CHAPTER 2: METHODOLOGY

2.1 Experimental Design

The entire research consists of two main experiments, which consisted of smaller, focused studies at specific spinal cord structures and/or neurotransmitters. Based on the objectives mentioned in Chapter 1, the first two, (1) to describe the gross morphology and general histology of the spinal cord of a locally available frog species, i.e. *Fejervarya limnocharis*, and (2) to provide cytoarchitectonic information on the spinal cord organization, were demonstrated in Experiment 1. This involved morphological and histological characterisation of the frog spinal cord. The last objective, which is to delineate the distribution of selected nociception-related neurotransmitters (enkephalin, substance P and serotonin) in the frog spinal cord, was achieved through Experiment 2 that emphasized on immunohistochemical localisation of selected nociception-related neurotransmitters. At the point when the research was carried out in year 2009, the animal ethics approval for non-mammalians was not required. Nevertheless, an application has been sent in and approval is being sought in line with the University's new regulation.

2.2   Handling of Experimental Animal

Experiments were performed on adult Rice-field Frogs (*F. limnocharis*) of both sexes, weighing in the range of 4.9 to 6.0 grams. They were acquired from local commercial sources, costing approximately RM0.50 each. These animals were temporarily housed in a plastic aquarium at room temperature, given a shallow pool of water to allow gradual adaptation (Figure 2.1).

Figure 2.1: *Fejervarya limnocharis* in captivity

2.3 Experiment 1 - Morphological and Histological Characterisation of Frog Spinal Cord

Experiment 1 focused on the morphological and histological examination of the spinal cord excised from a locally available frog species, *Fejervarya limnocharis*, This part of the work enabled correlations to be made based upon the observation from these two aspects and hence, providing a clearer picture on the spinal cord cytoarchitectonic organisation of this investigated species.

2.3.1 Preparation of Chemical Solutions

Chemical solutions that were being used in the subsequent steps were prepared beforehand in accordance to the formula and protocols as provided in Appendix B**.** These chemicals included:

a) Tricaine methanesulfonate (MS-222) solution as anesthetic agent

b) 0.6% frog physiological saline, to perfuse the experimental animal during intracardiac perfusion

c) 10% neutral buffered formalin, which was used as fixative

d) A series of alcohol (70%, 85%, 95% and absolute alcohol) for dehydration purposes

e) Mayer's albumin as adhesive agent for mounting of tissue sections

f) Other chemicals for specific staining techniques (i.e. H&E, Nissl, Lillie's Variant of the Weil-Weigert and modification of Golgi staining methods)

2.3.2 Animal Sacrifice and Perfusion

Prior to perfusion, the frogs were anesthetised with tricaine methanesulfonate (MS-222) (dosage: 350mg/L) by means of immersion for approximately 30 minutes. Amphibian skin is extremely permeable and thus, water-diluted anesthetic agent could be easily permeated through the skin (Cakir and Strauch, 2005). Shortly after reaching the desired level of anesthesia (a condition in which the animals is unconscious and unresponsive to painful stimuli), body weight (g) and snout-to-vent length (cm) of the frogs were recorded. The snout-vent length is a standard measurement of body length (Berry, 1975). The measurement is from the tip of the nose (snout) to the anus (vent) (Appendix A).

Fixation by intracardiac perfusion was conducted to ensure that excised spinal cord tissue was well fixed. The anesthetised frogs were dissected on its ventral side to expose the heart. The left side of the heart was pierced to allow the efflux of body fluid during perfusion. The frogs were intracardially perfused with 0.6% frog physiological saline followed by 10% neutral buffered formalin as fixative. Jerking movements and twitching of muscles by the frogs indicated that the fixative solution had reached the different parts of the body and diffused into the tissue area. The process of fixation was considered complete when the body became stiff. Promptly, the vertebral columns, which housed the spinal cord were carefully dissected out and immersed in 10% neutral buffered formalin until the next step of removing the excess tissues and bones was conducted. After that, the excised spinal cords were then placed in the fixative solution for at least 24 hours.

2.3.3   Gross Morphological Observation and Measurement of Spinal Cord

While in fixative, the spinal cord tissues were observed at the gross morphological level under a stereomicroscope. Physical features as well as measurements, i.e. weight and length of tissue sample, were recorded for the purpose of data compilation.

2.3.4   Histological Processing of Spinal Cord Tissue

The fixed tissues were processed according to the basic histological tissue processing method in which the end product, i.e. histological slides, were later observed through light microscopy to review the cytoarchitecture. Preparation of histological slides involved several steps as described in subchapters 2.3.4.1 to 2.3.4.6.

