CHAPTER III
MATERIALS AND METHODS

1. Animals and Experimental procedures

1.1 Animals

Forty one male Wistar rats (from the National Laboratory Animal Centre, Mahidol University,Thailand) weighing 200 to 250 g at the beginning of the experiment were used and housed in aluminium cages on a 12-h light /12-h dark cycle with free access to water and food. Room temperature was maintained at $25\pm2^{\circ}$C. Animals were allowed a 1-week acclimatisation period before use in the experiment.

The procedures involving animals were approved by the institutional ethical committee and done according to the guide declared by the national research council of Thailand. All efforts were made to minimize animal suffering and to reduce the number of animal used.

1.2 Experimental procedures

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1.2.1 Experimental groups

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Taxol-treated group (P group)

Paclitaxel (Taxol®, Bristol–Myers Squibb, France), in vehicle (Cremophor EL, Sigma) (6.0 mg/ml) was diluted in normal saline (NaCl 0.9%) just before an administration to the animals with a final concentration of 1.2 mg/ ml.

Taxol 16 mg/kg was administered intraperitoneal once a week for five consecutive weeks, giving a cumulative dose of 80 mg/kg/rat. This treatment protocol has been previously shown to induce neuropathy in the treated rats (Authier et al., 2000).

Control groups (C group and V group)

Control rats were divided into 2 groups, non-injected (C group) and vehicle injected (V group).

The vehicle, composed of Cremophor EL (Sigma) and absolute ethanol 1:1 v/v, was diluted at the time of injection with normal saline (NaCl 0.9%), in the same proportion as in the Taxol -treated group. Injected volumes of the vehicle were calculated according to the body weight of each rat. The vehicle was administered intraperitoneal once a week for 5 consecutive weeks, similar to the paclitaxel-treated group.

1.2.2 Tail flick analgesic test

Introduction

This method measures reaction time of animals to heat produced by focusing of bright light focusing on the tail. The apparatus includes 150-watt infrared bulb producing bright light which is converted by a parabolic reflector to a photocell. The rat tail is placed between the light source and photocell. The foot switch is then pressed triggering the timer to start and the bulb to be energized. The elapsed time is expressed in seconds. When the rat tail flicks, indicating its pain threshold, it uncovers the photocell. This activation of photocell turns off the timer and energy source. Reaction time is then recorded.

Procedure

Rats were restrained and placed on the tail-flick apparatus [Tail Flick Analgesia Meter (Harvard Apparatus, UK)]. The light source positioned above the tail was focused on a point 4-6 cm rostral to the tip of the tail. Deflection of the tail activated a photocell and automatically terminated the test. The tail-flick latency represents the period of time from the beginning of the test until the tail deflection. Light intensity was adjusted to obtain baseline tail-flick latency of less than 5 sec in control rats. A cut-off time of 30 sec was used to prevent skin burn. Three mesurements were done with an interval of at least 30 min, and the average reaction time was calculated for each rat. A baseline reaction time was obtained for each animal prior to the administration of the drug and then later at each week before the injection and after the last session of the treatment (week 5) of the drug. Before testing in the first week, the animals were exposed to the tail-flick apparatus to familiarize with the procedure.

1.2.3 Hind paw analgesic test

Introduction

This method measures the reaction time from when a heat stimulus is applied to the plantar surface of the rat hind paw until the animal licks its hind paw in response to the heat.

Procedure

Each rat was placed on a hot plate (Ugo Basile, Italy) where a constant temperature of 55°C was maintained. This temperature has been tested before and is considered suitable since it is low enough to avoid burning the skin, but is high

enough to induce the hind paw licking response. A transparent plastic cage was placed around the hot plate to prevent the animal from leaving the plate. Then, the START/STOP button was pushed and the elapsed time in control rats with in 30 was shown on the screen. When the investigator observed temperature discomfort in the rat (i.e., licking of hind paws), the START/STOP button is pressed; then, the animal was immediately removed from the hot plate. The reaction time was then recorded. The cut-off duration was 30 sec to ensure that burn injury did not occur.

