CHAPTER 1: INTRODUCTION

1.1 Research Questions and Objectives

The existing information on the spinal cord organisation is well-established as far as the mammalian system is concerned. Suffice to say, far too little attention has been paid to establish the non-mammalian model system, let alone the amphibians'. Despite the needs to amplify the limited knowledge regarding their neural structures in correlation to the successive behaviour throughout their significant evolutions, yet very few researchers were keen on dealing with such tissues. Owing to the intricacy of processing neural tissue of non-mammalian through histological and immunohistochemical procedures, there are no standard protocols in delivering satisfactory results that could be replicated across different labs. It is no surprise that the established reports on the amphibians are not as abundant as for the diverse reports on mammalians. Out of these few reported works, majority made use of the non-local species such as *Rana esculenta* (Lorez and Kemali, 1981), *Rana pipiens* (Halpern, 1972; Adli et al., 1988, 1999; Adli and Cruce, 1995a, 1995b; Stuesse et al., 2001) and *Rana catesbeiana* (Sasaki, 1977; Mensah and Thompson, 1978; Ueda et al., 1984; Partata et al., 2002; Guedes et al., 2004) as experimental animals. Reports on preliminary analysis on the central nervous system of the local species have been few, for examples *Bufo melanostictus* (Azlinda, 1996), *Polypedates leucomystax* (Rohaya, 1996) and *Microhyla heymonsi* by Tang (2008; Tang et al., 2010).

In addition, to counter the overemphasis of mammalian model in neurobiological researches, this study also serves as an initial effort in maximizing the use of locally available species of Amphibian. With neuroanatomical techniques (i.e. histology and immunohistochemistry, *Fejervarya limnocharis* or the Rice-field Frog was put under scrutiny as an alternative model system to guide the characterisation of spinal cord by

covering the aspects of cytoarchitectural and chemoarchitectural features. The spinal cord chemocytoarchitecture was demonstrated via the localisation of the common nociception-related neurotransmitters (enkephalin, substance P and serotonin). By deducing the distribution pattern of these neurotransmitters, the findings could shed considerable light on the fundamentals of spinal cord neurochemistry and the nociceptive mechanism, as well as the overall anatomical organisation of the frog spinal cord.

Thus, this research was conducted with three main objectives in the spirit of supporting a diversity of biological models.

1) To describe the gross morphology and general histology of the spinal cord of a locally available frog species, i.e. *Fejervarya limnocharis*.
2) To provide cytoarchitectonic information on the spinal cord organisation of *Fejervarya limnocharis* using basic neurohistological staining and immunohistochemistry.
3) To delineate the distribution of selected nociception-related neurotransmitters (enkephalin, substance P and serotonin) in the frog spinal cord via immunohistochemical techniques

1.2 *Fejervarya limnocharis* as Experimental Model

The use of amphibian model offers a comparative approach as to providing the fundamental basis for general comprehension of the nervous system and its circuitry. With a thorough analysis of information based upon several species coming from the same class, it is only then extrapolation and generalization could be deduced.

A locally available Anuran species with the given scientific name of *Fejervarya limnocharis* (Gravenhorst, 1829) was chosen as the species of interest in this anatomical characterisation study. Part of the justification for selecting this animal species is the fact that it is one of the most common Anurans in Malaysia. Aside from its constant availability and also easy accessibility throughout the research period, using this species as experimental subjects also has the advantage of being cost-saving. Unlike most mammalian laboratory animals, *F. limnocharis* requires no special housing or specific animal needs other than a basic aquarium with water and food supply. Even so, this species shows robustness and resistance to environmental changes. Most important of all, this species justification was also based on its origin from the family of Ranidae, seeing that most of the experimental animals involving Anurans in the past studies were of the same family, such as *Rana esculenta*, the Edible frog, *Rana pipiens*, the Northern Leopard frog and *Rana catesbiana*, the Bullfrog. Therefore, this enabled easy interpretation and comparison of results within the species of the same family.

