RV stage	Protein information and function
CTDP1	**H-DE**	**CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) phosphatase, subunit 1;** Processively dephosphorylates 'Ser-2' and 'Ser-5' of the heptad repeats YSPTSPS in the C-terminal domain of the largest RNA polymerase II subunit. This promotes the activity of RNA polymerase II. Plays a role in the exit from mitosis by dephosphorylating crucial mitotic substrates (USP44, CDC20 and WEE1) that are required for M-phase-promoting factor (MPF)/CDK1 inactivation **GO: 00330957** Tat protein binding Interacting selectively and non-covalently with Tat, a viral transactivating regulatory protein from the human immunodeficiency virus, or the equivalent protein from another virus. **GO: 0043923**- positive regulation by host of viral transcription Any process in which a host organism activates or increases the frequency, rate or extent of viral transcription, the synthesis of either RNA on a template of DNA or DNA on a template of RNA. modulation by host of viral transcription
CIITA	**H-DE**	**Class II, major histocompatibility complex, transactivator;** Essential for transcriptional activity of the HLA class II promoter; activation is via the proximal promoter. No DNA binding of in vitro translated CIITA was detected. May act in a coactivator-like fashion through protein-protein interactions by contacting factors binding to the proximal MHC class II promoter, to elements of the transcription machinery, or both. Alternatively it may activate HLA class II transcription by modifying proteins that bind to the MHC class II promoter **GO:0060333**- interferon-gamma-mediated signaling pathway A series of molecular signals initiated by the binding of interferon-gamma to a receptor on the surface of a cell, and ending with regulation of a downstream cellular process, e.g. transcription. Interferon gamma is the only member of the type II interferon found so far. **GO:0045348** - positive regulation of MHC class II biosynthetic process Any process that activates or increases the frequency, rate or extent of the chemical reactions and pathways resulting in the formation of MHC class II.

CHAPTER V

DISCUSSION AND CONCLUSION

There are differences in the clinical manifestations of furious and paralytic rabies in animals, including humans (Laothamatas et al., 2008). Natural infection in dog is a suitable model for studying the pathogenesis of rabies. From the results, there were difference in the expressed proteins found in furious and paralytic rabid dogs, especially in the early stages of infection in a rabid dog's CNS, namely, the hippocampus, parietal lobe, brainstem and cervical parts of the spinal cord. These proteins were associated with biological processes of host and rabies infection.

Previous studies found that RV spread was lesser in the cerebral hemisphere in the early stages of paralytic rabies in dogs, and was associated with prominent inflammation at the brainstem level when compared with the same stage in furious form (Hemachudha et al., 2013). The average survival rate of rabies infection in dogs in the furious form was less than paralytic form (Hemachudha et al., 2013). The viral antigen distribution in dogs is generally the same as in human cases. However, antigen distribution and RNA levels could be discordant, possibly due the difference between the stage of infection and the time of animal sacrifice (Shuangshoti et al., 2016). Proteomic data in rabies infected dogs can elucidate the biological processes underlying rabies pathogenesis.

From our result, Protein S100-A8 was found in the spinal cord in the early stages of paralytic dogs. This protein plays a role in the regulation of inflammatory process and immune response. Its function also includes leukocyte trafficking, modulation of the tubulin-dependent cytoskeleton during migration of phagocyte. This is supported by its presence in other diseases such as HIV-1, rhinovirus in COPD, tuberculosis and pregnancy loss (Baines et al., 2013; Marais et al., 2014; Nair, Khanna, & Singh, 2013). On the other hand, in the spinal cord area, PH domain leucine-rich repeat-containing protein phosphatase 2 (PHLPP2) was found in the early stages of furious dogs. This

phosphatase protein regulates the balance between cell survival and apoptosis through pro- and antiapoptotic gene transcriptional cascade (X. Li et al., 2014; J. Liu, Stevens, Li, Schmidt, & Gao, 2011; Qiao et al., 2010). PHLPP2 protein activated by RV resulted in the decrease of apoptotic process, and led to the increase in viral replication and movement from spinal cord. Moreover in the brainstem, RelA-associated inhibitor (PPP1R13L) protein levels was higher in the early stage than late stage of furious dogs. This protein may plays a central role in the regulation of apoptosis and transcription via its interaction with NF-kappa-B and p53/TP53 proteins (Takada et al., 2002). This protein may inhibit P53/TP53 function and thereby suppressing the subsequent activation of apoptosis.

Oxidative stress is also related to neuronal dysfunction (Jackson, Kammouni, Zherebitskaya, & Fernyhough, 2010; Kammouni et al., 2012; Kammouni et al., 2015). In dying mice, the swelling of mitochondria was recognized which might lead to the beading and fragmentation of axons and dendrites (Scott, Rossiter, Andrew, & Jackson, 2008). Oxidative stress injury-associated mitochondria and endoplasmic reticulum may play an important role in disrupting tract integrity and cytoskeletal networks. Attenuated RV strain induced more autophagosome accumulation than pathogenic RV strain *in vitro* model. The proteins were rapamycin (mTOR) signaling, p70S6K signaling, nuclear factor erythroid 2-related factor 2 (NRF2)-mediated oxidative stress and superoxide radical degradation (L. Li et al., 2017). This evidence is supported by the fact that rabid animals with the paralytic form have autophagic process and slow or decrease of viral pathogenesis. Another report also showed autophagosome in dorsal root ganglion neurons infected with CVS strain of RV in adult mice (Rossiter, Hsu, & Jackson, 2009). Lastly, a report showed that matrix protein of attenuated RV strain played an indispensable role in the process of autophagy to neurocytes, the preferential target of RV (Peng et al., 2016).

