Increased synapsin I expression in cerebral malaria

Klairoong Thonsranoi1, Supattra Giaharn1, Chuchard Punsawad2, Urai Chaisri1, Srivicha Krudsood3, Parnpen Viriyavejakul1,4

1Department of Tropical Pathology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand; 2School of Medicine, Walailak University, Nakhon Si Thammarat 80161, Thailand; 3Department of Tropical Hygiene, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand; 4Center for Emerging and Neglected Infectious Diseases, Mahidol University, Salaya, Nakhon Pathom 73170, Thailand

Received September 10, 2015; Accepted October 22, 2015; Epub November 1, 2015; Published November 15, 2015

Abstract: Synapsin I is a neuronal phosphoprotein contained in the synaptic vesicles of mammalian central and peripheral nervous systems. It regulates both neurotransmitter release and synaptic formation. Variations in synapsin I expression in the brain have been reported to cause brain malfunction. In severe malaria, neurological complications, such as convulsion, delirium and coma, suggest abnormalities in the release of neurotransmitters. This study evaluated synapsin I expression in cerebral malaria (CM). An immunohistochemical method was used to study the semi-quantitative and qualitative expression of synapsin I in the brain of CM patients (10 cases) who died with Plasmodium falciparum, compared with non-cerebral malaria (NCM) (4 cases), and control brain tissues (5). Synapsin I was expressed in the gray matter of the cerebral cortex and the molecular layer of the cerebellum, as a diffusely dense precipitate pattern in the neuropil, with no immunoreactivity in the neurons, neuronal dendrites, glial cells, endothelial cells, and Purkinje cells. The findings were similarly demonstrated in CM, NCM, and control brain tissues. However, in the granular layer of the cerebellum, a significant increase in synapsin I expression was observed in the granule cells, and the glomerular synaptic complex, from the CM group, compared with the NCM, and control brain tissues (all P < 0.05). Parasitemia showed a positive correlation with synapsin I expression in the granule cells (on admission: Spearman’s ρ = 0.600, P = 0.023) (before death: Spearman’s ρ = 0.678, P = 0.008), and glomerular synaptic complex (before death:  Spearman’s ρ = 0.571, P = 0.033). It was hypothesized that CM causes pre-synaptic excitation and eventually activation of synapsin I, leading to increased neurotransmitter release. Synapsin I inhibitor should be investigated further as a target for a therapeutic intervention to alleviate neurological symptoms in severe malaria.

Keywords: Malaria, Plasmodium falciparum, cerebral malaria, synapsin I, immunohistochemistry

Introduction

Cerebral malaria (CM) is one of the major fatal complications of Plasmodium falciparum infection. The condition is associated with a mortality rate of 15-20% in severe malaria cases [1]. According to the World Health Organization (WHO), CM is a clinical syndrome defined as a potentially reversible diffuse encephalopathy, characterized mainly by coma (unconsciousness state and inability to localize a painful stimulus), and the presence of asexual forms of P. falciparum parasites in the peripheral blood smears, in the absence of other causes of encephalopathy [2]. CM can cause variations in clinical presentation, including cognitive, behavioral and motor dysfunctions, seizures and coma [3]. The release of chemical mediators, immunological responses, and the process of cytoadhesion/sequestration are the main pathologic hypotheses for severe malaria, particularly CM. A dominant hypothesis of CM is the sequestration of parasitized red blood cells (PRBCs) to the cerebral microvascular endothelium, causing multifocal abnormalities in cerebral blood flow, resulting in hypoxemia, acidosis, hypoglycemia, and other metabolic derangements affecting brain function [4, 5]. The underlying pathogenic mechanism of CM is still incompletely understood. Previous study suggests that during P. falciparum malaria infection, the axons are vulnerable to damage. Axonal transport may be disrupted within the nerve fibers, and may lead to neurological dysfunction in CM [1]. Among the neuronal phosphoproteins, synapsin I plays an important role...
in the regulation of neurotransmitter release [6-8]. A recent study found a significant increase in glutamate level (the major excitatory neurotransmitter in the mammalian central nervous system) in the cerebral cortex of mice infected with *P. berghei* ANKA [3].

The physiologic neuro-regulating process in the pre-synaptic terminal is multifactorial, and occurs rapidly. Synapsin proteins may be involved in the process of acute infection with neurological symptoms, such as in severe malaria. This study investigated the expression of synapsin I in post-mortem CM brain tissues, compared with non-cerebral malaria (NCM), and normal brain samples.