The nomenclature of *Fejervarya limnocharis* is as below:

Kingdom	: Animalia
Phylum	: Chordata
Subphylum	: Vertebrata
Class	: Amphibia
Order	: Anura
Family	: Ranidae
Genus	: *Fejervarya*
Species	: *F. limnocharis*

1.2.1 Habitat and Distribution

To assist the species identification process, information gathered from various monographs of Amphibians (Berry, 1975; Inger and Stuebing, 2005) has extensively described its habitats and distribution, general morphology as well as ecological behaviour. As suggested by its common names like Rice-field Frog and Asian Grass Frog, *F. limnocharis* inhabits disturbed and also cleared areas that are often associated with the activities of man. This includes paddy fields, vegetable gardens and even roadside lawns. This species is widely distributed across the tropical Asia, which covers the west of Malaysia, India, Sri Lanka, Thailand, southern China, Japan, Taiwan, Singapore and the major Indonesian islands (Ecology Asia, 2008). According to Berry (1975), Sungai Tekam in Pahang, Field Study Centre of Universiti Malaya in Gombak, Lake Chenderoh in Perak, Lake Gardens in Kuala Lumpur and Templers Park in Selangor are some of the known localities in the Peninsular Malaysia where *F. limnocharis* could be found.

1.2.2 Morphology and Physical Attribute

From the external morphological aspect, its prominent features comprised of: (a) the distinct warty or finely pebbled skin surface, (b) conspicuous dark bars on the limbs and mouth, (c) the usual presence of a yellow vertebral stripe from the nose extending to the anus, over the overall rusty brown or greyish colour of its body, which is also covered with darker blotches or spots on the back (Berry, 1975; Inger and Stuebing, 2005) (Figure 1). Such features, especially the indistinguishable colour scheme of its body that could easily blend in with the environment and enables effective camouflage, provide a good mean to avoid predation. With the average snout-to-vent length that ranges from 32 to 50 mm for males and measures slightly longer at 49 to 60 mm for females, *F. limnocharis* is relatively small in size in comparison to other species of Ranidae.

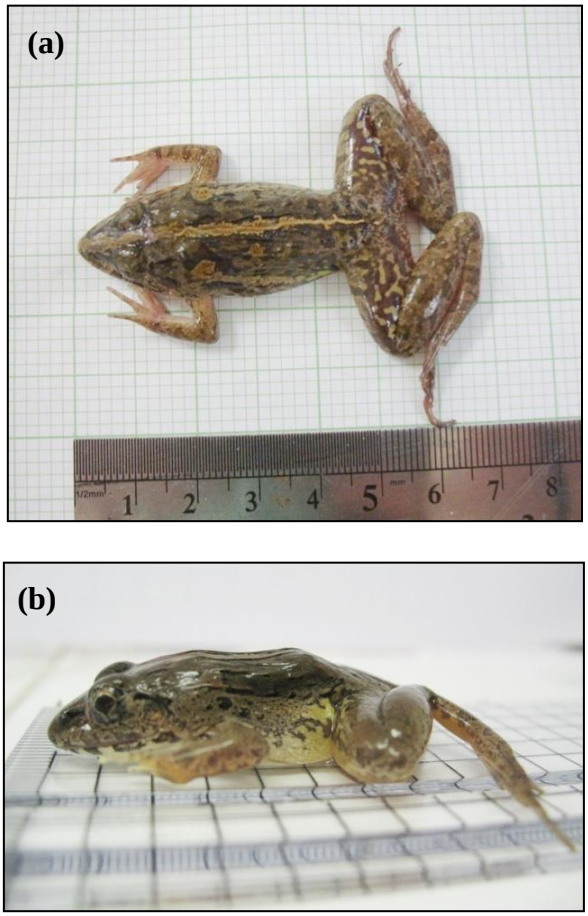

Figure 1.1: Adult frog, *Fejervarya limnocharis*; (a) dorsal view, (b) lateral view.

1.3 Overview of Spinal Cord

Spinal cord is one of the main components of the central nervous system (Kandel and Schwartz, 1985; Hendelman, 2000; Inderbir, 2006). It serves two functions in general. Firstly, it acts as a communication doorway connecting the brain and the lower parts of the body by carrying incoming sensory information from the body to the brain for interpretation and descending motor information away from the brain to the body, which resulted in body's response. The spinal cord also functions as a reflex centre that mediates reflex responses via interneuronal circuitry. The afferent fibres carries sensory impulses while the efferent fibres send out motoric outputs to the corresponding regions of the body. Similar to the brain, the spinal cord consists of a network of the basic functional units known as neurons that conduct electro-chemical signals. Also found among the neurons are the glial cells. They are non-excitable and primarily function to provide structural and trophic support for the neurons.