For each rat, the procedure was repeated every 5-10 min and at least 4 values were obtained. Generally, if the first reaction time was anomalously long, the value was discarded. There was no selection toward right or left sides since the timer was stopped when any hind paw was licked.

1.2.4 Electrophysiological measurement

Introduction

Nerve conduction studies are performed to diagnose disorders of the peripheral nervous system and help delineate the nature and distribution of the neural lesion which cover two main aspects: demyelination and axonal degeneration. With this technique, electrical stimulation of the nerve initiates an impulse, which travels along motor, sensory, or mixed nerves. The assessment of conduction response depends on the analysis of compound nerve action potentials recorded from the muscle in the study of motor fibers and sensory action potential from the nerve itself in the case of sensory fibers.

The conduction velocity is derived from the ratio between the distance between two points of stimulation and the corresponding latency difference which is the time the nerve impulse takes to travel between those two points. Latency is defined

as the time from the stimulation of the nerve to the peak of CAMP or action potential (Figure 3). Therefore, in case of the stimulation at two points (point 1 and 2), latency difference is equal to the difference between latency of the stimulation at point 1 (L_1) and latency of the stimulation at point 2 (L_2) (figure 3). The stimulation point must be as close as possible to the nerve.

$$\text{Conduction velocity} = \frac{D\,(\text{mm})}{L_2 - L_1\,(\text{ms})} = \frac{D}{L_2 - L_1} \quad \text{m/s}$$

D is the distance between the two stimulation points (point 1 and 2)in millimeters and L_1 and L_2 are the latencies of the stimulation at point 1 and 2 in milliseconds, respectively.

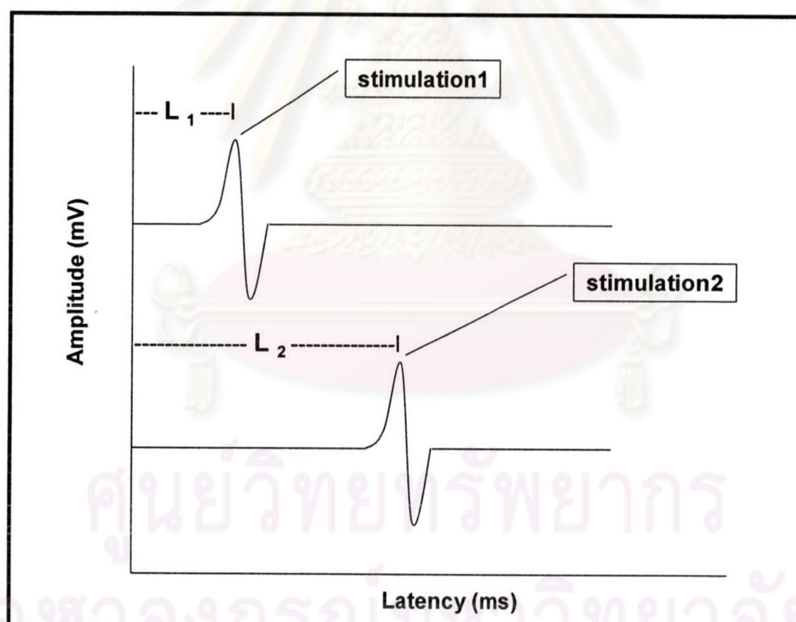

Figure 3 CMAP recording after two-point nerve stimulation where point 1 is closer to the recording position than point 2

L_1 = Latency of the stimulation at point 1, L_2 = Latency of the stimulation at point 2

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Procedure

The rats were anesthetized by 4% halothane with 1 l/min oxygen for 2-3 min until the animal was in a completely unconscious state confirmed by absence of a withdrawal reflex after toe-pinch test. The concentration of halothane was decreased to 1.5 – 2% with 500 ml/min oxygen and level of anaesthesia was checked again by the toe-pinch test. The animal was placed on heated blanket.