**Materials and methods**

**Specimen collection**

Brain specimens from fatal *P. falciparum* malaria patients and control brain samples were obtained from the Department of Tropical Pathology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. All tissues were embedded in paraffin blocks. The specimens were divided into 3 groups–cerebral malaria (CM), non-cerebral malaria (NCM), and control. CM diagnosis was based on clinical presentation in the patient’s chart and autopsy findings of cytoadhesion and sequestration in the blood vessels of the brain tissue. Patients were grouped into NCM when no cerebral involvement was documented in both clinical and autopsy reports. Control brain tissues were collected from patients who died from accidents with no brain injury. Histologically, control brain tissues showed normal neurons and supporting glial cells, with no sign of infection or hemorrhage. The use of left-over brain specimens was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (MUTM 2011-046-1).

**Immunohistochemical study of synapsin I in brain tissues**

The paraffin-embedded specimens were re-embedded with new paraffin medium, and sectioned with microtome at 4 µm thickness for hematoxylin and eosin (H&E) staining and immunohistochemical study. The primary antibody, synapsin I rabbit monoclonal antibody, detects endogenous levels of total synapsin proteins, which covered human synapsin-la and synapsin-lb (Cell Signaling Technology, Inc., USA). The brain-tissue sections were placed on an adhesive slide coated with poly-L-lysine (Sigma, St. Louis, CA), and deparaffinized with a series of xylene, then re-hydrated with graded alcohol. In the antigen retrieval technique, sections were treated with 0.1 M sodium citrate buffer (pH 6.0), and heated in a microwave (Samsung MW71B) at high power (800 W) for 20 mins, modified from previous study [9]. Then, endogenous peroxidase activity was blocked by treating sections with 3% hydrogen peroxide (H$_2$O$_2$) in distilled water (dH$_2$O) for 30 mins at 37°C, and rinsed in running tap-water for 10 mins. Then, the sections were incubated with goat serum for 30 mins at room temperature to block non-specific staining, and washed twice with phosphate buffered saline (PBS). The sections were then incubated with primary antibody, synapsin I rabbit monoclonal antibody (Cell Signaling Technology, Inc., USA) (1:200 dilution) overnight at 4°C. The following day, the brain sections were washed with PBS, and overlaid with secondary antibody, biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc., USA), and incubated for 30 mins at room temperature. After washing with PBS, the sections were incubated with avidin-biotin peroxidase complex (ABC) conjugated with horseradish peroxidase (HRP) (Vector Laboratories, Inc., USA) or alkaline phosphatase (AP) (Vector Laboratories, Inc., USA) for 30 mins. The enzymatic reaction was visualized by adding 3,3’-diaminobenzidine (DAB) (Vector Laboratories, Inc., USA) or Vector® Red substrate kit (Vector Laboratories, Inc., USA), resulting in the formation of a brown (DAB) or red (Vector® Red) color at the antigen sites. Mouse brain and eyeball (retina) were used as the positive control, to validate the specificity of the primary antibody.

**Evaluation of immunohistochemical staining**

Positive cells showed characteristic granular brown or bright-red stained synapsin I in the cytoplasm, nucleus, or both, or formed into complexes called glomerular synaptic complex in the granular layer of the cerebellum. The number of immunopositive cells and staining intensity for synapsin I were evaluated in the granular layer of the cerebellum. Each slide was examined at 1,000 × magnification in 10 ran-
Table 1. Clinical data of malaria patients

<table>
<thead>
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<th>Cerebral malaria (n = 10)</th>
<th>Non-cerebral malaria (n = 4)</th>
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<tr>
<td>Male: Female</td>
<td>7:3</td>
<td>3:1</td>
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<tr>
<td>Age (P = 0.539)</td>
<td>31.7 ± 3.38</td>
<td>28.75 ± 5.91</td>
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<tr>
<td>Hemoglobin (g/dl) (P = 0.304)</td>
<td>11.82 ± 0.49</td>
<td>12.58 ± 0.33</td>
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<tr>
<td>White blood cell count (P = 0.304)</td>
<td>11,992.40 ± 1115.37</td>
<td>14,988.50 ± 2,332.76</td>
</tr>
<tr>
<td>Parasite count (on admission) (parasites/µl) (P = 0.002)</td>
<td>601,668.77 ± 68,153.77</td>
<td>63,215.25 ± 16,748.60</td>
</tr>
<tr>
<td>Last parasite count (parasites/µl) (P = 0.002)</td>
<td>54,721.80 ± 11,707.06</td>
<td>0</td>
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Figure 1. Histopathologic changes of the brain in CM. Representative images showing petechial hemorrhages (arrowheads) in the cerebral cortex (A) (200 ×), and sequestration of PRBCs (arrows) in the cerebral blood vessels (BV) (B) (400 ×). Hematoxylin and eosin stain.