Autophagy is a function, an innate immunity, to prevent infections from intracellular microbial pathogens. Autophagy can be triggered by factors such as nutrient depletion, endoplasmic reticulum stress, oxidative stress, mitochondrial damage and microbial infection (Kammouni, Wood, & Jackson, 2017; Kroemer, Marino, & Levine, 2010). AMP-activated protein kinase (AMPK) was reported to

activate autophagy functions mainly through the inhibitions of MTOR, or by directly inhibiting the phosphorylation of its downstream target, RPS6KB (ribosomal protein S6 kinase) (Cheng, Fryer, Carling, & Shepherd, 2004; Kimura et al., 2003). Autophagy can inhibit apoptosis by maintaining cellular homeostasis, but autophagy and apoptosis may also cooperatively induce cell death (Eisenberg-Lerner, Bialik, Simon, & Kimchi, 2009). RV infection can activate the autophagy pathway, and can induce the formation of autophagosome. As we found S100A8 protein in paralytic rabies, especially in early stage of infection in our study, it may be that S100A8 was a part of programmed cell death occurring through the cross-talk between mitochondria and lysosomes via reactive oxygen species (ROS), the process involving Bcl2-famiy member BNIP3 (Ghavami et al., 2010). Moreover, EP300 protein was found in paralytic dogs in late stages of RV infection in this study. EP300 is histone acetyltransferase p300 that regulates transcription via chromatin remodeling, activation, and phosphorylation of CREB protein, and indirectly increase the transcriptional activity of TP53 through acetylation. In other viruses, EP300-associated virus immune evasion, where the virus evades the host immune response, requires a lot of viral proteins to associate with and inhibits cellular proteins with an antiviral function. Interaction between viral proteins with EP300 can prevent p53 acetylation, p53-dependent transcription and apoptosis induction. This suggests that RV interacts with host cellular pathways to achieve their replication cycle. Virus can enter the host cell and travel to replication sites (Kim, Sharma, & Nyborg, 2008; Muench et al., 2010; Qian et al., 2016).

The cytoskeleton of cells is made of microtubules, actin filaments and intermediate filament. These structures give the cell its shape and help organize the cells. They also aid intracellular transport and segregates chromosomes during cell division. The proteomic data found in this study strongly indicates that cytoskeleton plays an important role in the progression of rabies infection in the CNS tissues. In the furious form of rabies during the early stages of infection in our study, keratin type II cytoskeletal 6B (KRT6) was found in the brainstem region, and dynein heavy chain 9 (DNAH9) was expressed dominantly. Further, in the late stages of furious form of rabies in the same region, keratin type II cytoskeleton 1 (KRT1) and keratin, type I cytoskeleton 10 (KRT10) were found. In addition, protocheherin-10 (PCDH10) and

interleukin-1 receptor accessory protein (IL1RAP) were found dominantly in the early stage of furious dogs in the hippocampus. Our previous neuroimaging studies of rabid dogs during early stage of infection, showed tract integrity but macro-structural damage in the brainstem of paralytic form, while structural damage was found in the cerebral cortex of furious rabies (Laothamatas et al., 2011). These might be the result of reorganization of cytoskeleton assembly in the CNS as part of the host response to the CNS infection, which also depends on the viral load and the inflammatory response in the CNS tissues.

Although neurons are highly specialized, longest living cells, basal levels of neuronal autophagy may be especially important in neuroprotection. A more recent study in viral encephalitis models, Japanese encephalitis virus (JEV)-induced autophagy was found to negatively influenced virus replication in mouse neuronal cells and embryonic fibroblasts, delaying virus-induced cell death. JEV-infected cells further enhanced autophagy and reduced virus titers *in vitro* model (Sharma et al., 2017). After treating duck embryo fibroblast (DEF) cells with rapamycin, an inducer of autophagy, Duck enteritis virus (DEV) replication increased. These results indicated that DEV infection induced autophagy in DEF cells and autophagy-facilitated DEV replication (Yin et al., 2017). Further, autophagy levels in both the herpes simplex virus type 1 (HSV-1) and Sindbis virus directly correlated with decreased viral titers in infected brains (Liang, E, & Jung, 2008; Orvedahl & Levine, 2008). For negative-sense ssRNA virus, the vesicular stomatitis virus (VSV), deletion of autophagy genes increased viral replication and decreased survival *in vitro* cell lines (Shelly, Lukinova, Bambina, Berman, & Cherry, 2009). In an influenza virus (IAV) study, autophagy deficiency led to a transient increase in phosphorylation of mTOR and its downstream targets including 4E-BP1 and S6 at a very early stage of IAV infection, and markedly suppressed p70S6K phosphorylation at the late stage of IAV infection. Furthermore, autophagy deficiency resulted in the impairment of Hsp90 induction in response to IAV infection (G. Liu et al., 2016).