Orthodromic motor conduction study of the tail nerve was performed. The stimulating electrode was placed approximate 3 cm distal to the base of the tail (figure 4). Then, the active recording electrode as placed distally 2 cm from the stimulating electrode. The reference recording electrode was placed approximate 1 cm more distally to the active recording electrodes.

Finally, the ground electrode was placed between the stimulating and recording electrodes. All the electrodes were connected to the oscilloscope (Neurostar, Oxford instrument) and the nerve was stimulated with a supramaximal stimulus at least 5 times and the average CMAP was shown using the function in the oscilloscope. After that, the latency designated as Ld (= L_1 in figure 3), was calculated and recorded from this CMAP. Then, the stimulating electrode was moved to the more proximal part of the tail 20 mm from the previous position. The stimulation was again repeated and the latency (Lp = L_2 in figure 3) was calculated. According to the equation above, the distance 20 mm was divided by the difference between Lp and Ld.

All rats were subjected to the tail nerve conduction measurement before the start of treatment and then at the end of each week for 5 weeks.

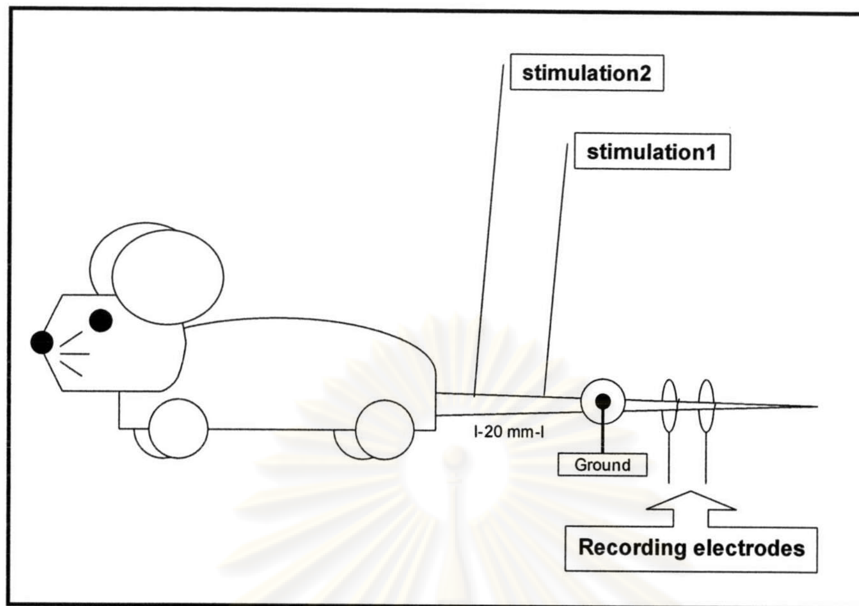

Figure 4 Diagram explaining positions of stimulating and recording electrode in NCV study

1.2.5 Sacrifice

At the end of the experiment which is week 5 since the first injection, all animals were sacrificed by intraperitoneal injection of overdose pentobarbital. Dorsal root ganglia at the levels of L4 and L5 including sciatic nerve were removed bilaterally and immediately snap-frozen in dry ice. The tissues were then transferred to -70 ^{o}C freezer and kept there until use.

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2. Western blot analysis

2.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.1.1 Introduction

Electrophoresis is the study of the movement of charged molecules in an electric field. The generally used support medium is cellulose or thin gels of polyacrylamide or agarose.

Because the mobility of a molecule in the gel is influenced by both charge and size, biological samples must be treated so that they have a uniform charge. After this treatment, electrophoretic mobility then depends primarily on size.

SDS disrupts the secondary, tertiary and quaternary structure of the protein to produce a linear polypeptide chain coated with negatively charged SDS molecules. Mercaptoethanol assists the protein denaturation by disrupting all disulfide bonds. Hence, SDS and mercaptoethanol are generally added in the samples during the preparation.