2.3.4.1   Dehydration

Dehydration was initially carried out to remove all the water from the tissue samples. They were placed in specimen vials filled with 70% alcohol for 24 hours and followed by sequential transfer through a series of alcohol solutions with ascending concentration; 70% alcohol, 85% alcohol, 95% alcohol (I), 95% alcohol (II), absolute alcohol (I) and absolute alcohol (II), for approximately 45 minutes each.

2.3.4.2   Clearing

Clearing involved the process of removing the remaining alcohol in the tissue by using toluene as the clearing agent. Tissue samples were submerged in a reasonable amount of toluene for two changes; toluene (I) and toluene (II), with the time duration of 45 minutes each.

2.3.4.3   Infiltration and Embedding

After the second change of toluene, spinal cord tissue samples were transferred into a mixture of toluene and paraffin (1:1) for an hour. This mixture was heated at 60°C in an oven beforehand. To ensure that the melted paraffin had completely infiltrated into the tissue samples, the tissues were passed through three changes of melted paraffin at 60°C, each step for an hour (Figure 2.2 and Figure 2.3). Subsequent to infiltration, the tissue samples, together with their labels were placed in embedding molds filled with pure melted paraffin. The moulds were initially marked with a coloured wax pencil to help in aligning the tissues. This step was important so as to obtain the ideal symmetrical sections, as well as in locating the tissues after the paraffin had solidified. The paraffin blocks containing embedded tissues were then left to cool in water (at room temperature) and ready to be further processed.

Figure 2.2: Melted paraffin kept in the oven at 60°C.

Figure 2.3: Oven for paraffin infiltration and embedding.

2.3.4.4 Treating of Slides and Cover Slips

Slides and cover slips (24×50mm) were cleaned to ensure that they were free from any traces of oil and dust. This was done by soaking them in methylated spirit, wiped dry with paper towels and then stored in an enclosed box until further use.

2.3.4.5 Sectioning and Mounting of Tissue Sections

The solidified blocks of tissue samples were removed from the moulds and carefully trimmed into smaller pieces by using a razor blade. Then, a heated spatula was placed between the paraffin block and a block holder causing the paraffin to melt and thus, enabling the block to be affixed onto the block holder (Figure 2.4) (Gurr, 1956). Such set-up would allow steady gripping of paraffin block in the specimen holder of a microtome during the sectioning process. The block was trimmed into the shape of trapezium before it was sectioned. The paraffin blocks of embedded tissue were transversely cut into series of 10µm sections. The sectioning process was carefully done using a rotary microtome (Figure 2.5).

Selected sections of tissue from the long ribbon were promptly mounted on Mayer's albumin-coated slides, with two to three drops of distilled water on the surface to help the sections to expand. They were then laid onto the 'Electrothermal' slide warmer at 45°C to avoid wrinkles and infolding of the sections (Figure 2.6). Excess water was blotted using dry paper towel and the slides were allowed to dry. Finally, the slides were arranged in glass slide baskets and stored in the oven at 40°C before proceeding to the staining step (Figure 2.7).

Figure 2.4: Burner and spatula used in affixing paraffin block to block holder.
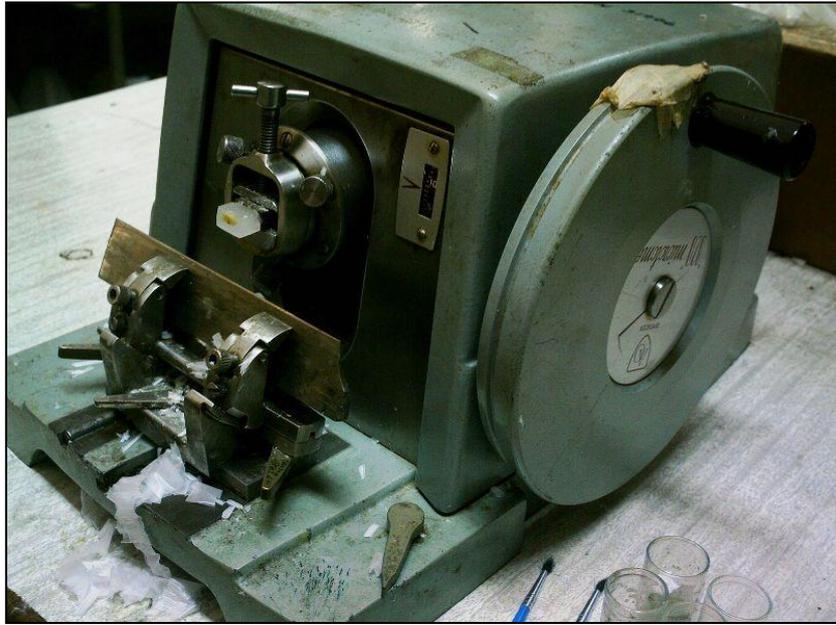
Figure 2.5: Rotary microtome for tissue sectioning.