Results

**Summary of clinical data**

Fourteen cases of patients who died from *P. falciparum* malaria were included in the study. Brain specimens consisted of 10 cases of CM and 4 cases of NCM. Normal brain tissues (5 cases) were used as control. A clinical summary of the malaria cases is shown in Table 1. Other complications in CM included anemia (4/10, 40%), jaundice (8/10, 80%), pneumonia 5/10, (50%), shock (2/10, 20%), pulmonary edema (4/10, 40%), disseminated intravascular coagulation (DIC) (4/10, 40%), acute kidney injury
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(AKI) (6/10, 60%), hypoglycemia (2/10, 20%), and acidosis (2/10, 20%). Among the NCM, the causes of death were respiratory distress syndrome with pulmonary edema (1), septicemia (2), and various organ failures (1).

Histopathological evaluation of brain tissues

Brain tissues from CM cases generally showed congestion, minute petechial hemorrhages, hemosiderin pigment deposits, parasitized red blood cells in the blood vessels, and a degree of sequestration. The brain tissues from NCM cases showed unremarkable findings, similar to the normal brain. Representative images of changes in the brain tissue in CM are illustrated in Figure 1.

Cerebrum

The neuropil of the grey matter showed similar immunoexpression of synapsin I in CM, NCM, and control brain tissues. Generalized uniform intense staining in the neuropil of the gray matter was demonstrated. Quantification of immunoexpression was not possible due to diffused immunostaining of synapsin I protein in the gray matter. Synapsin I expression was absent in the neurons, dendrites of neurons, glial cells, and endothelial cells in both white and gray matters (Figure 2).

Cerebellum

The cerebellum consists of the cerebellar cortex (gray matter) and an inner, white matter. The cerebellar cortex can be distinguished into 3 layers--molecular, Purkinje cell, and granular layers. For the molecular and Purkinje cell layers, the expression of synapsin I was similar in CM, NCM, and control brain tissues. In the molecular layer, intense precipitate was scattered diffusely in the neuropil (Figure 3). There was no synapsin I expression in the body of neurons, dendrites of neurons, glial cells, or endothelial cells. This pattern was similar to the findings in the cerebral cortex. The Purkinje cells and associated dendrites in the Purkinje

Figure 2. Synapsin I expression in the cerebral cortex, illustrating strong immunostaining in the gray matter (GM), and no staining in the white matter (WM) (A) (400 ×). At higher magnification (B), the junction between gray and white matter is shown. In the pyramidal cell layer (C), neuropil (Np) shows dispersed, strong immunostaining for synapsin I, while immunostaining is absent from the neurons (arrows), dendrites (asterisks), glial cells (arrowheads), and blood vessels (BV) (avidin-biotin peroxidase complex technique).
cell layer also showed no immunoreactivity to synapsin I (Figure 3). The granular layer consists of granule cells, Golgi type II cells, and small, irregular dispersed, faint staining, clear spaces called the glomeruli [14]. Within the granule cells, many fine punctate precipitates of synapsin I were observed in the nuclei and cytoplasm. The glomerular synaptic complex demonstrated clusters of immunoreactive areas (Figures 3C and 4). The small granule cells in the granular layer of the cerebellum showed differential synapsin I expression in CM, NCM, and control brain tissues. Synapsin I expression was distinct in the nuclei and cytoplasm of the granule cells. CM cases showed significantly increase in mean percentage immunopositive cells (27.85 ± 2.93) and total score (TS) (72.68 ± 8.94) of synapsin I in the granule cells of the cerebellar cortex, compared with NCM cases (4.99 ± 1.84, TS = 9.46 ± 3.98, P = 0.002) and control brains (6.08 ± 2.61, TS = 11.18 ± 5.41, P = 0.001), (NCM vs. control brain: P = 0.905) (Figure 5A). The spaces between the granule cells contain the glomerular synaptic complex. Although the intensity of synapsin I expression was similar in all 3 groups, the density of immunoreactive glomeruli per high power field was significantly increased in CM (80.44 ± 5.57), compared with NCM (16.45 ± 2.64, P = 0.002) and control groups (14.62 ± 1.84, P = 0.001), (NCM vs. control brain: P = 0.73) (Figure 5B).

Correlations between parasitemia and synapsin I expression

Parasitemia was positively correlated with synapsin I expression in the granule cells (On admission: Spearman’s ρ = 0.600, P = 0.023) (before death: Spearman’s ρ = 0.678, P = 0.008), and glomerular synaptic complex (before death: Spearman’s ρ = 0.571, P = 0.033) (Figure 6).

Discussion

Synapsin I is a neuronal phosphoprotein bound to the synaptic vesicles, which regulates the
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Figure 4. Synapsin I expression in the granular layer of the cerebellar cortex. The granular cells in the control (A) and NCM (B) groups show few immunopositive cells compared to CM (C) brain tissues (arrowheads). Increased synapsin I expression in the glomeruli synaptic complex (arrows) was demonstrated in the CM (C). All images are 1,000 × magnification, Bar = 10 μm (avidin-biotin alkaline phosphatase complex technique).

