CHAPTER 4: DISCUSSION

4.1 General Morphology of Frog Spinal Cord

Primarily, the spinal cord serves two functions. First, it acts as a mediator of signal transmission between the brain and the body. The spinal cord carries sensory impulses to the brain where the processing events take place, and sends motor signals to the body in which the consequential response is to be executed. Secondly, it plays a role as a reflex center where reflex responses are generated within the spinal cord solely by means of interneuronal circuitry (Ville et al., 1978). Taking into account of the entire interconnecting central nervous system, the significant difference between anuran species and other amniotes (mammals, reptiles and birds) would be the perceptible absence of the telencephalo-spinal pathway or simply the direct cerebral projection to the cord in frogs (Ebbeson 1976; Llinas and Precht, 1976; Stevens, 2004).

From the gross morphological aspect, the frog spinal cord was relatively comparable to the features commonly seen in those of higher vertebrates. In adult *Fejervarya limnocharis*, the spinal cord ended before the termination of vertebral column and this characteristic was also displayed in other Anurans (Oksche and Ueck, 1976). The spinal cord was not fully extended throughout the column based upon two possible grounds; (A) atrophy of the structure of the caudal segment after metamorphosis, leaving remnant that forms the filum terminale, and (B) continuous growth of the vertebral column even after the spinal cord has achieved its maximum length (Sarnat and Netsky, 1981). Hence, the shorter spinal cord relative to the length of the column. Extending from the most caudal end of spinal cord or conus medullaris was a threadlike prolongation, known as the filum terminale. Similar condition could also be observed in the spinal cords of many fishes and mammals. On the other hand, this structure is deemed missing for avian and reptilian. The spinal cord and its column of these animals appeared to have

equal lengths, where the cord extends as far as the free vertebra (Sarnat and Netsky, 1981; Marshall, 1961; Schaeffer and Waters, 1996).

This long, tubular mass of nervous tissue exhibited noticeable enlargements at the cervical and lumbar regions. This was reflecting the massive nerve innervations at the corresponding level of the fore- and hindlimbs of the animal (Ebbeson, 1976; Sarnat and Netsky, 1981; Hochman, 2007). While the remaining two regions, i.e. thoracic (found in between cervical and lumbar regions) and sacral (located caudally from the lumbar region) were of reduced diameters in general. As lesser control mechanism was involved for the upper trunk and lower part of the hindlimbs, hence fewer neurons are required to innervate these parts of the body. Similarly, the features observed at the gross morphological level were also reflected in the spinal cord structure when analysed histologically. Though cervical and lumbar intumescences are prominently found in most of the amphibian, mammalian and avian spinal cords, however, such feature is lacking in fishes and limbless reptilians like snakes and legless lizards. This is very much associated with the absence of extremities in these animals (Sarnat and Netsky, 1981; Cameron et al., 1990; Schaeffer and Waters, 1996).

4.2 Histology of Frog Spinal Cord

In the transverse section, the spinal cord demonstrated a classical organisation in which the white matter superficially encircles the core ‘butterfly-shaped’ grey matter. As their names implied, the spinal cord tissue gave the appearance of two contrasting layers. The inner grey mass was made up of neuronal cell bodies and their dendrites whereas the outer mass of white matter was composed of bundles of myelinated axons that lie parallel along the length of the cord. The cervical and lumbar intumescences occurred in the cord was consistent to the histomorphometric analysis that revealed greater overall

cross-sectional diameter and area as well as the grey matter area. These general features were no different than those as seen in other amniotes, although not as defined as in mammals and birds.

In each half section, the grey matter was simply differentiated into 3 regions: dorsal horn, intermediate zone and ventral horn. Dorsal horn is the site where the afferent terminals that carry sensory information was terminated in the spinal cord. On the contrary, the ventral horn comprised of motor neurons. These neurons are one of the largest cells in the central nervous system and also known as the 'final common pathway' for motor processing (Hochman, 2007). These motor neurons or efferent neurons that innervate the muscles send out nerve impulses to the effector organs in response to the command from the brain and resulted in muscle movements. The intermediate zone that lies between the dorsal and ventral horns contained numerous interneurons. Besides relaying impulses between the afferent and efferent neurons, interneurons were often related to the body's reflex function.

Overall, greater area of grey matter occurred in the regions of enlargements (cervical and lumbar) in contrast to the other levels. This is in correlation with the massive nerve innervations to the respective fore and hindlimbs. The thoracic and sacral levels were mainly involved in the innervations to the thoracic and abdominal areas of the body where movements were more restricted and hence fewer muscles are innervated. Thus, this would clarify the decrease in the grey matter area due to lesser extent of functional involvement.

Likewise, the white matter was subdivided into dorsal, lateral and ventral funiculi on each side of a cross section. These funiculi were made up of myelinated fiber tracts

subserving a common function. Linking the brain and the spinal cord are tracts generally known as the ascending and descending spinal tracts. The ascending tracts enable sensory information to be transmitted towards the brain for interpretation and as opposed to that, the descending tracts carry commands caudally from the brain to the spinal cord. Specific ascending and descending tracts would be found connecting the spinal cord and to the brainstem structures like cerebellum and reticular nuclei. In the transverse sections of four spinal segments of the frog, the white matter showed a gradual reduction in proportion as it descends rostrocaudally. Segments from the upper levels have thicker masses of white matter owing to the collected axons from the ascending and descending tracts going to and from the entire body. Going down the cord, more and more descending axons make an exit while ascending axons enter the pathway to the brain. Hence, this explains the progressively smaller number of axons seen at the lower segmental levels, especially at the sacral level (Kandel and Schwartz, 1985).

At the center of the grey matter is the central canal; an extension from the rostrally located ventricular system of the brain in which the cerebrospinal fluid (CSF) circulates. The CSF carries nutrients to the neural tissue and waste from it and eventually returns to the blood circulatory system where it originated from. Apart from nourishing the nervous tissue, it also provides physical support to both the brain and the spinal cord by cushioning impacts upon the structure that could possibly lead to tissue damage. The ependymal cells that layer the canal contribute to the formation of the cerebrospinal fluid, although the main source would possibly come from the choroid plexus (Oksche and Ueck, 1976).

From the observation, the four main segmental levels of the spinal cord could be distinguished from one another based on their distinctive histological features. The first level, i.e. cervical was the largest of all segments as reflected by the localised enlargement of this region. The cervical spinal transverse sections have the most grey and white matter compared to the other four levels. High accumulation of large motor neurons was observed at the spinal grey's ventral horns, particularly at the ventrolateral field of this level. All of these attributes were in relation to nerve innervations to the forelimbs. Situated between the cervical and lumbar regions is the thoracic level, where cross sections from this region could be differentiated by a sudden reduction in diameter from the size of the cervical segments. The grey matter contained lower density of neuronal cell bodies than the cervical and lumbar regions. Motor neurons from this level primarily resided at the medial motor field. The lumbar level is where the second enlargement was reported. Nerves supplying to the muscles of hindlimbs in this region enable the frog to execute saltatory movements like jumping and leaping on firm grounds. Also evidently observed was the presence of motor neurons pooling at the lateral motor field of ventral horn. Despite the greater area of grey matter within the cervical and lumbar intumescences, the spinal segments of the latter contained relatively less white matter than those in the former. The last segment at the caudal of the spinal cord is the sacral level. Segments from this region have the least share of white and grey matter areas aside from being the smallest sections if the transverse and vertical diameters were to be measured. Likewise for the sacral spinal segments, they too have a lower concentration of neuronal cell bodies.