Figure 2.6: 'Electrothermal' slide warmer.

Figure 2.7: Oven for storing slides-containing slide baskets.

2.3.4.6   Histological Staining

Five types of neurohistological staining methods were utilised in highlighting cytoarchitectonic features of the spinal cord sections; Hematoxylin and Eosin (H&E), Nissl, modification of Golgi, Thionin and Lillie's Variant of the Weil-Weigert staining techniques. The use of Hematoxylin and Eosin (H&E), Nissl and modification of Golgi staining techniques (Appendix C) allows the identification of neuronal soma, while Thionin and Lillie's Variant of the Weil-Weigert staining techniques enables the visualisation of the myelinated axons (Table 2.1). The outcome of these five methods served as a general reference in characterising the spinal cord cytoarchitectonic organisation. All staining procedures were carried out in a fume cupboard for safety precautions (Figure 2.8).

Figure 2.8: The staining platform in fume cupboard

Table 2.1: Staining techniques and their expected observation

Staining technique	Purpose	Expected results
1) Hematoxylin and Eosin (H&E)	Visualisation of neuronal soma	a) Nuclei - purple to blue b) Cytoplasm - pink
2) Nissl		a) Cell bodies - blue
3) Modification of Golgi		b) Neurons and processes - black
4) Thionin	Visualisation of myelinated axons	a) Nuclei - blue b) Fibre tracts - red to violet
5) Lillie's Variant of the Weil-Weigert		a) Myelin sheaths - bluish black b) Nuclei - red

2.3.4.6.1 Hematoxylin and Eosin (H&E) Staining Method (Gurr, 1956)

The slides with spinal cord sections were processed for H&E staining method, which involved several steps. The steps included deparaffinization in changes of xylene, hydration process involving a series of alcohol solution with descending concentrations, tissue staining using Harris's Alum Hematoxylin and Eosin as dyes and finally, the dehydration of tissue in changes of alcohol of ascending concentrations and clearing sections from dehydrant in changes of xylene. The stained tissues were mounted on glass slides in Canada balsam.

The H&E staining method displayed colour contrast between the stained nuclei and cytoplasm within a tissue section. The expected results showed the shade of purple to blue for the nuclei stained by the hematoxylin dye, whereas the cytoplasm would be stained in pink by the eosin dye.

2.3.4.6.2 Nissl Staining Method (Gurr, 1956)

The Nissl staining technique involved deparaffinization of paraffin section in xylene transfers, hydration process by running the slides in a series of alcohol solutions with descending concentrations, staining them in cresyl violet, dehydration process in three changes of tertiary butyl alcohol (TBA) and lastly, clearing sections from dehydrant in changes of xylene. Sections were then mounted on glass slides in DPX.

This staining method highlighted the visualisation of cell bodies solely, leaving out their axonal and dendritic projections that remained unstained except for the parts close to the soma. Utilising cresyl violet as dye, the cell bodies would appear blue after the staining process. RNA-containing Nissl bodies would be displayed in the neuronal soma, showing tigroid appreance.

2.3.4.6.3 Thionin (Gurr, 1956)

This method was slightly different from H&E and Nissl as the whole tissue undergone postfixation-cum-staining prior to tissue processing into paraffin blocks. First, it involved simultaneous fixation and staining for 5 to 7 days in a specially-prepared solution, i.e. 0.5% thionin in 10% formalin. The subsequent steps to produce a paraffin block were as usual (Subchapter 2.3.4) and the tissues were mounted under a cover slip in DPX. The thionin stain revealed blue-coloured nuclei and fibre tracts stained in red-to-violet colour.