A historical review published in the year of 2008 by Pearce has provided a selective compilation of knowledge progression concerning the anatomy of spinal cord. As mentioned in the article, a physician named Herophilus made an initial discovery of the cord in 325 to 260 B.C., referring it as the caudal extension from the hindbrain and thus, led to the naming of this structure as what we presently known; the 'spinal cord'. In the second century A.D., Galen's study based on animal's dissection brought forward the explicit anatomical details of the vertebral column, spinal cord and nerve roots. From there onwards, more subsequent advancement in the studies of this structure began to arise from different approaches that varied from the anatomical findings such as, the works of Blasius, Huber, Clarke and Lissauer, to the technical aspect (Pearce, 2008). Stiling's self-developed technique through the introduction of microtome hence, marked the keystone in the investigation of spinal cord by histological method. Subsequently,

Rexed's proposal to divide the cord grey matter into ten laminae in relation to the neuronal distribution that were of similar sizes, served as one of the efforts in bridging both the anatomical and physiological aspects. The latter came into focus as a result of the growing of knowledge throughout the centuries. Regardless of many other discoveries and advances in scientific studies of the spinal cord thereafter, the ongoing pursue to characterise and further understand this structure at the fundamental level should not be disregarded.

1.3.1 Frog Spinal Cord

Frog spinal cord is reckoned to be a good model system for the study of various spinal mechanisms due to its relatively shortness in length (11 segments), technical accessibility and its close resemblance to those in mammals (Ebbeson, 1976). As opposed to many amniotes including birds and reptiles, direct forebrain projection to the spinal cord of amphibians is nonexistent (Halpern, 1972; Kuhlenbeck, 1975; Ebbeson, 1976; Mensah and Thompson, 1978; Stevens, 2004). This projection is also termed as the corticospinal tract in mammals; its function closely associated to voluntary skilled movements. The uniqueness of this condition in the amphibians therefore, marks the importance of the spinal cord in animals deriving from this class as the activity modulation may likely to occur in the lower structures of the central nervous system, i.e. the brainstem and the spinal cord, itself.

The main emphasis of many studies concerning the spinal cord is on the grey matter. Initial effort by Rexed to demarcate this region into laminar arrangement was presented in the cat spinal cord (Rexed, 1952). Since then, the intrinsic organisation based upon the classical lamination scheme is widely accepted not only in mammals including rats (McClung and Castro, 1978; Molander et al., 1984; Grant and Koerber, 2004),

monkeys (Ralston, 1979; Zhang and Craig, 1997) and rabbits (Kirmani et al., 2011) but also in other animal models including birds (Brinkman and Martin, 1973; Leonard and Cohen, 1975), reptiles (Kusuma et al., 1979; Wolters et al., 1986; Fernández et al., 1992), and fishes (Cameron et al., 1990) (Table 1.1). The segregration of the spinal grey matter is conventionally based upon cytological features, projection patterns, neurochemical distributions as well as the functional aspects (Heise and Kayalioglu, 2009). By complying with the same cytoarchitectonic boundaries, the efforts to parcellate the spinal grey of the frog so far have been less than successful due to the absence of well-defined cellular subdivisions (Ebbeson, 1976; Sasaki, 1977; Adli et al., 1988). Such obscurity indirectly leads to the insufficiency of detailed information regarding the intrinsic organisation in literature at present; but nonetheless these anatomical data are important in identifying and interpreting homologous structures for studies within or across species.

Table 1.1: Adaptation of Rexed lamina for spinal grey

Animal Classes	**Reference**
Mammals	
1) Cat	Rexed (1952); Galhardo and Lima (1999)
2) Rat	McClung and Castro (1978); Molander et al. (1984); Grant and Koerber (2004)
3) Rabbit	Kirmani et al. (2011)
4) Monkey	Ralston (1979); Zhang and Craig (1997)
Birds	
1) Domestic fowl	Brinkman and Martin (1973)
2) Pigeon	Leonard and Cohen (1975)
Reptiles	
1) Lizard	Kusuma et al. (1979); Wolters et al. (1986)
2) Turtle	Kusuma et al. (1979); Fernández et al. (1992)
Fishes	
1) Elasmobranch fish	Cameron et al. (1990)

1.4 Pain and Nociception

'Pain' is a term generally applied on human due to our ability to verbalise the different degree and types of sensation involved. 'Pain' is considered to be a very subjective matter as it involves a wider spectrum of definition, by taking into account the complex perceptual as well as both physical and emotional components (Stevens, 2004). Thus, 'nociception' is usually preferred as the more precise term for non-human animals to depict the unpleasant sensation associated with actual or potential tissue damage in response to noxious stimuli, without making assumption as to the capacity of pain experienced.