4.2.1 Cytoarchitecture of Frog Spinal Grey

Grey matter of the spinal cord especially those of mammalian species, has long been studied by numerous neuroanatomists and neurophysiologists. Over the years, adaptations have been made from the classical Rexed laminae scheme that was initially demonstrated on cats (Rexed, 1952; Galhardo and Lima, 1999), to fit various animal models. This also involved other mammalian species, for example rats (McClung and Castro, 1978), rabbits (Kirmani et al., 2011) and monkeys (Ralston, 1979; Zhang and Craig, 1997), as well as non-mammalian species such as domestic fowls (Brinkman and Martin, 1973), pigeons (Leonard and Cohen, 1975), elasmobranch fishes (Cameron et al., 1990), snakes (Kusuma et al., 1979), turtles (Kusuma et al., 1979; Fernández et al., 1992) and lizards (Cruce, 1979; Kusuma et al., 1979; Wolters et al., 1986) (Table 1.1). The proposed schemes for different models have brought about the continuous overflow of varied nomenclatures with arbitrary definitions in describing the distinctive areas in the spinal grey following the integration of Rexed's parcellation.

As described in this study, cytoarchitecture of the frog spinal grey showed poor differentiation of cell groups and therefore, it was difficult to delimit the whole of grey matter into specific laminae or cell columns if one were to abide by the suggested laminar guidelines. Also, the margins that separate various areas in the frog grey matter could not be defined precisely but rather identified as transition zones. Nonetheless, the basis of the cytological characteristics of the spinal schemes was incorporated in a loose way, into the Ebbeson's field system (Ebbeson, 1976), which was first introduced to *Rana catesbiana* and *Rana pipiens* of the Anurans. Further discussion in the delineation of the cytoarchitectonic subdivisions of the frog spinal grey and its comparison of such across different species was hoped to improve the understanding of their homologies and hence, serve as an attempt to lessen the confusion over various terminologies with

coinciding fundamental characters. The findings of this study was being put side by side against past studies by various authors on different animal models (Table 4.1); cats (Rexed, 1952; Galhardo and Lima, 1999), rats (McClung and Castro, 1978), rabbits (Kirmani et al., 2011), monkeys (Tashiro et al., 1990; Zhang and Craig, 1997), domestic fowls (Brinkman and Martin, 1973), elasmobranch fishes (Cameron et al., 1990), snakes (Kusuma et al., 1979), turtles (Kusuma et al., 1979; Fernández et al., 1992) and lizards (Cruce, 1979; Kusuma et al., 1979). To avoid extensive repetition of authors, citations for the reported information concerning a particular animal model in the following subchapters were referred to as mentioned earlier.

Table 4.1: Past studies on the spinal grey cytoarchitectonic subdivisions using different animal models.

Animal species	Reference
1) Cat	Rexed (1952); Galhardo and Lima (1999)
2) Rat	McClung and Castro (1978)
3) Rabbit	Kirmani et al. (2011)
4) Monkey	Tashiro et al. (1990); Zhang and Craig (1997)
5) Domestic fowl	Brinkman and Martin (1973)
6) Elasmobranch fishes	Cameron et al. (1990)
7) Snake	Kusuma et al. (1979)
8) Turtle	Kusuma et al. (1979); Fernández et al. (1992)
9) Lizard	Cruce (1979); Kusuma et al. (1979)

4.2.1.1 Dorsal Field

Dorsal field of the frog spinal grey covered a large portion of the anterior grey matter, in which the projection of dorsal root fibres were found to be restricted within it (Adli et al., 1988; Stevens, 2004). The dorsal field is the presumable representation of the dorsal horn. This study demonstrated that the field was composed of loosely packed, primarily smaller cells (4–11 × 8–25μm) though occasionally large cells (12–19 × 26–44μm) were also present. Most commonly found were neuronal somas of spindle- and triangular-shaped, of which similar types have also been demonstrated in the spinal grey dorsal region of other species including cats, monkeys and elasmobranch fishes. It is assumed that the afferent fibers would be making synapses with these neurons.

In the work of Prescott and De Konick (2002) involving neuronal cell types of dorsal horn lamina I of rats (mammals), it has been revealed that there was a significant correlation between the neuronal soma morphology and the signal processing characteristics of each type. Fusiform or spindle-shaped neurons were categorised as tonic cells, those in pyramidal or triangular form were phasic cells and, delayed onset and single spike cells were multipolar or polygonal in shape. These intrinsic physiological functions of these neuronal types were determined through their response to stimulus trains and synaptic input that was introduced via somatic current injection. Thus, from the results obtained, the cells of the dorsal field were likely of tonic and phasic cells. Moreover, the correlation between morphological profile and functional properties has also been reported. An example of this is the response from different morphological cell types towards a particular stimulus, where fusiform and multipolar cells were classified as nociceptive whereas those responding to innocuous cold were pyramidal (Grant and Koerber, 2004).

Referring to the Rexed laminar arrangement, lamina I to IV are predominantly found within the dorsal horn. The first lamina or the marginal zone caps a thin layer of the dorsal horn apex with loosely distributed cells (Grant and Koerber, 2004; Heise and Kayalioglu, 2009). It was reported that this layer to be one-cell thick in most cases (mammals, birds and reptiles) but seemingly indiscernible in both elasmobranch and frog spinal cord as seen in the findings of this current study. The presence of various somatal morphologies like spindle, pyramidal, flattened and multipolar, was also noted (Zhang and Craig, 1997; Galhardo and Lima, 1999). Lying beneath lamina I is the lamina II or commonly known as the substantia gelatinosa. This lamina is characterised by its high neuronal density of small cells, as seen in Nissl stained sections. In mammals, lamina III and IV are located ventrally to lamina II. They were composed of heterogenous cells of larger size in a more diffuse organisation compared to those in lamina II. In frogs however, the anterior aspect of the dorsal field was composed of sparsely distributed smalls-sized somas and was occasionally found to be almost neuron-free. The differential cell compactness that displayed the alternation of loosely and densely packed cells through lamina I to IV could barely be ascertained in the dorsal field as Nissl-stained somas were randomly scattered within the whole area of this area. Despite the differences between frog and those abiding the Rexed's scheme, the finding of this study corroborated the idea of Ebbeson (1976). It was reported that dorsal field was equivalent to the Rexed laminae I to IV. These laminae are often associated to exteroceptive sensation arising from the periphery (Dafny, 1997) and the dorsal field could possibly be functionally similar since Adli and coworkers (1988) showed that axons of different neurons 'terminate' in the region.