2.3.4.6.4 Lillie's Variant of the Weil-Weigert Method (Lillie, 1954)

There was a slight difference in this method compared to the previous two. Tissue samples were initially fixed in 10% neutral buffered formalin before they were transferred to 2.5% potassium dichromate to complete a total of 4 days' chromation. The subsequent steps were similar to the procedure mentioned earlier in subchapter 2.3.4. Prior to staining of tissue sections, dehydration in a series of alcohol solutions with descending concentrations were carried out. Staining involved chemicals including iron alum, alcoholic hematoxylin, borax, potassium ferricyanide and safranin O as the counterstain. To finish, tissue sections were dehydrated in acetone and cleared from dehydrant in xylene before mounting them in DPX.

The predicted observations from this method would stain the myelin sheaths bluish black, the nuclei red with pink background. This technique helped to emphasize the white matter where myelinated axons were predominantly found in contrast to their cell bodies, which were arranged in the inner grey layer.

2.3.4.6.5 Modification of Golgi Method (Gurr, 1956)

The modified Golgi staining protocol started off with the immersion of tissue samples in 3% potassium bichromate for 3 to 4 days before transferring them to 2% silver nitrate for another 2 days. Both steps were carried out at room temperature. Following that, the tissues were processed as usual and no further staining of sections were required. Tissues were mounted under a cover slip in DPX.

This rapid method helped in delineating the neurons and their processes, in which they would be observed as black elements after the staining procedure. However, the Golgi method also exhibited a limitation of not being able to differentiate the nucleoli and the rest of the perikarya as both were also darkly stained, and thus hindering the process of cell calculation and analysis.

2.4 Experiment 2 - Immunohistochemical Localisation of Selected Nociception-related Neurotransmitters in Frog Spinal Cord

With the frog spinal cord structural basis ascertained in Experiment 1, this facilitated the subsequent work in the second part of the research to be carried out in Experiment 2, which involved immunohistochemical localisation of selected nociception-related neurotransmitters in the frog spinal cord. Immunohistochemical protocol performed in this study was applicable to the routine formalin-fixed, paraffin embedded tissues. The chemical solution preparation steps and the simplified immunohistochemical protocol are presented in Appendix D and Appendix E.

2.4.1 Colchicine Administration

Colchicine administration blocked axonal transport and thus, enhanced the concentration of the investigated neurotransmitter immunoreactivities in the cell bodies. A few batches of the experimental frogs were subjected to colchicine injection. This was done for the comparison of results in the spinal cord sections of both colchicine treated and non-treated animals for three nociception-related neurotransmitters, which were later localised immunohistochemically.

Firstly, temporary anesthetization was induced by immersion of animal in tricaine methanesulfonate (MS-222) (dosage: 250mg/ℓ) for approximately 30 minutes. Under the influence of anesthesia, each of the immobilised animals was injected with 0.05mg colchicine in 50µl 60% frog physiological saline through the foramen magnum 24 hours prior to perfusion. By running down the dissecting needle along the midline of the head to the intersection between the skull and vertebral column, the foramen magnum was located where a slight depression was felt, i.e. roughly 2 to 3 mm behind the posterior border of the tympanum. After the injection, the frogs were washed under running tap water to rinse off the MS-222 solution in order for recovery.

2.4.2 Slide Coating

For immunohistochemical work, it was advisable to use specialised pre-coated slides in silane. However, to save on cost, normal glass slides were also usable, provided that they were pre-treated with a layer of adhesive agent such as silane (3-triethoxysilylpropylamine) or Poly-L-lysine to improve section adhesion. The slide-coating procedure was carried out according to the instructions given by the kit and they were usually allowed to dry in the oven for 2 hours or in room temperature overnight before proceeding to the next step. Silanized slides could be manually prepared by dipping clean slides in 2% silane adhesive solution in acetone for 2 minutes and followed by slide-washing in two changes of distilled water before placing them in the oven to dry. To strengthen the tissue adhesion on slides, Miller (2001) suggested 'double-dipping' of slides in the silane solution.

2.4.3 Perfusion and Tissue Fixation

Tricaine methanesulfonate (MS-222) (dosage: 250mg/L) was again administered to the frogs via immersion. The measurements of the animals including body weight (g) and snout-to-vent length (cm) were recorded, shortly after the reaching of the desired anesthetic level (approximately after 30 minutes).

Dissection was carried out on the ventral side of the animal to expose the heart. The left side of the heart was slightly snipped in order for the blood to flow out during perfusion. Using a peristaltic pump, the animal was intracardially perfused with 0.6% frog physiological saline until the fluid was clear. Subsequently, this was followed by 10% buffered formalin as fixative. Jerking movements and twitching of muscles by the experimental animal indicated that the fixative solution had reached the different parts of the body and diffused into the tissue area. The process of fixation was completed

when the body became stiff. Promptly, the vertebral column, which housed the spinal cord was carefully dissected out and post-fixed in 10% neutral buffered formalin until the next step of removing the excess tissues and bones was conducted. After that, the abstracted spinal cord was post-fixed in the same solution for not more than 24 hours before placing the tissue in 70% alcohol overnight.