2.1.2 Sample preparation

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The L4/5 DRG were homogenized in the homogenisation solution [0.1 mmol/l PIPES pH 6.9, 5 mmol/l magnesium chloride, 5 mmol/l EGTA, 0.5% Triton X-100, 20% glycerol, 10 mmol/l sodium fluoride plus 1 mmol/l PMSF, 2 mmol/l sodium orthovanadate and protease inhibitor cocktail (1 μg/ml pepstatin A, 1 μg/ml leupeptin, 10 μg/ml benzoyl-L-arginine methyl ester, 10 μg/ml p-tosyl-L-arginine methyl ester, 10 μg/ml L-1-tosylamide-2-phenylethylchloromethyl

ketone, 10 μg/ml trypsin inhibitor and 10 μg/ml aprotinin); all from Sigma] using a microhomogenizer (IKA®-WERKE, Germany). Sample buffer (5 x) (0.25 M Tris pH 6.8, 10% glycerol, 0.01% bromophenol blue, 10 mM dithiothreitol, 2% SDS and 2% β-mercaptoethanol; all from Sigma) was added, and samples were boiled and protein concentration determined using the Bramhall protein assay (see section 2.1.3). The samples were then kept at -20°C until use.

2.1.3 Bramhall protein assay

The technique is the Bamberg modification of the method reported by Bramhall and coworker (Bramhall et al., 1969). Based on the principle that Coomassie Blue (Coomassie Brilliant Blue G, Sigma) binds to the protein in an acidic medium, the amount of Coomassie dye bound to the sample is proportional to the amount of protein in that sample. By comparing with the protein standard, the protein concentration in the sample can be determined.

Procedures

A sheet of Whatman No.1 filter paper was divided into equal squares (1.5 x 1.5 cm). Bovine serum albumin (BSA) protein standard (1 mg/ml, Pierce) was applied to each square from 0.5 to 8 μl in triplicate. Subsequently, 3 μl of homogenization solution (blank) or 3 μl of each sample was applied to each square in triplicate. To remove other substances from the paper which might interfere with the dye-binding reaction, the paper was allowed to dry and washed briefly with absolute methanol. The paper was stained for at least 30 min in a solution of 0.5 % Coomassie Blue (dilution of 5% stock by 7% acetic acid) after drying. Then, it was washed with several changes of 7% acetic acid to remove non-specific binding and allowed to dry. The filter paper was then cut into individual

squares and each square was put in a 2 ml Eppendorf tube. Next, 1.4 ml of elute solution (66% methanol, 33% distilled water and 1% ammonium hydroxide) was added in each tube to elute the bound stain.

After vortexing, the tubes were left at room temperature for 15 min and vortexed again to ensure the complete removal of dye stain. Standards, blanks and samples (200 μl) were transferred to a 96-well plate and a plate reader (Multiskan Ex, ThermoLabsystems) was used to read the absorbance at 600 nm. Values of the blanks were subtracted from those of the standards and samples. Standard curve was plotted from the values of 9 standards and protein concentration in the samples was calculated by referring to the standard curve.

2.1.4 Electrophoresis

Running gel (separating gel, 10% polyacrylamide) and stacking gel were prepared using wide vertical gel apparatus (wide Format Mini COC system) which can be used with 24-well combs. First, the running gel was prepared (see the formula in appendix 1) and N,N,N′,N′-tetramethylethylene diamine (TEMED) was added immediately prior to use. TEMED was added to catalyse the release of free radicals from ammonium persulphate (APS) resulting in accelerating acrylamide polymerization. The running gel was poured into the space between 2 glass plates and allowed to set for at least 15 min. Then the stacking gel was prepared (see the formula in appendix 1) and poured on the top of running gel. The comb was immediately inserted into the stacking gel and the gel was left to polymerize for 15 min. After the comb was removed, the samples were loaded in the volumes equivalent to 10 μg of protein in each well following brief boiling of samples. The first well of the gel was loaded with the protein marker or standard (Kaleidoscope Prestained Standards, Biorad). Subsequent to adding running buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3, Sigma) on the stacking and running gels, the

voltage was applied at 90 V until the proteins concentrated to form a sharp line and passed into the running gel and, then, the voltage was increased to 160 V. The electrophoresis was run for approximately 60 min.