4.2.1.2 Lateral Field

In the frog spinal grey, lateral field was positioned ventrally to the dorsal field and adjacent to the anterior central field. The lateral field constituted within the posterior portion of the dorsal horn and extended to the intermediate zone of the grey matter. Consistent to Ebbeson's finding (1976), segregation of this field into lateral zone and medial zone was observed although the separating margin was not apparent. The prominent feature of this field was marked by the great amount of large cells (14–23 × 24–42µm) in the lateral zone and they were characterised as heterogenous in shape and size, while the medial zone contained smaller sized cells (5–13 × 6–23µm). The loosely arranged content of the lateral region may be attributable to the penetration of nerve fibre bundles. This is very much comparable to the characteristics of lamina V as seen in rats, cats, domestic fowls, elasmobranch fishes and lizards. Ebbeson (1976) suggested the correlation between lateral field and the Rexed lamina V to VI that majorly concerned the proprioceptive stimuli from muscles, tendons and joints with regard to movement or position of the body (Dafny, 1997). However, lamina VI was not readily demonstrated in this case, as opposed to many tetrapod species seeing that this definitive band of tightly packed neurons was exclusively present at the cervical and lumbar enlargements. Lamina VI has been revealed in lizards with a similar division into lateral and medial zones as in lamina V (Cruce, 1979). Thus, it is also possible that both aspects of these laminae were misguidedly integrated together within the same field in frogs due to the ambiguous borderlines among different fields. Also to be noted is the possibility of large neuronal somas found in the lateral zone of lateral field to be homologous to the interneurons in lamina VII (Ebbeson, 1976).

4.2.1.3 Central Field

The central field was equivalent to the Rexed's lamina X or the substantia grisea centralis with associated somatosensation function (Miller and Seybold, 1987). It constituted the anterior and posterior grey commissures as where the decussating fibers of some descending pathways were found (Cruce, 1979). This region has been comparably seen in the spinal cords of cats, rats and birds. However, it was indistinct in lizards and not demonstrated in the elasmobranch fishes. The cells were small and chiefly triangular- and spindle-shaped. This is consistent with the findings of this study where spindle-shaped neuronal somas were commonly found at the dorsal part of central field or presumably the posterior grey commissure, although none of the identifiable somas were in triangular form. These cells could possibly be linked to the commissural neurons, in which their axons project to the contralateral side and thus, allowing interaction between both sides of the spinal cord (Cruce, 1979; Carlin et al., 2006).

4.2.1.4 Ventrolateral Field

In terms of position, the ventrolateral field corresponded to Rexed's lamina VII as they both showed medial extension to the posterior part of central field and constituted the largest area in the spinal grey that filled the intermediate zone and parts of the ventral horn. Lamina VII is known as the intermediary for nerve impulse transmission between muscles and the midbrain (Dafny, 1997). Also demonstrated in mammals, elasmobranches and domestic fowls, the cells in this field were thinly scattered, with occasional large somas being found. It was proposed that they might function as interneurons that link to the motor neurons (Heise and Kayalioglu, 2009). However, this was in contrary to lizards where this area was reported to contain large polygonal cells

in abundance. Ebbeson (1976) was able to show the supraspinal input, which possibly originates from the reticular formation, into the ventrolateral field.

4.2.1.5 Ventromedial Field

Ventromedial field has been demonstrated in frogs as to being the termination site for tectospinal, and vestibulospinal fibres as well as some reticulospinal axons (Ebbeson, 1976). The ventromedial field is a probable reminiscent of Rexed lamina VIII as displayed in many amniotes including mammals, reptiles and birds and anamniotes like the elasmobranch fishes. It is possible that the cells present in this area either served the purpose as interneurons that bridge the connection between other cells in lamina VIII to those in medial lamina IX, or as commissural cells, which connect lamina VIII and medial column of lamina IX with their corresponding cells on the contralateral side of the spinal cord (Cruce, 1979). The spinal commissural neurons in lamina VIII are important for coordinating motor activity on two sides of the body (Heise and Kayalioglu, 2009).

4.2.1.6 Lateral Motor Field and Medial Motor Field

Lateral motor field and medial motor field were located at the base of ventral horn. They consisted of somas, which were mostly larger and more variable in shape compared to the presumptive sensory neurons found in the dorsal and lateral fields. The large somas were most likely identified as motor neurons due to its heavily stained Nissl content. The location of motor neuron pools varied at different segmental levels. In cervical and lumbar spinal sections, motor neurons with diagonally-oriented axis were seemingly clustered in the lateral motor field of the ventral horn. On the other hand, motor neurons with horizontally-oriented axis were more commonly seen to reside at the medial motor field of the thoracic and sacral spinal segments. In frogs, at larvae or

tadpole stage, it was found that the trunk and tail muscles receive motor innervations from both medial and lateral motor pools whereas the limb muscle was also innervated by additional cells from the lateral pool (McDiarmid and Altig, 1999). As for fully developed adult frogs, the medial and lateral motor neuron groups supply the thigh and foot muscles, respectively (Cruce, 1974; Mensah and Thompson, 1976).

The presence of large multipolar neuronal somas in clusters is a prominent feature in the ventral horn of mammals, significantly at Rexed's lamina IX. Neurons of lamina IX played major roles in the motor activity. This lamina could be further divided into two motor columns, one at the medial part and another covers the lateral area. Collectively, the ventrolateral field and ventromedial field in the frog spinal grey are possibly analogous to cytoarchitectonic lamina IX. Also, these areas could be linked to the equivalent region of the retrodorsolateral column, dorsolateral column and ventrolateral column judging by the position of the nuclear groups (FitzGerald, 1985). As supported by Kirmani and coworkers (2011) via reconstruction of serial transverse sections from different segmental levels, the organisation of motor neuronal somata was said to be arranged into vertical columns instead of being randomly scattered. In amniotes including mammals, lizards, turtles and domestic fowls, neurons from the medial motor column innervate the axial musculature while those at the lateral area of the ventral horn innervate the extremity musculature (Nishikawa et al., 1991). Thus, this is in agreement with the conception of the motor neuron pool at lateral motor field and medial motor field as seen in this study. Similarly for fishes, though they do not possess limb extensions, it has been shown that motor neurons situated medially innervated the depressor muscle of pectoral fins while laterally found neurons supplied the elevator muscle group (Droge and Leonard, 1981; Cameron et al., 1990).

In mammals, large motor neurons are termed as α-motoneurons and they are responsible for innervating the extrafusal or skeletal muscle fibres that are involved in movement while those smaller cells that mingled among the motor neuron pool might represent the γ-motoneurons, innervating intrafusal muscle fibres or the muscle spindles to control muscle tone (Kusuma et al., 1979; Hochman, 2007; Heise and Kayalioglu, 2009). Emergence of extensive transverse dendritic arborisation appeared to be projecting from these motor neurons (Light and Metz, 1978). In frogs however, Gray (1957) have demonstrated the lack of γ-motoneurons in the spinal cord.