However, there is a questionable view of whether animals are able to perceive pain as humans do and hence, countless efforts such as behavioural assays were conducted to prove the credibility of the statement. Most commonly the tail-flick test, flinch-jump test, pinch test, hot plate test and recently, the formalin test, were introduced to measure the response of an animal subjected to noxious stimulus (Oyadeyi et al., 2007). As proposed by Singer (1990) and Machin (1999), the existence of such sensory capability in animals is evident as this vital ability enables them to detect harmful signals to their wellbeing and hence, enhances their prospect of survival. Behavioural motor responses demonstrated by non-mammalians such as rapid startle reactions, affective responses like vocalization, coordinated reactions such as the act of biting the source of pain and the development of avoidance behaviour, were all suggesting discomforts in response to nociceptive stimuli (Machin, 1999; Maccio-Hage, 2005). These had provided substantial evidence for the existence of pain perception in animals.

Free nerve endings or primarily known as nociceptors are sensory receptors involved in pain detection. Found beneath the skin are the fibres associated to cutaneous pain

whereby the duration of pain is shorter. Pain persists longer for somatic and visceral pain as detected by nociceptive afferent fibers in tendons and joint, and body organs respectively. In mammals, three general classes of primary afferent are identified: A-beta, A-delta and C fibers. However, only A-delta and C fibers are classified as nociceptive afferents fibers while A-beta fibers are said to be unspecific for detecting pain stimuli (Lautenbacher and Fillingim, 2004). Similarly in amphibians, the corresponding primary afferent groups are: A, B and C fibers (Stevens, 2004). Upon stimulation or activation of these primary afferent fibers at the site of insult, pain-signaling neurotransmitters are released.

The three endogenous neurochemicals considered in this research namely enkephalin, substance P and serotonin have been closely associated with nociception processing. Localisation mapping of the neurotransmitters has greatly assisted in the effort to understand their functional involvements in relation to the location where they are generally present. Substance P elicits pain due to its release corresponding to tissue damage or injury (Kantner et al., 1985). Whilst serotonin was generally thought to exhibit inhibitory effect but later discovered that it could also be an excitatory transmitter in lamina I-III of the dorsal horn as microinjections of this substance resulted in algesia (Stamford, 1995). In contrast to substance P, an opioid peptide, enkephalin was found accountable for the cause of diminished pain, relieving the nociceptive sensation as it interacts with the opioid receptors located near the synapses of the dorsal horn (Glazer and Basbaum, 1983).

1.5 Review of Neurotransmitters

Most intercellular communication between the central nervous system and the rest of the body is achieved by the release of chemical transmitters or neurotransmitters at the chemical synapses (Ottoson, 1983). Neurotransmitters are generically termed as endogenous chemicals that mediate the transmission of signals across a synaptic cleft, exhibiting an activating or inhibiting function of the neighbouring cells. Over the years, there are numerous types of transmitter being identified at various sites in the nervous system. However, each chemical is bound to fulfill several rigorous criteria before recognised to such status. The main determining conditions are as follows (Ottoson, 1983; FitzGerald et al., 2007): (a) the substance must be synthesized endogenously within a neuron, (b) the release of this substance must be followed by depolarization of the pre-synaptic neuron, which in turn induces the influx of calcium ions, (c) specific receptor must be present on the post-synaptic membrane to alter the membrane potential of the target neuron, (d) local administration of the isolated substance must exhibit the same effect as pre-synaptic activity, (e) antagonistic substances must block the effect of the putative transmitter, and (f) the biochemical pathway for terminating or inactivating the transmitter effect must be identified.

Neurotransmitters are encapsulated within vesicles and mainly located in the synaptic region prior to its release from the pre-synaptic neuron and attachment to the appropriate post-synaptic receptor. As action potential occurs, depolarization of the axon terminal will trigger the influx of calcium ions. These ions induce the release of neurotransmitter molecules via exocytosis from its vesicle into the synaptic cleft. Consequently, this will lead to interactions between the released endogenous chemicals with the specific receptors on the post-synaptic cells, eliciting post-synaptic effects. Depending on the type of corresponding receptor they bind to, it can result in either

excitatory or inhibitory post-synaptic potentials. To prevent the continuous action of the neurotransmitters on the post-synaptic cell, they will be either removed from the cleft thru reuptake or undergo degradation by analogous enzymes. The article by Kanner and Schuldiner (1987) can be referred to for a review of neurotransmitter synaptic activities.

