Eurycoma longifolia Jack (Tongkat Ali) induced c-Fos Expression in Sensory and Motor Neurons of the Rat Brain Nervous System.

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ARTICLE INFO

Article history:
Received 13 June 2015
Accepted 28 July 2015
Available online 5 August 2015

Keywords:
Eurycoma longifolia Jack, c-Fos, rats, immunohistochemistry, CNS

ABSTRACT

Background: Extracts of the plant Eurycoma longifolia Jack (EL) have been shown to possess cytotoxic, antimalarial, anti-ulcer, antipyretic activities. The roots have been frequently prescribed for the treatment of persistent fever, tertian malaria, sexual insufficiency, dysentery, glandular swelling, antipyretic, complications after childbirth. However, there is little data concerning the ability of Eurycoma longifolia Jack on antinociception. A ethnomedical report strongly indicated its potential therapeutic effects in facial and spinal pain modulation. The functional neuroanatomical map of EL in spinal cord nuclei is not available. Objective: The aim of this study was to investigate the neuroanatomical functional site of the EL extracts (root) in rat spinal nucleus by means of c-Fos immunohistochemistry. Results: Our present study demonstrated that i.p., administration of EL (Tongkat Ali, 8mg/kg) significantly (p<0.01) increase in the number of c-Fos immunoreactive neurons in the dorsal and ventral nuclei of the rat. Conclusion: The result of the present study strongly indicates that Tongkat Ali has neuronalmodulatory effects on sensory and motor neurons. To the best of our knowledge, it is the first report which indicates that EL might play crucial roles in motor and sensory neuro modulation. Our findings warrant further study to reveal the underlying molecular mechanisms of EL induced sensory and motor neuro modulation. Background: write background about topic of paper. Objective: write the main objective for your paper. Results: write the main and most important results for your paper. Conclusion: write the main conclusion for your paper.

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INTRODUCTION

Eurycoma longifolia is known as Tongkat Ali in Malaysia and Singapore, Pasak Bumi in Indonesia, Cay Ba Binh in Vietnam and Tung Saw in Thailand [1-6]. The roots of this plant have been frequently prescribed for its potential herbal remedies for the treatment of aches, persistent fever, tertian malaria, sexual insufficiency, dysentery, glandular swelling, antipyretic, complications after childbirth [1, 2-10], or as health supplements [1, 7]. E. longifolia is known to be a promising natural source of biologically-active compounds (2). A wide variety of chemical compounds such as eurycomamide, tannins, high molecular weight polysaccharides, glycoproteins, and mucopolysaccharides, alkaloids of the quassinoid group has been identified from its root [2, 4-21]. The root of the EL is consumed as a tea in the traditional way. Root extracts have also been used traditionally to reduce blood pressure, fevers (mainly due to the presence of quassinoids) and fatigue. Root extracts of EL has been shown to enhance serum testosterone levels [22] and also increased muscle mass and strength [22,23,24]. It is noteworthy to mention that, most of the studies on Tongkat Ali and its therapeutic effect focused on male /female fertility, sexual power [25-34] which are mainly control by the region of lumbar and sacral spinal nuclei. However it’s mechanism of neuromodulatory function not known. Most importantly, ethnomedical studies reported that EL was frequently prescribed for its potential therapeutic effects in headache, muscle pain, bone pain and body pain [35-37]. However, to the best of our knowledge, there are no studies that have focused on the underlying molecular mechanism of action of EL in anti-nociception, therefore the pain modulating action of E. longifolia has not been investigated. Thus although numerous ethno-medicinal studies have reported the therapeutic properties of EL on pain modulation, the mode of action of its anti-nociceptive activity has not
been elucidated. Spinal cord dorsal horn nucleus is a critical area in relaying and modulating pain [38,39]. Moreover, Motor neurons are located in the ventral horn of the spinal cord and control effectors muscles in the periphery. Understanding the underlying neuromodulatory functional mechanisms of EL on Spinal motor and sensory neurons is among the fundamental steps required to elaborate the exact therapeutic potential of EL. Most importantly, the functional neuroanatomical map of EL in spinal cord nuclei is not available.

The immediate early genes (IEGs) encoded protein c-Fos is considered to be a transcription factor. c-Fos is expressed at low or undetectable levels in most cell types, but can be rapidly and transiently induced by many types of stimuli, including tissue trauma. C-Fos is a functional anatomical marker of activated neurones within the central nervous system. Monitoring the nuclear expression of c-Fos, the protein product of the c-fos gene, is an established reliable anatomical technique for the functional mapping of the neuronal activity and can be helpful to examine the ability of neurons to react with changes in gene expression to external stimulation under physiological, pathological and pharmacological challenges (34, 35, 63).