4.3 Localisation of Selected Nociception-related Neurotransmitters

The distribution of ENK-, SP- and 5HT-like immunoreactivities in the frog spinal cord was studied using an indirect immunohistochemical technique. All of them were found present in the representative sections of the major segmental levels (cervical, thoracic, lumbar and sacral); each set revealing a relatively consistent intensity and distribution pattern of immunoreactivity throughout the four levels surveyed. The current observations generally agree with the findings as reported by Lorez and Kemali (1981), Adli et al. (1988), Partata et al. (2002) whose researches also involved frogs (*Rana esculenta*, *Rana pipiens* and *Rana catesbiana* respectively*)*. However, localisation maps and the detailed immunoreactive features differed to some extent from those in other amniotes as well as anamniotes, for examples, the work of Seybold and Elde (1980) on rats, Tashiro et al. (1990) on monkeys, Wolters et al. (1986) on lizards, LaValley and Ho (1983) on domestic fowls and Cameron et al. (1990) on elasmobranch fishes (Table 4.2), just to name a few.

Table 4.2: Past studies on the immunohistochemical localization of nociception-related neurotransmitters in spinal cord using different animal models.

Reference	Animal species	Neurotransmitter
1) Lorez and Kemali (1981)	Frog	ENK, SP
2) Adli et al. (1988)	Frog	ENK, SP, 5HT
3) Partata et al. (2002)	Frog	SP
4) Seybold and Elde (1980)	Rat	ENK, SP
5) Tashiro et al. (1990)	Monkey	ENK, 5HT
6) Wolters et al. (1986)	Lizard	ENK, SP
7) LaValley and Ho (1983)	Domestic fowl	ENK, SP
8) Cameron et al. (1990)	Elasmobranch fishes	ENK, SP, 5HT

4.3.1 Immunoreactive Fibres and Varicosities

In general, ENK- and SP-like immunoreactivities occurred in a somewhat similar pattern but the former covered a greater extent than the latter, which appeared in a narrower region with labeled elements appearing more dispersed. The distribution pattern of 5HT-like immunoreactivity on the other hand, was distinctly different and appeared lighter from the other two.

Most of the studies on amniotes have described a high density of ENK- and SP-like immunoreactive fibres and terminals in the spinal lamina I and II of the dorsal horn while the ventral horn received sparse innervations of both substances (Hökfelt et al., 1977; LaValley, 1980; LaValley and Ho, 1983; Wolters et al., 1986; Reiner, 1987; Du and Dubois, 1988; Sakamoto and Atsumi, 1989; Cameron et al., 1990). These laminae are the supposed recipient of cutaneous primary afferent fibres and closely associated to the nociceptive sensory modulation (Rexed, 1952; Adli et al., 1988; Brinkman and Martin, 1973; Tashiro et al., 1990). Surprisingly, immunreactivity of ENK and SP in the frog spinal cord revealed an unusual localisation pattern where their fibres and varicosities were found richest along the mediolateral band (MLB). Referring to Adli and coworkers (1988), MLB is a region devoid of primary afferent fibres, lying between

the dorsal terminal field and ventral terminal field. This band was comparably located at the base of the dorsal field, stretching towards the lateral field, and it also entered the central field. Observation of such was also reported in the past studiess that used frogs as their experimental animals (Lorez and Kemali, 1981; Adli et al., 1988; Partata et al., 2002). Such differences might be attributable to the peculiar cytoarchitectonic parcellation of the frog spinal grey that appeared to be less differentiated than that of other amniotes.

Despite the difference in the ENK- and SP-like immunoreactivity patterns in frog compared to other amniotes, distribution of 5HT-like immunoreactivity was relatively consistent with the immunoreactive regions of most animal species. In frogs, 5HT-like immunoreactive fibres and varicosities were mainly concentrated at the top part of dorsal field and possibly the homolog of the superficial laminae in the dorsal horn of rats (Hadjiconstantinou et al., 1984; Wessendorf and Elde, 1987), monkeys (Kojima et al., 1983; Tashiro et al., 1990) domestic fowl (Ho and LaValley, 1984) and pigeons (Davis and Cabot, 1984). Also similarly revealed in other frog species, i.e. *Rana pipiens* (Adli et al., 1988), the density of positive 5HT immunoreactivity was much lower as compared to those of ENK's and SP's. Conversely, there were conflicting notes from past studies, stating that the dorsal horn contained the rarest 5HT immunoreactive fibres while the densest area was situated at the ventral horn instead (Wessendorf and Elde, 1985; 1987). As did frogs, it was common to find 5HT-like immunoreactive elements on the perikarya of motor neurons in the ventral horn among amniotes, which suggests the contribution of 5HT in the motor function. Likewise, ENK and SP are not likely to have any direct interactions with motor neurons due to the absence of immunoreactivity in these areas.

In frogs, the distribution of ENK- and SP-labeled varicosities in the central field are more perceptibly seen in the posterior grey commissure as well as the lateral region of the field, which was the extension of MLB. Comparatively, 5HT immunoreactivity was sparser and primarily localised in the anterior grey commissure. These findings were also consistently observed in lamina X of primate spinal cord, which corresponds to the termination site of somatic and visceral primary afferents (Honda and Lee, 1985; LaMotte, 1988). Aside from the spinal grey, immunoreactivities of the investigated neurotransmitters also were localised in the white matter area as did most of other species. The occurrence of fibres and terminals with ENK, SP and 5HT labels in the lateral funiculus including the Lissauer's tract, and ventral funiculus suggests that they were probably supraspinal in origin, for example arising from the descending pathways of the brain stem raphe nuclei (Conrath-Verrier et al., 1983; Wolter et al., 1985; Sergeyev et al., 1999).

Changes in the level of a particular neurotransmitter following lesion infliction may provide possible indication of its origin or source as to being propriospinal or supraspinal. Dorsal rhizotomy in previous immunohistochemical investigations has resulted in no alteration of ENK-like immunoreactivity of the rat spinal cord (Hökfelt et al., 1977; Seybold and Elde, 1980). Such observation consequently implied that the ENK-like immunoreactive elements are possibly contained in the interneurons or in the intrinsic spinal neurons, to say the least (LaValley, 1980; LaValley and Ho, 1983; Wolters et al., 1986). Another plausible explanation to clarify such occurrence is the existence of an ENK immunoreactive system that originated in the lower medulla oblongata and with projections to the spinal cord (Hökfelt et al., 1979).