2.4.4 Histological Processing of Spinal Cord Tissue

The spinal cord tissues were processed according to the standard histological tissue processing procedures as described in Chapter 2.3.4 to produce paraffin section slides prior to immunostaining.

After formalin fixation, the tissue was subjected to dehydration in graded alcohols, alcohol clearing procedure, paraffin infiltration and embedding, similarly as explained in earlier subchapters. The paraffin block with embedded tissue sample, could be temporarily stored in a cool place for later use or proceed to sectioning using a microtome (refer to Chapter 2.3.4.5). Each section had the thickness of approximately 6µm to 7µm for immunohistochemical staining. Once mounted onto pre-coated slides, tissue sections were allowed to dry before they were kept in the oven at 40°C overnight.

2.4.5 Deparaffinization and Rehydration

As a precaution step to increase the adhesion of tissue sections, slides were warmed on a hot plate at 50°C for 20 minutes. Prior to staining, paraffin from the mounted tissue sections was removed by soaking the slides in xylene for 10 minutes. This was followed by transferring the slides into a series of alcohol solutions with descending concentration; absolute alcohol for 10 minutes, 95% alcohol for 3 minutes, 70% alcohol for 3 minutes, 50% alcohol for 3 minutes. The slides were then rinsed in distilled water for 2 minutes.

2.4.6   Epitope Retrieval

Tissue fixation could cause the masking of antigenic sites by formation of protein cross-links, especially if tissue samples were exposed to formalin for a prolonged period of time. Therefore, it was necessary to conduct the heat-induced epitope retrieval step by incubating the slides in Tris/EDTA pH 9.0 buffer solution via the water bath method. Recovering these masked epitopes would therefore enhance the staining intensity, giving a satisfactory observation of antigen immunoreactivity under study.

A container filled with Tris/EDTA pH 9.0 buffer solution in sufficient quantity to cover the tissue sections, was placed in a water bath. The water bath was then heated to 70°C to 75°C. The slides were introduced to the pre-heated Tris/EDTA pH 9.0 buffer solution and incubation was carried out for 20 to 30 minutes. Then, the container with slides was removed from the water bath and allowed to cool for another 20 minutes to room temperature. The sections were rinsed with a wash buffer, Tris-buffered saline (TBS) with the detergent, Tween 20 and ready for the subsequent step.

2.4.7   Immunohistochemical Staining

The experimental work was conducted following the indirect immunoenzyme method (Boenisch, 2001). The steps were outlined as follows.

2.4.7.1   Blocking of Endogenous Peroxidase

False-negative reactivity might occur due to endogenous peroxidase activity within every cell. Therefore, pre-incubating the sections in ready-to-use peroxidase-blocking solution containing hydrogen peroxide (Dako) for 30 minutes at room temperature could reduce unspecific background staining. This was followed by rinsing the slides in TBS-Tween 20 wash buffer with gentle agitation for 5 minutes before blotting dry the

areas surrounding the sections using paper towel. This step was carried out rapidly and then, immediately proceeded to the next normal serum incubation step to avoid the sections from drying out.

2.4.7.2   Normal Serum Blocking

A step involving blocking serum, i.e. normal whole serum derived from the animal of which the secondary antibody was raised, was employed before the application of primary antibody. In this case, sections were pre-treated with 10% normal goat serum in TBS (100µl normal goat serum to 1ml TBS) for 30 minutes at room temperature to inhibit cross-reaction of secondary antibody with endogenous immunoglobulin in the tissue. Slides were then dried by blotting the sides of the sections with paper towel.

2.4.7.3   Primary Antibody Incubation

Adjacent sections from all representative levels were processed separately for enkephalin, substance P and serotonin localisation. Primary antibody (Abcam), diluted in antibody diluent (Dako) at its optimal titer was applied to the tissue sections. Rabbit enkephalin, substance P and serotonin antisera were diluted at 1:500, 1:500 and 1:200, respectively. Incubation was then carried out for 30 minutes for the former two and 40 minutes for the latter, at room temperature. As a routine step, slides were rinsed in TBS-Tween 20 wash buffer twice for 5 minutes each, under gentle agitation, after the incubation process.