Investigating the molecular mechanisms of EL induced nuromodulation in sensory and in the motor nuclei might open up new research avenue for EL and its therapeutics potential in pain and motor disorder system.

Therefore, this study we investigated the neuroanatomical functional site of the EL extracts (root) in rat spinal nucleus by means of c-Fos immunohistochemistry.

**Methods:**

We obtained approval from the Institutional Animal Care and Use Committee for this study. All experimental procedures were carried out in accordance with CARE Universiti Teknologi MARA (UiTM), guidelines.

Male Sprague–Dawley rats (180–200 g), 5–6 per group, were used in all experiments. Animals were housed in groups of two, and had free access to food and water at all times. Animals were on a fixed 12 h light–dark cycle. In order to minimize stress, all animals were brought to the laboratory and acclimatized once daily for at least three days by exposure to the general handling and anaesthesia procedures. The surgical procedure was based on that described previously [62]. Briefly, rats were anaesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg) (CEVA Sante Animale, France). *Eurycoma longifolia* Jack (Tongkat Ali) powder form, water extracted was purchased from Universiti Teknologi Malaysia, Institute Bio product Development, Skudai, Johor, Malaysia and dissolved in distilled water. A single intraperitoneal injection (i.p.) of EL (8 mg/kg) was given [61]. In control group (intact) i.p. normal saline (saline treated) was administered. In all cases the dosing volume was 1 ml. Rats of all groups were kept under deep anesthesia until sacrifice to minimize the pain perception. At 90 minutes after EL/ saline administration the deeply anesthetized rats were perfused transcardially with heparinized physiological saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, containing 0.2% picric acid for 20 min. The cervical part of the spinal cords were removed and immersed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 4 hrs at 4°C. The spinal cords were cryoprotected in 25% sucrose phosphate-buffered saline (PBS). Serial 40 μm thick sections were cut with a freezing microtome and immersed in PBS. The immunocytochemical detection of c-Fos protein was performed using the peroxidase-antiperoxidase (PAP) detection protocol [62]. To insure penetration of antibodies, the sections were pre-incubated in a solution containing 0.3% Triton X-100 in PBS after blocking endogenous peroxidase. Free-floating sections were then incubated for 3 days at 4°C with rabbit antibody to c-Fos protein (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:4,000 in PBS containing 0.3% Triton X-100. The sections were incubated in a 1:2,000 dilution of goat anti-rabbit IgG (E-Y Laboratories Inc., San Mateo, CA) for 1 day at 4°C and followed by the tertiary antibody, peroxidase-antiperoxidase (PAP) in a 1: 2,000 dilution and incubated for 1 day at 4°C. The primary, secondary and tertiary antibodies were diluted to appropriate concentrations in 0.3% bovine serum albumin and 1% normal goat serum and 0.05% sodium azide. Between the incubation steps, sections were thoroughly washed with PBS. The DAB method for visualization of peroxidase was used. The sections were treated for 20 min. at room temperature with 0.02% diaminobenzidine in 0.003% hydrogen peroxide. After the final rinses in 0.05 M Tris-HCl- buffer, pH 7.6, the sections were mounted onto gelatin-chrome alum-coated glass slides, air dried, dehydrated with ethanol, cleared in xylene, and coverslipped. The specificity of the immune reaction was assessed by omitting the primary antibody in which no specific immunostaining was detected.

The sections were analyzed under a microscope and the labeled c-Fos positive neurons per section were counted. Counts were obtained from a 1 in 2 series of sections and 6 sections per animal were used. Then, the mean number of c-Fos positive neurons (± standard deviation; S. D.) for each time point was calculated. The statistical analysis was performed using a paired t test and differences at p<0.001 were considered significant.

**Results:**

In this study at 90 minutes after the *Eurycoma longifolia* (EL) administration(i.p.), a robust increase in Fos expression was found bilaterally in the dorsal (Fig.1 A & B) and ventral nuclei (Fig.2, B & C) of the spinal cord.
As shown in Fig. 1, compare to intact the higher numbers of densely labelled c-Fos positive neurons were observed bilaterally in the dorsal nucleus at 90 minutes after *Eurycoma longifolia* administration (8 mg/kg, i.p.). The semi-quantitative analysis of the number of the c-Fos-positive nerve somata per section revealed 1.8± 0.5 in the right side and 2±.06 in the left side of the dorsal nucleus in the intact rats. Compared with the intact rats (p<0.001), the number of c-Fos immunoreactive cell bodies was 34.8 ± 2.9 per section in the left side and 36 ± 2.5 per section in the right one in the dorsal nucleus. EL administration increased Fos expression by 17 folds (Fig. 1C).