Neurotransmitters can be classified into three main chemical groups; amines, amino acids and polypeptides (Ottoson, 1983). Coming from the first group of amines are acetylcholine and other biogenic amines such as serotonin, epinephrine, norepinephrine and histamine, whereas the amino acid transmitters include of examples like glycine, glutamate and gamma-aminobutyric acid (GABA). Both of these two principal classes have been generally referred to as small-molecule transmitter substances due their low molecular weight as suggested by their name. The third class comprises a diverse group of polypeptides such as enkephalin, substance P, endorphin and somatostatin. Commonly termed as neuropeptides, they have relatively short chains of amino acids linked by peptide bonds. In specific regions, neuropeptides were co-localised within the same neuron as amino acids and amines neurotransmitters; hence, the term ‘neuromodulator’ was derived to outline the function of these peptides. Their co-existence with the classical neurochemicals was believed to have a connection as they alter or mediate the release or actions of established neurotransmitters (Adli et al., 1988; Crossman and Neary, 2005).

1.5.1 Enkephalin

Enkephalin was classified as an endogenous opioid peptide based on its opium-like effect in relation to analgesia (Goodman et al., 1983). Discovered back in 1975 by Hughes and his co-workers, there were two forms of this pentapeptide that have been isolated, Methionine-enkephalin (Met-enkephalin) and Leucine-enkephalin (Leu-enkephalin) (Miller and Pickel, 1980; Ottoson, 1983; Goodman et al., 1983). Both types differed by only an amino acid. The structure of Met-enkephalin and Leu-enkephalin are as shown:

Met-enkephalin: Tyr-Gly-Gly-Phe-Met

Leu-enkephalin: Tyr-Gly-Gly-Phe-Leu

Many past studies have reported the close association of enkephalin-immunoreactive sites to immunohistochemical localisation of opiate receptors (Simantov et al., 1977; Goodman et al., 1983; Ottoson, 1983). These findings gave hints to the role played by this pentapeptide in the integration of nociception perception at the spinal cord level; inhibiting the release of nociception-related transmitters such as substance P. However, Met-enkephalin is of higher interest in this research were of higher interest due to previous report (Hökfelt et al., 1977) where the considered neurotransmitter occurred 3 to 4 times greater in amount than its counterpart peptide, Leu-enkephalin.

1.5.2 Substance P

The discovery of substance P by von Euler and Gaddum back in 1931 in tissue extract and later study by Lembeck in 1953, had put forward the idea of substance P having excitatory effect at the sensory synapse in the spinal cord (Ottoson, 1983; Aronin et al., 1983). Also generally referred to as a tachykinin neuropeptide, substance P is made up of a short polypeptide chain with 11 amino acids in the sequence of [Arg-Pro-Lys-Pro-

Gln-Gln-Phe-Phe-Gly-Leu-Met]. This neuropeptide and its specialized receptor, neurokinin-1 (NK-1) receptor were widely localised in various brain and spinal cord regions associated to modulation of nociception (Kandel and Schwartz, 1985). It was believed that it is released from the terminal of peripheral sensory nerve fibres into the spinal dorsal horn as a result of inflammation in response to tissue damage or injury, which lead to a increased sensitivity towards nociception (Lembeck et al., 1981; Kuraishi et al., 1985; De Felipe et al., 1998). In addition, its involvement in nociception transmission was further supported as administration of capsaicin, a noxious stimuli, in rats induces the release of substance P from the primary sensory neurons (Gamse et al., 1979; 1981; Purkiss et al., 2000). Mapping substance P in the central nervous system could therefore useful in assisting the work of developing potential analgesic drug in therapeutic treatment via administration of receptor antagonists.