Figure 5. Comparative findings of synapsin I expression in the cerebellum, showing a significant increase in immune-reactivity against synapsin I antibody for granule cells (A) and glomerular synaptic complex (B) in CM group. (*P < 0.001, **P < 0.002, NS not significant).

exocytosis of the synaptic vesicles and neurotransmitter release, neurite elongation, and synaptogenesis [15]. Synapsin I immunoreactivity has been used as an indirect indicator of synapses in the central nervous system [15-18]. The present work used immunohistochemical methods to demonstrate the expression of synapsin I in terms of distribution and intensity in the cerebrum and cerebellum of *P. falciparum* malaria patients. Synapsin I and II have
been demonstrated to be widely distributed in
the central nervous system, including the olfac-
tory bulb, cerebral cortex, hippocampus, basal
ganglia, thalamus, hypothalamus, cerebellum,
pons-medulla, spinal cord and posterior pitu-
itary gland [19]. For the cerebrum, the present
study found that the immunostaining pattern
was similar to previous reports, where a dif-
fused precipitate was observed in the neuropil,
but neurons and dendrites showed no or mini-
mal immunoreactivity [15, 18]. Synapsin I
expression was found in the gray matter (neuro-
pil) of the cerebral cortex, the molecular layer
and the granular layer of the cerebellar cortex.
Moreover, synapsin I was observed in the
nucleus and cytoplasm of granule cells, and in
the glomerular synaptic complex of the cerebel-
larum. Glomeruli are specialized structures where
mossy fibers and Golgi cells are connected
together synthetically [20]. In the CM group, the
glomerular synaptic complex expressed an
increase in synapsin I protein compared with
NCM and normal brain tissues, which may indi-
cate that CM can induce synapsin I activation.

Synapsin I expression has been associated
with ischemia. A study in the hippocampus of
Mongolian gerbils after transient forebrain isch-
emia showed a strong increase in synapsin I
immunoreactivity in the hilus of dentate gyrus
and in the mossy fiber layer of the hippocam-
pus, suggesting a role for synapsin I in plastic
adaptation of the hippocampus following injury
[15]. In CM, cytoadhesion and sequestration of
parasitized red blood cells are important
mechanical processes that lead to transient
obstruction and possibly brain ischemia.
Increased synapsin I expression in CM could
stimulate the activation of pre-synaptic ves-
icles and modulate neurotransmitter release. A
previous study has shown an association
between synapsin I and brain spectrin in neu-
rotransmitter release [21]. In an animal model,
vesicular glutamate transporter (VGLUT) 1,
VGLUT 2, and the vesicular GABA transporter
(VGAT), were decreased in mouse forebrains
devoid of synapsin I and II [22]. It would be use-
ful to determine the neurotransmitter level and
its correlation with synapsin I activation in cere-
bral malaria. Synapsin I has not been studied in
the infectious process with neurological
involvement. So far, research on synapsin I has
been linked to neurodegenerative diseases,
such as Alzheimer’s disease [23]. An impair-
ment in synapsin I has also been documented
in epileptic seizures in human [24] and animal
models [25-28], in behavioral alteration [29],
learning deficit [30], and Huntington’s disease
[31]. Moreover, synapsin protein has been iden-
tified as a novel drug target for the treatment of
refractory hypertension, a common side effect
of cyclosporine A (CsA), the immunosuppres-
sive drug used in transplant patients and as a
treatment for autoimmune diseases [32].

A murine model study of CM found an increase
in glutamate levels in the cerebral cortex and
cerebrospinal fluid, suggesting a role for gluta-
mate in the central nervous system dysfunction
found in CM [3]. In addition, the metabolites
of the kynurenine pathway, particularly quinolinic
acid, has been linked to convulsion in CM [33],
and has been detected at a high level in the
cerebrospinal fluid of Kenyan children with CM
[34]. It can be speculated that synapsin I is
involved in neurotransmitter release in CM. The
findings illustrate a functional correlation between synapsin I level and clinical severity. Further investigations of synapsin I inhibition might eventually help alleviate CM symptoms.

Acknowledgements

This work was supported by the Office of the Higher Education Commission and Mahidol University under the National Research Universities Initiative, Thailand. Printing of the manuscript was partially supported by the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. The authors would like to thank Dr. Mario Riganti for the use of left-over specimen.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Parnpen Viriyavejakul, Department of Tropical Pathology, Faculty of Tropical Medicine, Mahidol University, 420/6 Rajvithi Road, Bangkok 10400, Thailand. Tel: +66 2 3069184; Fax: +66 2 3069184; E-mail: parnpen.vir@mahidol.ac.th

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