As proposed in the work of Tashiro and Ruda (1988) in cats, the dense fibre plexus of SP-like immunoreactivity in the superficial laminae of the dorsal horn was more likely to derive from either the primary afferent axons or those of intrinsic dorsal horn neurons, rather than the terminal projection in the spinal cord that originates from the brain stem. This is supported by the effect of dorsal rhizotomy that exhibited the disappearance of SP labels in the rat spinal dorsal horn (Seybold and Elde, 1980). Sciatic nerve transection in frogs (Partata et al., 2002) and other amniotes such as cats (Tesslers et al., 1985), rats (Villar et al., 1991) and turtles (Partata et al., 2003) has similarly led to a decrease in SP-like immunoreactivity on the ipsilateral side where the lesion was inflicted. In addition, it is likely that the speculated actions of this neuropeptide are fairly similar in these investigated animals (Partata et al., 2002; 2003). In the spinal cord of paraplegic cats, accumulation of SP-like labeled elements below the lesion, particularly in the dorsolateral part of the dorsal horn was seen, which indicated the rostral or upward direction by axoplasmic flow of this peptide (Naftchi et al., 1978). In the same experiment, SP fibres in the ventral horn were suggested to be involved in spinal segment transmission as their immunoreactivity, although in small amount, showed no changes in sections above and below the transected lesion (Naftchi et al., 1978).

Following lesion experiments in rats by Hadjiconstantinou and his coworkers (1984), they postulated that spinal cord 5HT did not originate only from the brain as results showed a reduction in immunoreactivity instead of being entirely lost after complete cord transection. Another contradicting finding by Micevych et al. (1986) had reported that both SP- and 5HT-like immunoreactivity was significantly depleted while no apparent changes on ENK-like immunoreactivity in the lumbar ventral horn of rats upon total spinal transection. This led to the proposition of SP- and 5HT-like materials in the

ventral horn being derived from supraspinal tract while those of ENK originated from intrinsic neuronal cell bodies of the spinal cord. In contrast to the earlier findings, Tashiro et al. (1990) demonstrated a complete loss of both ENK- and 5HT-like immunoreactivity in the lumbar spinal segments of monkeys after thoracic cordotomy that disabled the sensation of pain. Hence, this showed implication that the spinal axons of these two neurotransmitters had supraspinal origin, most likely to arise from the brainstem, especially the raphe nuclei. Up to now, the existing data with efforts to elucidate the origins of neuronal elements that contain these neurotransmitters are very much puzzling.

Co-localisation of ENK- and SP-like immunoreactivities in fibres and terminals was seen present in the work of Tashiro et al. (1987) and Ribeiro-da-Silva et al. (1991) on cats and rats, respectively. Findings from these studies were contradicting as the former reported a sparse distribution over the superficial part of the dorsal horn while the latter expressed disagreement for the laminae I to III was distinctively intense with both labeled components in axonal varicosities and cell bodies. Senba et al. (1988) proposed that interaction between these neuropeptides occurred at the axon terminals of the substantia gelatinosa neurons with regard to their sites of co-localisation. Coexistence of 5HT-like immunoreactivity with SP-like immunoreactivity in fibres and terminals within the spinal cord of rats and cats has been demonstrated via immunoflorescent double labeling (Wessendorf and Elde, 1985; 1987; Tashiro and Ruda, 1988). Such coexistence was most common in the ventral horn and therefore they both are believed to play greater role in motor function, specifically with somatic and sympathetic autonomic motor neurons. For ENK- and 5HT-like immunoreactivity, double-labeled elements of these two neurotransmitters were distributed mainly in the dorsal horn; lamina I and II (Tashiro et al., 1990), which suggested their involvement in sensory

functions. It was verified by Wessendorf and coworkers (1990) that the co-localisation of 5HT with ENK was much less common than the coexistence of 5HT with SP in both brainstem and spinal cord of rats.

4.3.2 Immunoreactive Cell Bodies

Among the three investigated neurotransmitters, only ENK-containing cell bodies have been reported in the frog spinal cord at all levels whereas none of such for SP and 5HT in both normal and colchicine-treated animals. As did Lorez and Kemali (1981) as well as Adli and coworkers. (1988), this experiment also observed ENK cells at all levels, located within areas that were prominently concentrated with immunoreactive elements, specifically MLB as well as the central field. They also appeared in the dorsal horn of rats (Johansson and Hökfelt, 1980; Lima et al., 1993) but not in human (Lanerolle and LaMotte, 1982), lizards (Wolters et al., 1986), domestic fowls (LaValley and Ho, 1983), and rainbow trouts (Vecino et al., 1992). The occurrence of cell bodies that contained ENK-like immunoreactivity within the spinal grey implied the existence of local spinal enkephalinergic neurons (Hökfelt et al., 1977; Lorez and Kemali, 1981; Wolters et al., 1986). It has been demonstrated that majority of the nociceptive neurons in the dorsal horn contained ENK-like immunoreactivity (Ma et al., 1997). Thus, there is a likelihood that analgesia, as associated with the function of ENK, may be produced by the neuronal circuitry at the spinal cord level (Hökfelt et al., 1977; Seybold and Elde, 1980).

Also in agreement with the present findings, no SP-labeled cell bodies in the spinal grey was reported in human (Lanerolle and LaMotte, 1982), rats (Hökfelt et al., 1977; Seybold and Elde, 1980), frogs *Rana catesbiana* (Partata et al., 2002), lizards (Wolters et al., 1986), domestic fowls (LaValley and Ho, 1983), elasmobranch fishes (Cameron et al., 1990). In contrast, some past studies stated the opposite, such as in rats (Chan-

Palay and Palay, 1977; Hökfelt et al., 1977) and turtle (Partata et al., 2003), where positive neuronal cell bodies were identified in the lateral column of the dorsal horn.

5HT label was not found in any cell body of the frog spinal cord in this present study and some of previous reports in domestic fowls (Ho and LaValley, 1984), elasmobranch fishes (Cameron et al., 1990) and lizards (Wolters et al., 1985). Regardless, neurons with 5HT-like immunoreactivity have been demonstrated in lamina VII and X of rats (Newton et al., 1986) as well as in the anterior grey commissure of frogs *Rana pipiens* (Adli et al., 1988), which was solely detected within the thoracic spinal segments, rendering them to be functionally involved in sympathetic regulation. This group of 5HT cells are said to be a probable extension from those of the brainstem.

4.3.3 Effects of Colchicine Administration

This study has revealed the presence of immunoreactivities of the investigated neurotransmitters in the frog spinal sections of normal and colchicine-treated frogs. Intraventricular colchicine administration was done by means of injection into the foramen magnum area. Synthesis of neurochemicals, especially neuropeptides, occurs in the cell bodies and they were then rapidly transported to the nerve terminals. Colchicine administration addressed the problem of low content of a particular immunoreactive neurochemical in the cell bodies, which might result in the difficulty in staining them. Such misinterpretation as they failed be detected, was avoided by blocking the axonal transport with colchicines. This helped in increasing neurochemical-containing granules in the cell bodies and therefore, enhancing its selected detection (Palkovits, 1985). The findings revealed that colchicines treatment increased the concentration of neuronal cell bodies staining in the case of spinal ENK. Concomitantly, the treatment also showed a decrease in overall number of fibres and varicosities in all ENK-, SP- and 5HT-labeled

spinal cord sections. This observation was in agreement with past studies that worked on the localisation of various neurochemicals for example, somatostatin (Dubé and Pelletier, 1979; Seybold and Elde, 1980), substance P (Seybold and Elde, 1980; Triepel et al., 1985), enkephalin (Seybold and Elde, 1980) in selected central nervous system structures.