Chaperone proteins or heat shock proteins (Hsp) have previously been shown to incorporate itself into rabies, influenza, vesicular stomatitis and Newcastle disease viruses (Sagara & Kawai, 1992). Hsp are a family of proteins that are produced by cells

in response to exposure to stressful condition. Rabies infection associated with Hsp70, a proviral protein, suggests that Hsp70 is involved in the viral life cycle (Lahaye et al., 2012). Pathway of co-chaperone Cdc37/Hsp90, a non-kinase target, affected the RV life cycle directly (W. Xu & Neckers, 2012; Y. Xu et al., 2016). In our study, DNJ (Hsp40) homolog, subfamily B, member 1 (DNAJB1) was found in the early stage of paralytic rabies. DNAJB1 protein, a negative regulation of inclusion body assembly, interacts with HSP70 and can stimulate ATPase activity. This stimulation is associated with heat shock cognate protein (HSC70) that regulates transcriptional process (R. Zhang et al., 2017). In other viruses such as IAV, Hsp40/DNAJB1 promotes viral replication by assisting nuclear import of viral nucleoprotein (Batra et al., 2016; Cao et al., 2014; Rauch & Gestwicki, 2014).

In the hippocampus region, there were 21 expressed proteins specifically in the early stage of paralytic dogs. After analysis of proteins and inhibitors in STITCH (Figure 15), there were many specifically expressed proteins such as Cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1). There was a report that correlated neuronal death and epileptic activity to CYP1A1 protein. The clinical manifestation, such as seizure or epileptic behavior, was found in mouse hippocampus (Anderson, Hasan, Yin, Qadri, & Quattrochi, 2006; Chali et al., 2015; Sudenga et al., 2014). CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) phosphatase subunit 1 (CTDP1), this protein increased the frequency or extent of viral transcription. The expression of CTDP1 was reduced in cell infected with lentivirus, and suppressed cell growth, induced G0/G1 phase arrest and repressed cell colony formation (Zhong et al., 2016). And Class II, major histocompatibility complex, transactivator (CIITA). CIITA protein is essential to the transcriptional activity of HLA class II promoter. CIITA plays a role in the control of the cellular immune response through the interferon gamma-mediated signaling pathway and regulation of MHC class II biosynthetic process (Hake et al., 2000). The behavioral change may occur from limbic system activity disorder in hippocampus area (Bartsch, 2012). Street rabies virus (MRV strain) could cause dendritic spine injury and F-actin depolymerization in mice hippocampus (Song et al., 2013). However, infected mouse brain showed very few morphological changes in the neuronal bodies and process. The structural change was found and could partially

explain the severe clinical disease. Proteomic analysis of rabies-infected hippocampus showed the CYP1A1, DNAJB1, CTDP1 and CIITA proteins that expressed only in early stage of paralytic might play role virus clearance and help to decrease pathogenesis and prolong survival time.

In previous proteomic studies of RV infection, there have been many different *in vitro/in vivo* host model systems and clinical specimens. The limitations of proteomic technique such as 2D-DIGE with mass spectrometry (MS) and 1D-PAGE LC-MS/MS should be noted. Cell lines commonly used *in vitro* models included Vero cells (Kluge et al., 2013), CCL131 cells (Zandi et al., 2009), N2a cells (X. Wang et al., 2011) and BHK21 cell (Zandi et al., 2009). Mice have been the common animal model used for proteomic study, due to its small size, easy management and the large amounts of existing database available. Mice species such BALC, NMRI and ICR were used with different viruses and techniques (Dhingra et al., 2007; L. Li et al., 2017; Vaziri et al., 2012). However, dog model was used in this study as it composed of both early and late stage of natural rabies infection. As for clinical specimens, there have been reports from human and canine brains naturally infected from dog RV strain (Farahtaj et al., 2013; Thanomsridetchai et al., 2011; Venugopal et al., 2013). On the other hand, different strains of RV, such as CVS, CVS-B2C, BD06, SRV9 and human Louis Pasteur 2061, have been used in previous studies. Attenuated CVS-B2C strains of SHBRV showed the expressed proteins involved in the induction of apoptosis in mouse brains (Dhingra et al., 2007). In this study, the dogs were naturally wild-type canine rabies infection that suited to elucidate the pathogenesis. Different proteomic techniques, such as one and two directional polyacrylamide electrophoresis and several kinds of mass spectrometry have previously been used, despite its limitations. Integration of systems of several different sample types and hosts, RV strains, machine types, and experimental designs, including modern and updated computational programs collaboratively are excellent tools to help generate a more wholesome, clearer picture of the underlying mechanisms and pathogenesis of both forms of rabies, in animals and in humans, at different stages of infection.

Conclusion

In summary, we report, for the first time, of differences in proteome in CNS tissues from the two rabies-infected forms, in the early and late stages of infection. This data will be useful for better understanding the molecular mechanism during the early and late stages of both furious and paralytic rabies.

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