In the ventral nucleus, compared with the intact rats (p<0.001), the number of c-Fos immunoreactive cell bodies was 54.8 ± 5.2 per section in the left side and 53.8 ± 5.5 per section in the right side. In the ventral nucleus EL administration increased Fos expression by 25 folds (Fig. 2D). These results suggested EL pretreatment induced activation of ventral nucleus of the spinal cord.

**Fig. 1**: Photomicrographs showing the expression of c-Fos protein in the dorsal nucleus of the spinal cord after administration (8 mg/kg, i.p.) of *Eurycoma longifolia* Jack (Tongkat Ali), a significantly increased number of c-Fos positive neurons is observed at 90 minutes El administration (B & C). (B) is the higher magnification of (A). Fig. 1. (C), A graphic showing the semi-quantitative analysis of c-Fos positive neurons (p<0.001). Scale bar, 50 μm.

**Fig. 2**: Photomicrographs showing the expression of c-Fos protein in the ventral nucleus of the spinal Cord after administration (8 mg/kg, i.p.) of *Eurycoma longifolia* Jack (Tongkat Ali), a significantly increased number of c-Fos positive neurons is observed at 90 minutes El administration (B, C & D). (C) is the higher magnification of (B). Fig. 1. (D), A graphic showing the semi-quantitative analysis of c-Fos positive neurons (p<0.001). Scale bar, 50 μm.

**Discussion**:

The result of this study demonstrated that EL administration (8mg/kg) induced increase in c-Fos expression in the dorsal and ventral nucleus of the spinal cord (Fig. 1 & 2 ). The central pain processing circuits for nociceptive information begin at the level of the spinal cord dorsal horn. Interneuronal networks in the dorsal horn are responsible not only for the transmission of nociceptive information but also help modulate that information and pass it on to other spinal cord neurons, therefore, certain patterns of stimulation can lead to increase nociceptive transmission or inputs result in the inhibition of projection neurons. The balance of these excitatory and inhibitory processes is the mechanism of modulation of pain in the spinal cord dorsal horn [38,39]. The excitatory neurotransmitters in the dorsal horn include amino acids, particularly glutamate, as well as neuropeptides, such as SP, CGRP, VIP, somatostatin, and others [40].
For example, peripheral nerve damage, can lead to an up regulation or down regulation of these. Another example is the increased stores of glutamate in the dorsal horn that occurs after the development of experimental arthritis [41].

The excitatory neurotransmitters in the dorsal horn are mediated by a number of neurotransmitters, including inhibitory amino acids, such as GABA and glycine, as well as neuropeptides, such as encephalin [42]. In the light of these studies, the increased in c-Fos expression in present study suggest that EL might have neuromodulatory function on spinal dorsal neurons.

Motor neurons are located in the ventral horn of the spinal cord and control effectors muscles in the periphery. To ensure fine coordination, motor neurons acquire and retain the identity of muscles they innervate and integrate the functional neuronal circuitry of movements. Disorders in the spinal motor neurons lead to atrophy and/or spasticity of the associated musculatures. For example, the motor symptoms of the Parkinson disease are involved in spinal motor neurons [43]. Therefore, the EL induced increased in c-Fos expression in our study strongly indicated that understanding the underlying neuromodulatory functional mechanisms of EL on Spinal motor and sensory neurons is among the fundamental steps required to elaborate the exact therapeutic potential of EL.

Previous study reported that EL increased cAMP levels [44]. cAMP is one of the most common and universal second messengers. The cAMP regulate highly diverse physiologic processes, metabolism, secretion, calcium homeostasis, muscle contraction, cell fate, and gene transcription, thereby to permit delicate adaptations of biologic responses [45]. Many studies have shown that cAMP-inducing agents demonstrated adaptive effects on skeletal muscle by increasing myofiber size and promoting fiber-type transitions to glycolytic fibers. Moreover, reports showed that cAMP signaling improved muscle function and ameliorated atrophy in several rodent models, including disuse, denervation, aging, and muscular dystrophy. In addition to functional adaptation cAMP signaling participates in muscle precursor cell differentiation, migration, and fusion. In adult muscle, stimulation of cAMP production showed slow degeneration or promoted regeneration in rodent models of necrotic muscle injury and Duchenne's muscular dystrophy [45-48]. Reports suggested that cAMP may not only upregulated the expression of growth-promoting genes, but also limited the expression of genes that negatively impact axonal regeneration [49].

cAMP-responsive element-binding protein (CREB) phosphorylation mediated c-fos expression in response to agents that increase intracellular concentrations of cAMP or Ca++. Similarly, nerve growth factor appeared to stimulate c-fos transcription via phosphorylation of CREB. CREB-binding sites have been found in the promoter regions of immediate-early genes such as c-fos. The c-AMP-responsive element binding protein (CREB) and its phosphorylated product (P-CREB) are nuclear proteins expressed after stimulation of pain-producing areas of the spinal cord [50,51, 52]. Therefore our present result strongly suggests further study to investigate the EL regulated neuromodulation in a spinal model of inflammation and hyperalgesia.