4.3.4 Nociception-related Functional Implications

The investigated neurotransmitters, i.e. ENK, SP and 5HT, are known to be implicated in nociceptive transmission in the spinal cord. While the role of ENK and 5HT have been shown to exert inhibitory effect upon nociceptive input in the dorsal horn, SP oppositely appeared to be excitatory, mediating the expression of nociceptive stimuli (Messing and Lytle, 1977; Glazer and Basbaum, 1981; Kantner et al., 1985; Fürst, 1999).

Opiate receptors have been elucidated within the substantia gelatinosa of the spinal cord and the localised ENK labels corresponded closely to the distribution of those sites (Atweh and Kuhar, 1977; Simantov et al., 1977). Furthermore, Cesselin and coworkers. (1989) reported that segmental release of ENK in areas of the spinal cord that received nociceptive inputs was induced in response to noxious thermal stimuli. These findings, thus, suggest the idea of ENK participation in the integration of pain perception. Studies have revealed that enkephalinergic neurons in the dorsal horn influenced the nociceptive modulation via postsynaptic mechanism and involved in local inhibitory feedback loop in a different pathway from the previously hypothesised decreasing content of substance P release from primary afferent terminals (Glazer and Basbaum, 1983; Ma et al., 1997). A direct correlation between SP and the specific nociceptive response in neurons of the spinal dorsal horn was verified by De Koninck and colleagues (1992) through combined

methods that involved intracellular recordings of dorsal horn neurons *in vivo*, intracellular labeling via horseradish peroxidase injection and immunocytochemical analysis of SP at the electron microscopic level. This structural-functional evidence showed that dorsal horn neurons responding to noxious stimulation of the skin with slow and prolonged excitatory postsynaptic potential were contacted by a large number of SP-containing varicosities. Considering the overlapping immunoreactive sites between ENK and SP in the frog spinal cord, it is very likely that they are functionally interconnected. However, the involvement of these neurotransmitters might differ to some extent from those of other amniotes in terms of functional role and mechanism of action due to their dissimilarity in the localisation pattern. For 5HT, its role in nociceptive modulation has been speculated in relation to the regions of positive immunoreactivity in the superficial portion of the spinal grey matter, especially the substantia gelatinosa (Hancock, 1982; Tashiro et al., 1990). Also, the capability of 5HT to express dual effects depending on its receptor subtypes was demonstrated in physiological experiments on formalin-induced nociception in rat spinal cord (Oyama et al., 1996). Activation of 5-HT_3 receptors facilitated nociception whilst 5-HT_{1A} and 5-HT_2 receptors mediated spinal antinociceptive effect of 5HT (Crisp et al., 1991; Oyama et al., 1996; Zeitz et al., 2002). Despite the ample information concerning the individual roles of ENK, SP and 5HT in modulation of nociception, the interactions among the neuropeptides (ENK and SP) and the monoamine (5HT) in the colocalised sites of spinal cord are still not fully understood.

4.4 Technical Considerations

There is no one-fit-all standard protocol that could accommodate all types of tissue, be it histology or immunohistochemistry. Tweaking and fine-tuning are common routines to enable a ‘standard’ protocol to work optimally by taking into account numerous factors. Modification in histological and immunohistochemical techniques across different laboratories may vary from the species of experimental animal used, thickness of tissue sections, types of fixatives, embedding medium and other reagents to types of staining protocols to be employed for visualisation of different structure within a tissue section and also the environment setting of the laboratory. Hence, these factors could inadvertently causes inconsistency in the overall results but most often than not, the dissimilarities were not drastic provided that the general guidelines of a particular procedure were followed. Technical predicaments and justification of modification in this study are further discussed in this subchapter.

4.4.1 Gross Anatomical Study

Experimental animal used in this study was *F. limnocharis*, a small-sized common Field Frog which measured about 50 to 70mm from snout to vent. Thus, it has been an initial challenge to handle animal of such small size, especially in performing vascular perfusion with fixatives and removing the spinal cord tissue from the bony vertebral column, prior to post-fixation of tissue sample. The problem of operating on such diminutive tissue was resolvedby using stereo microscope and delicate surgical instruments. Even so, these meticulous tasks required more than just steady hands and skilful techniques but also utmost patience to accomplish successful perfusion-cum-fixation and harvested tissues that were undamaged for the subsequent histological procedures.

Unfortunately, removal of spinal cord with complete spinal nerve remained intact was proven to be a tricky task. Therefore, instead of using spinal segments based on the corresponding spinal nerve roots, this study opted to highlight the four main segmental levels of the spinal cord as externally observed in differentiation of cord thickness where the cervical and lumbar portions were marked by the sudden enlargements. Selected sections were taken from the middle of each segment to ensure correct representation of the segments.

4.4.2 Histological Analysis

Defining the basic cytoarchitectonic structure of the frog spinal cord was made possible through the employment of histological stainings. Staining methods involved in this study were H&E, Nissl, the modification of the Golgi method, Thionin and Lillie's variant modification of Weil-Weigert method. Except for H&E, the rest of the methods are considered as common stains designed for nervous tissue. The H&E staining technique is a standard staining procedure for tissue histology that provides colorimetric contrasts to distinguish the purplish nuclei and the pink cytoplasm. However, H&E did not offer a good visualisation for neuronal somas especially for non-mammalian neural tissues. Therefore, Nissl and Golgi staining techniques were introduced to this part of the work to assist the identification of neuronal profiles in the spinal sections. Nissl staining method was the main solution in delineating the neuronal somas, where the cresyl violet dye strictly highlighted the Nissl substances and ribosomes that were found within the somas. The Golgi method allowed the entirety of the neuronal structure to be stained through impregnation with potassium dichromate and silver nitrate. But the downside was that the neurons were labeled in a random manner. Such unpredictable qualities have been noted by De Carlos and Borell (2007), and Peters (2007), which therefore render the unsuitability of this method for quantitative analysis. Most of the

time, incomplete impregnation due to technical variation resulted in the failure of displaying the full neuronal branching pattern. The Lillie's variant modification of Weil-Weigert technique and thionin stain on the other hand, revealed the axonal fibers as darkly stained myelin sheaths and thus, gave a better contrast to the white matter. All of the mentioned staining techniques were found to work well in staining the frog spinal cord tissue given that minor modifications were made, such as adjustment in staining duration for particular chemicals and dyes. In general, these methods had proven a satisfactory colorimetric differentiation of certain structures within the frog spinal tissue sections with good contrast and clarity of details.

During the trial-and-error period in searching for suitable staining techniques for the non-mammalian nervous tissue, there were some unreported staining trials, for instance the Masson's trichrome stains, which also showed quality results. It was rather surprising for this method to be compatibly working for spinal cord tissue in delineating the neuronal fibers insulated by the myelin layer. Masson's trichrome is traditionally suited for connective tissues, rendering red-stained keratin and muscle fibres as well as blue-stained collagen and bone. Neurokeratin, found among the components of the myelin layer, was believed to have been highlighted in this staining method, which resulted in the appearance of fibrous network of the white matter (Figure 4.1). Therefore, the Masson's trichrome staining technique should be considered as one of the potential alternatives for visualisation of the fibre tracts in the frog nervous system in the future work.