1.5.3 Serotonin

Serotonin is one of most widely studied classical neurotransmitters for its psychological functions (Sahelian, 2005). The effects of this neurotransmitter were discovered in regulation of mood, anxiety, aggression as well as the sleep and wake cycle. Serotonin, derived from the amine group, is synthesized from the amino acid precursor tryptophan. The synthesis involves two enzymes, i.e. tryptophan hydroxylase and 5-hydroxytrytophan decarboxylase, producing the final product of serotonin or chemically known as 5-hydroxytryptamine (5HT) (Kandel and Schwartz, 1985). In humans and animals, groups of serotonergic neurons are readily located in both central nervous system and also enterochromaffin cells of the gastrointestinal tract. The presence of serotonergic cells in the central nervous system were found grouped into two main sources: (a) caudal system in medulla where its axons descend in the lateral columns of spinal cord, appearing to mediate the control of spinal transmission of pain impulses

(Ottoson, 1983), and (b) rostral system in the raphe nuclei of midbrain, innervating the forebrain structure (Waymire, 1997). Existence of 5HT in spinal cord has been reported, but not by many (Hancock, 1982; Hadjiconstantinou et al., 1984; Cameron et al., 1990). Sommer (2006) reported that serotonin may exert dual roles, i.e. analgesic and hyperalgesic effects depending on the site of action and the type of receptors the neurotransmitter reacts with. For instance, its release from the brainstem would produce analgesic action by inhibiting pain at the spinal sites. On the contrary, when injury is inflicted at the periphery level, the increase of serotonin content would result in excitation of afferent nerve fibres and hence, contribute to hyperalgesia (Sommer, 2006). Studies have shown that the coexistence of serotonin and substance P in the central nervous system, for example in rats and frogs, through immunohisto- and immunocytochemistry, and autoradiography (Chan-Palay et al., 1978 and Adli et al., 1988). Hence, this suggests the involvement of both transmitters in related role especially in the nociceptive system.

1.6 Review of Neuroanatomical Methods

In combination with light microscopy, neuroanatomical methods including neurohistological staining and immunhistochemistry are useful for evaluating the structural organisation of the spinal cord and thus, allowing the vague areas in the frog spinal grey to be addressed. The application of histology provides comparative visualization of different structures within a nervous tissue contingent upon the type of staining protocol used (Santafé et al., 2007). Components like neuronal soma, nerve fibres, myelin sheaths of axons and the neuronal Nissl bodies could be highlighted by means of neurohistological staining. Prior to microscopic examination, several intricate procedures are to be carried out in order to produce good histological slides. Since this study primarily focused on the preparation of formalin-fixed paraffin-embedded tissues,

the typical histological processing procedure would consist of: (a) tissue fixation to avoid cell degradation and to preserve the morphology of the tissue throughout the harsh conditions of the subsequent processing, (b) dehydration to eliminate water from the tissue sample, (c) clearing to remove the dehydrant, (e) infiltration and embedding to produce paraffin blocks of tissue, (f) tissue sectioning of the paraffin block and (g) histological staining of the tissue sections in order to observe the neuroanatomical features through microscopic analysis.

Immunohistochemical technique enables demonstration of cells and fibres immunoreactive to a specific neurochemical subpopulation within the spinal cord in respect to the chemoarchitecture of the homolog regions. It is an established routine to identify targeted cellular or tissue constituents termed as antigens via antigen-antibody interactions (Palkovits, 1985). These interactions are then visualised via immunohistochemical staining using a marker compound such as enzyme, colloidal metal, fluorescent label and radioactive element (Robinson et al., 1990). The present study involves the indirect immunohistochemistry method. The principle of this procedure involves the application of an unlabeled primary antibody that binds to the tissue antigen of interest (which in this case would be the investigated neurotransmitters) and the interaction forms the primary antibody-antigen complex. This is followed by the application of enzyme-labeled secondary antibody, raised in another animal host. The enzyme conjugated to the secondary antibody is then developed by an appropriate chromogen to produce colorimetric end product. Hence, the presence of coloured products in the tissue section indicates the presence of the antigen being investigated (Elde, 1985; Robinson et al., 1990).

Findings derived from this experiment could also indicate the functional involvement of a neurochemical with reference to the immunoreactive location. So far, immunohistochemistry has enabled the topographical localisation of many neurotransmitters in the nervous system (Palkovitz, 1985). Demonstration of this technique was shown in similar studies that aim to identify the immunoreactivities of the investigated nociception-related neurotransmitters in the nervous system of human (Lanerolle and LaMotte, 1982), monkeys (Tashiro et al. 1990), cats (Hökfelt et al., 2003) rats (Simantov et al., 1977; Seybold and Elde, 1980; Iritani et al., 2006), lizards (Wolters et al., 1986), elasmobranch fishes (Cameron et al.,1990), domestic fowls (LaValley and Ho, 1983) and also frogs (Adli et al., 1988; Adli and Cruce, 1995a, 1995b; Partata et al., 2002; and Guedes et al., 2004).