Report demonstrated that drug compound altered specific splicing patterns of gene (survival motor neuron gene, SMN2 gene lacks exon 7) which represented a new approach to modification of gene expression in disease treatment [53]. Previous study demonstrated that c-fos expression levels in alpha motoneurons were associated with the activity levels of their corresponding muscle [54].

Another report demonstrated that mating and mating or noncontact sociosexual interaction induced c-Fos expression in spinal neurons and co-localized with androgen-sensitive neurons. In addition, report showed that testosterone-treated rat induced c-Fos expression in the medial pre-optic nucleus. Therefore, our present result indicate that in order to reveal the exact functional role of EL in spinal motor and sensory nuclei further study is required to identify the nature of these c-Fos expression (e.g., neurotransmitter, receptors e.t.c.) observed in this study [55,56].

In spinal bulbocavernous (BC) motoneurons, combined treatment with both testosterone and brain-derived neurotrophic factor (BDNF) is more effective than treatment with either compound alone in the maintenance of androgen receptor immunoreactivity. Moreover, the effect of EL on BDNF protein regulation in SNB motoneurons not studied. Previous report observed that following mating to ejaculation induced c-Fos expression in BC, spinal sensory and motor neurons and co-localized with androgen receptor (AR) mRNA. In addition, it is documented that treatment with testosterone is protective in surviving motoneurons spinal cord injury perhaps through regulation of BDNF. Furthermore, BDNF expression is regulated through a calcium-dependent signaling pathway, involving CRE and CREB. Taken together, these findings along with our present results suggest that further study is required to reveal the underlying neuromodulatory mechanism of EL on spinal motor and sensory neurons. It is possible that that EL also neuromodulate BDNF [57-60]. In the light of the above mentioned reports our present results highlight the fact that further studies are clearly needed to elucidate the underlying functional neuromodulatory mechanisms that played by EL to induce c-Fos expression in the spinal sensory and motor neurons.

To our knowledge, the present study demonstrated for the first time that spinal sensory and motor neurons of rat CNS are possibly the neroanatomical functional sites of EL neuromodulation. Further studies are necessary to substantiate these observations, as spinal sensory and motor neurons contains a variety of peptides
often different biochemical and functional nature and not all activated neurons express c-Fos (67,68). Previous study indicated that c-Fos expression in the brain was induced by transsynaptic stimulation (69). Therefore, our present result indicate that in order to reveal the exact functional role of EL in spinal motor and sensory nuclei further study is required to identify the nature of these c-Fos expression (e.g., neurotransmitter, receptors e.t.c.) observed in this study.

A previous report demonstrated that c-fos expression and fMRI both methods detected similar brain nuclei in response to same stimulus pressure in anaesthetized rats [64]. More specifically, functional c-fos imaging has become available in vivo. Single-stranded phosphorothioate-modified oligonucleotides with sequences complementary to c-fos (sODNs) were coupled to superparamagnetic iron oxide nanoparticles (SPIONs) and injected intracerebroventricularly. These SPION-cfos constructs after hybridizing to stimulus-induced c-fos mRNA produced a magnetic resonance signal that can be detected in living animals by MRI [65,66]. These reports strongly support that c-Fos has become a valuable translational tool to bridge clinical and preclinical research in animals or in animal models. In the light of the above studies our present results indicate that EL might have wide range of unknown therapeutic functions.

Conclusion:
Our present findings demonstrated for the first time that Eurycoma longifolia Jack (Tongkat Ali) may modulate the c-Fos expression in the rat spinal sensory and motor neurons. Furthermore, EL might have wide ranges of unknown therapeutic potentials. The results obtained here will be useful for further studies to gain more insights into the EL induced underlying neuromodulatory mechanisms in spinal sensory and motor neurons of rat CNS.

ACKNOWLEDGMENTS
This work was supported by a grant form PENYELIDIKAN DANA PEMBUDAYAAN PENYELIDIKAN (RAGS), University Teknologi MARA, Grant # 600-RMI/RAGS5/3(124/2012)

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