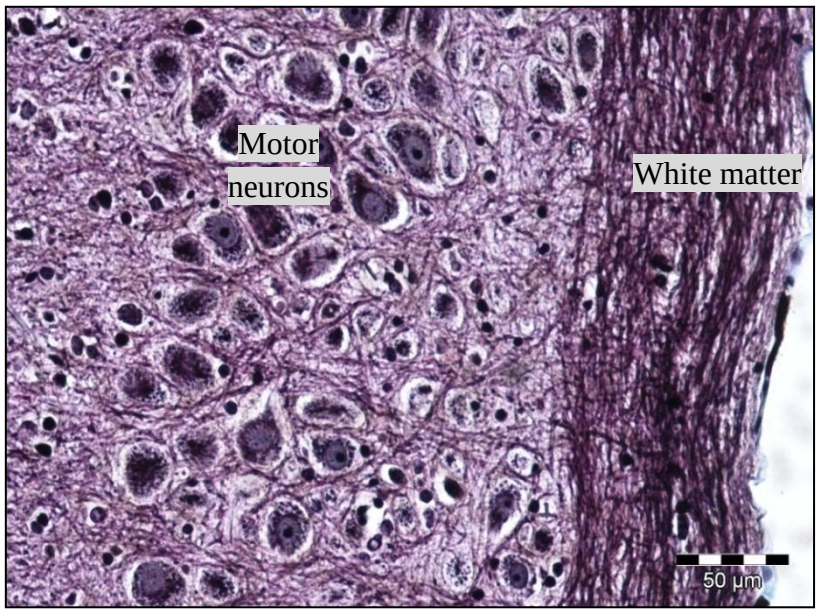

Figure 4.1: Longitudinal section at the lumbosacral segmental level showing accumulation of motor neuron at the ventral horn and the fibrous layer of white matter (Masson's trichrome, 200× magnification).

4.4.3 Immunohistochemical Technique

Immunohistochemistry has been widely used in the detection and mapping of neurochemicals in nervous tissues of various animal species. Even with the so-called 'standard' guidelines, the process of troubleshooting each staining session was painstakingly elaborate and this step is critical in order to achieve the optimal results for demonstrating the presence and location of the antigen being probed; in which this case would be the nociception-related ENK, SP and 5HT. The major setbacks encountered in the analysis of this technique revolved around the concerns of cellular morphological quality, antigenicity loss and masking, tissue section detachment as well as unspecific background staining. All of these problems could render interpretation of results obtained difficult.

This study basically dealt with easily-prepared formalin-fixed, paraffin sections for improved morphological details of the frog spinal cord besides other advantages such as the ability to give reproducible results, permanent preservation of staining, in addition to

the ease of storage and obtaining serial sections. However, if proper tissue processing steps were not strictly complied, such preparation was prone to the loss of antigenicity, which could lead to reduced immunoreactivity. Although the underlying mechanism remained unclear, it was revealed that minimisation of tissue to high heat, water exposure and long storage time would help in the antigen preservation in these tissue sections (Bertheau et al., 1998; Wester et al., 2000; Xie et al., 2011). Nonetheless, ENK-, SP- and 5HT-like immunoreactivities have been tested in the past studies using the application of formalin-fixed, paraffin-embedded sections (Naftchi et al., 1978; LaValley, 1980; Lanerolle and LaMotte, 1982; LaValley and Ho, 1983; Inman and Steward, 2003). This present study and those of paraffin sections showed comparable immunohistochemical findings to the other experimentations that employed the frozen section method. In the period of trial-and-error experimentation, it was found that frozen or cryostat sections were more difficult to work with in comparison with paraffin sections. They had higher tendency for formation of ice crystal artifacts that damaged the internal tissue structure. The density of immunopositive staining in the frog spinal cord was, however, slightly reduced, which could also be attributable to the thin sections that were being used for this analysis. A better option of immunohistochemical application for future work would most likely be vibratome sections, which do not undergo high temperature and the process of freezing.

Formalin or diluted formaldehyde solution in water is a common fixative in immunohistochemical work that allowed efficient preservation of morphological details in the tissue (Buchwalow and Böcker, 2010). However, overfixation or prolonged fixation in this solution, usually for more than 24 hours, would result in deleterious effect on the antigen-binding characteristics by the formation of methylene crosslinks and, thus, masking the epitopes. With the appropriate epitope retrieval method, the

immunoreactivity of antigens that is masked by formalin fixation can be restored. In this part of the experiment, the enzymatic digestion failed to perform as good as the heat-induced epitope retrieval method (HIER) in Tris/EDTA pH 9.0 buffer solution, which was therefore finalised into the routine. To reduce the probability of antigen denaturation under high temperature and tissue detachment, this step was carried out in 70 to 75°C with longer incubation time instead of 95°C to 99°C as suggested in the product manual. The optimal condition is crucial so that the epitopes could be recovered through heat, without having the rest of the polypeptide chain unraveled (Yamashita, 2007).

Beside the main concern of antigen damage in this experiment, there was also another problem regarding the detachment and 'wrinkling' of tissue sections on coated slides throughout the immunohistochemical procedure. Different kinds of adhesive agents had been tested and the strength of adherence, from highest to lowest, were concluded in the respective order: 3-aminopropyltriethoxysilane or commonly known as silane, Poly-L-lysine and Mayer's albumin. The latter two adhesives still allowed 'wrinkling' or loosening of tissue sections if not partial or whole tissue section detachment. Similar findings were also demonstrated by Holland et al. (1996). However, comparison was also made against the commercial pre-coated silanized slides, which evidently performed better but 'double-dipped' slides in silane was also equally good, although slight section fold-overs still occasionally occurred. The idea of 'double-dipping' was adapted from Miller (2001) as he has acquired satisfactory results from preparing his own silanized slides. There are also other causes that lead to tissues falling off slides, for examples, insufficient fixation, uneven spreading and incomplete drying of sections after they were mounted on slides, as well as incompatible epitope retrieval technique especially in high pH buffer solution. Moreover, it is important to note that even the

relatively trivial step of slide-rinsing in wash buffer should be looked into. As economic as it seems, the conventional method of jet-washing the slides using a wash bottle were not advisable in this case considering the tissue sections were prone to loosening. Instead, they were washed in a slide dish filled with wash buffer under gentle agitation on a platform rocker that produced seesaw motion. This manner of washing had resulted in better adhesion of tissue sections on slides.