2.4.7.4 Secondary Antibody Incubation

Sections were subsequently incubated in horseradish peroxidase (HRP)-coupled secondary antibody raised from goat, acquired from the Dako REAL™ Envision™ Detection System for an hour at room temperature. Slides were again washed in TBS-Tween 20 wash buffer thrice for 5 minutes each, under gentle agitation.

2.4.7.5 Incubation with Chromogen

To develop colourimetric reaction product, incubation of tissue sections in 3,3'-diaminobenzidine tetrahydrochloride (DAB), also from the Dako REAL™ Envision™ Detection System, was performed. The sections were incubated for 5 minutes at room temperature and followed by a quick rinse in distilled water for 1 minute. DAB was chosen as substrate chromogen for demonstrating peroxidase since it yielded brown products at the site of the target antigen. However, this reagent posed carcinogenic potential to human, thus it was handled with care to avoid skin contact.

2.4.7.6 Counterstaining

For better visualisation, selected sections were counterstained in Mayer's Hematoxylin by dipping them into the dye for 30 to 60 seconds before rinsing them thoroughly in distilled water. This was followed by a 10-second immersion into 0.2% ammonia (NH_4OH) for bluing effect and a final rinse in distilled water. Stained slides were then mounted in glycerin (Glycergel), which was pre-heated in hot water (50°C) for 3 to 5 minutes before use. Counterstaining tissue sections in Mayer's Hematoxylin would stain the nuclei blue that contrasted well with the dark brown DAB reaction product.

2.4.4.7 Controls

Conducting control tests were important to establish the validity of the immunohistochemical technique as well as the specificity of antibody used in the experiment. Hence, the staining process using adjacent sections taken from all representative levels were accompanied by the following control procedures.

a) Positive control: An experiment where immunohistochemical method was applied on a tissue sample known to express the protein in investigation. Kidney and liver tissues of the experimental animal were used to validate reagent and immunostaining procedure (Huang and Weiss, 1999; De Falco et al., 2002; Caamaño-Tubío et al., 2007).

b) Negative control: An experiment where treatment with the primary antibody was omitted to reveal non-specific binding of the HRP-labelled protein. This allowed the evaluation on nonspecific staining within the sections as well as the specificity of an antibody being tested.

2.5 Light Microscopic Observation and Histological Analysis

All slides produced from both experiments (Chapter 2.3, Chapter 2.4) were studied through light microscopy under the magnification power of 40×, 100×, 200× and 400×, respectively. From the selection of tissue sections, live images were captured and stored using a specialised camera attached to both light microscope (Olympus BX51) and computer setup (Figure 2.9). The Olympus analySIS LifeScience Research software was used to analyse the images. It enabled various functions to be performed on the images such as the measuring of a specific region within a tissue section and the merging of images into a complete montage of a particular neural structure. Aside from that, the DinoEye eyepiece and its corresponding software were also utilised for preliminary analysis of the work. The processed images were presented in the 'Results' chapter.

Figure 2.9: Olympus BX51 microscope

2.6　Analysis of Spinal Cord Cytoarchitecture

The morphology and distribution pattern of the neuronal soma in the spinal grey were evaluated from the analysis of 10 successive cross sections (each was 10μm in thickness) of the cervical, thoracic and lumbar spinal segments from a total of six adult frogs. The neuronal cells observed at various locations within a spinal cord section were primarily categorised based on the somatal shape. Only neuronal somas with unstained nuclei and the presence of prominent nucleoli at the centre were recorded to ensure data consistency. This was crucial to avoid double-counting error especially for neurons in the dorsal, lateral and central fields. Majority of the neuronal somas were as small as the supporting glial cells. This had caused difficulty to distinguish the neuronal soma from the glial cells. In contrary, identification of neurons in the ventrolateral, ventromedial, lateral motor and medial motor fields was more straightforward since the multipolar motor neurons were larger in somatal size and with greater cytoplasmic area. The Nissl

substances in the somas were seen as tigroid appearance in Nissl-stained spinal cord sections. Nonetheless, the morphological identification and classification of neurons was not critical seeing that most of the cells observed had indeterminate shaped cell bodies. Such somatal shape appearance might occur due to incomplete staining or cellular damage or the possibility of neurons being cut at odd angles. Though the absolute number of neurons might be underestimated, the relative proportion remains as the major concern of this analysis. Perikaryal dimension including the width and length of a cell, of each somatal shape were also recorded. Cell width was the measurement of vertical extent from side to side while cell length was measured as the longest horizontal extent of a neuronal soma.