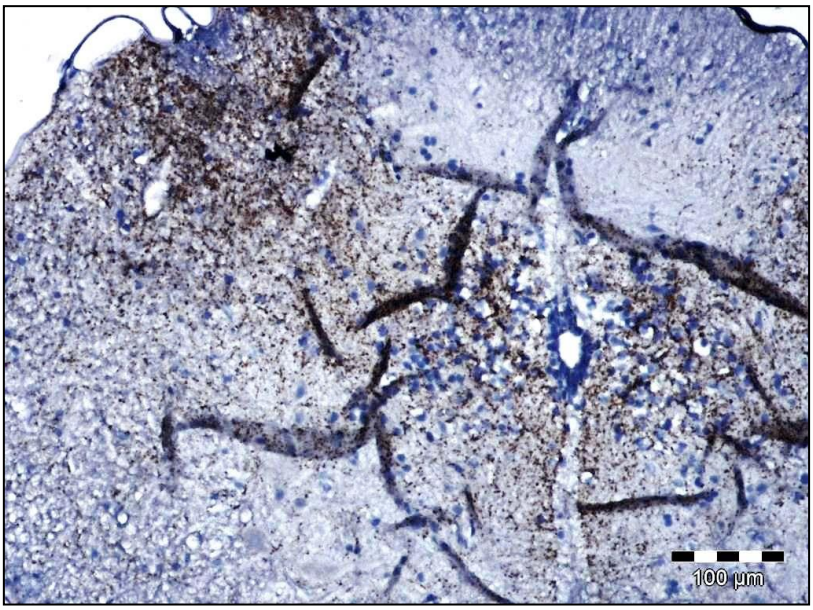

Figure 4.2: Loosening or ‘wrinkling’ of tissue section immunostained for ENK (100× magnification).

Background staining is also among the most frequently encountered problems in immunohistochemistry. Background staining often interferes the visualisation of immuoreactive elements within a tissue section. The common causes for such occurrence include incomplete deparaffinization of tissue section prior to immunostaining, poor washing technique that leaves traces of unbound antisera before the incubation in the subsequent reagent, drying out of sections at any point during the experiment and simply the fact that the antibody used is too concentrated. In order to reduce background staining, thorough washing is crucial. It is recommended to add Tween-20, which is a detergent, into the wash buffer and the rinsing step should be

repeated with at least two changes of 5 minutes each. Incubation of reagents is suggested to be conducted in a moist atmosphere by layering a damp tissue paper at the bottom of a covered staining trough, which acts as an incubation chamber. This would help in preventing evaporation of reagents during the incubation period.

Concentration of primary antibody does not only affect background staining; incorrect dilution could also lead to false-positive and false-negative results. The optimal antibody concentration is characterised as to having the maximum specific staining with the lowest non-specific background (Boenisch, 2001). This could be determined by titration in 2-fold dilutions, starting with the dilution level as suggested by the product manual and improvisation is then made according to the staining outcome.

At the final leg of the experiment, all the hard work comes down to results of the control slides, which are the key indicator to the validity of the immunohistochemical assay. Although the spinal cord, itself, is a positive tissue control on its own, an alternate tissue sample that is known to exhibit the antigen being tested would help in cross-checking the results. Positive tissue control in this experiment was derived from the kidney and liver tissues obtained from the same experimental animal. Likewise in many past reports (Huang and Weiss, 1999; De Falco et al., 2002; Caamaño-Tubío et al., 2007), they showed positive results for the expression of the three investigated neurotransmitters and thus, indicating that the immunostaining protocol is working. And for validation of antibody specificity, negative reagent control should reveal no immunostaining at all when primary antibody was omitted. Collectively, these controls were able to verify the workability and functionality of the immunohistochemical protocol.

The rest of the immunohistochemical procedure that are not mentioned in this section are involved in incubation of mostly ready-to-use reagents, such as the peroxidase-blocking solution, secondary antibody and DAB chromogen. Suffice to say, the current development in this field has undoubtedly simplified the overall procedure and shortened the time required for chemical preparation upon use. Nevertheless, all variable factors in the immunohistochemical protocol should be scrutinised as to facilitate the interpretation of immunoreactivity as accurate as possible.

4.5 Research Limitations and Suggested Future Works

Despite the effort to provide a thorough comparative analysis of the frog spinal cord organisation into a wider perspective, this study, nevertheless, has its shortcomings. First, it was considerably difficult to evaluate the poorly differentiated spinal grey of the frog owing to its nature as well as the subjectiveness in defining the neuronal architecture and further demarcating these contents into suggested arrangement pattern especially the laminar structures that stemmed from the mammalian species. The identification of the small-sized stained cells or 'microneurons' as described by Sasaki (1977) in the frog spinal cord especially in the dorsal half of the grey matter could not be precisely ascertained as they could either be neuronal or neuroglial cells. The same problem was also encountered by Ebbeson (1976) and Sasaki (1977) in their work on spinal cord cytoarchitectonic analysis of the Bullfrogs (*Rana catesbiana*) and the Northern Leopard Frogs (*Rana pipiens*). Sasaki (1977) has addressed this issue by means of light and electron microscopy and his findings summarised that these microneurons were exclusively restricted within the dorsal region of the dorsal horn and the region around the central canal. These areas concurred with Ebbeson's dorsal field and central field respectively. It was also concluded in his study that no differentiated laminar structures could be recognised in the frog spinal grey.

Additionally, comparison of results was made against previous studies that involved tissue sections of different thickness, varying from 10 to 100μm. Thus, the inconsistency in the section thickness could be one of the plausible factors that lead to a range of dissimilar characteristics of an investigated spinal grey region. Likewise for the choice of histological procedure and staining techniques used in similar works, though most of them including this study opted for the common Nissl and Golgi staining methods in general. As mentioned before, protocols across different labs, undoubtedly, involved a certain degree of modification depending on the chosen animal species as well as the suitability of conditions; i.e. availability of reagents or facilities, discrepancy of chemical formulations and etc. Hence, this renders varying results even though the main principles of a protocol were abided by.

As far as dendritic tree pattern of neurons in the spinal grey is concerned, this study is unable to demonstrate these structures in their whole extent due to physical limitation of the three-dimensional nature of the dendritic trees and the difficulties in staining the fine processes using the modification of Golgi method. This leads to the lack of elaborative data that highlights the features of the neuronal processes. Undeniably, most parts of the data provided in this study are focused on the frog spinal grey while information on the white matter area especially the ascending and descending pathways is still insufficient. To scrutinise this area, lesion experiments would be necessary.

The current investigation via immunohistochemistry was an attempt to map out the distribution of ENK-, SP- and 5HT-like immununreactivities in the frog spinal cord as separate entities. As per now, comparison of localisation maps only offers partial information on these neurotransmitters as to their origins and pathways, possible coexistence among the tested substances, in addition to the functional implications

especially in nociception processing. A great deal of information has been presented over the years, mainly in the mammalian model, that has provided direct and indirect evidences on the aspects mentioned previously. In spite of that, conclusion cannot be drawn as to whether these evidences are applicable in frogs as they are in mammals. To support these hypotheses, further investigations involving multiple approaches such as simultaneous multicolor immunohistochemical or immunofluorescence method in combination with electron microscopy to determine the coexistence of neurotransmitters, physiological evaluation via lesion studies to shed a light in identifying their origins and termination sites and electrophysiological testing coupled with ultrastructural analysis for the functionally characterised immunoreactive neurons for detailed cytological information.