2.2 Western blot analysis

2.2.1 Protein transfer

Transferring of the proteins separated by gel electrophoresis to nitrocellulose membrane, using an electroblotter (Bio-rad), allows further examination of individual proteins by immunological methods. Eight sheets of Whatman paper and nitrocellulose membrane (16.5 x 19 cm) were soaked in transfer buffer (5.82 g of Trizma base + 2.93 g of Glycine + 200 ml of methanol add distilled water to be 1 liter) for 15 min. Four sheets were placed on the bottom plate. Then, nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotechnologies) was placed on top. On the nitrocellulose, the acrylamide gel with separated proteins removed from the electrophoretic gel plates was placed. Finally, the remaining four sheets of paper were put on the top of the gel and air bubbles were eliminated by rolling a glass rod over the paper. The top plate attached to the lid was placed and locked in position. Electrical current was then applied at 0.35 A for 50 min for one gel or 70 min for two gels. After the current was turned off and the membrane (or blot) with the gel were removed from the electroblotter, blocking of nonspecific binding sites on the blot was processed by incubation with 5% non-fat milk in Tween buffer for approximately 2 hours at room temperature. The polyarylamide gel was stained with the gel staining solution (see appendix 2) and subsequently used to confirm the equal loading of protein.

2.2.2 Immunological detection

After the blocking process, the blot was incubated in a dilution of a primary antibody directed against the protein of interest, usually overnight at 4°C (see appendix 3). On the next day, the blot was washed with Tween buffer on a shaker three times (15 min each) and was then incubated with a secondary antibody linked with horse-radish peroxidase (HRP) (anti-rabbit or anti-mouse IgG HRP-linked antibody (see appendix 3), New England Biolabs) for 2 hours at room temperature. The blot was then washed with Tween buffer for 15 min x 3 on the shaker and the immune complexes formed on the blot were detected using enhanced chemiluminescence (LumiGLO chemiluminescent substrate, New England Biolabs). Basically, in the presence of hydrogen peroxide, HRP calatyzes the reaction that generates light from luminal, a chemiluminescent substrate. To detect the light emission, the blot was exposed to an X-ray film (Hyperfilm ECL, Amersham Pharmacia Biotechnologies) and the film was processed using an automatic developer in a dark room.

2.2.3 Densitometric analysis

The film was scanned using a scanner and imported as a gray-scale image to a microcomputer. Image analysis software (Image ProPlus) was used to do the densitometric analysis. Bands of interest were individually selected and spot density in each specified area was automatically analyzed by the program. The density data were exported to Microsoft Excel for further calculation. The values from backgrounds were subtracted from the values of the bands to obtain final values (density of the band without interference from the background). The density of the protein of interest is proportional to the amount of that protein in the sample and can be compared to other samples. Based on the fact that inactive MAPKs are phosphorylated by upstream regulators to become active, the ratio of

the phosphorylated (or active) form to the phospho-independent (or total) form of the enzymes was used in this thesis to indicate MAPK activity. This method has been shown to be comparable to the kinase assay which directly measures enzyme activity (Bokemeyer et al., 2000). Unless otherwise stated, all control values were normalized to 1 to allow for easier interpretation.

3. Statistical analysis

All data were analysed using SPSS for Windows version 10 for statistical analysis. Statistically significant differences were shown when p values were less than 0.05, unless otherwise stated.

3.1 Comparison between 2 independent groups

When normal distribution was observed in 2 independent groups, student's t-test, performed in Microsoft Excel, was used to compare means. To check whether the distribution was normal, the data were analyzed using SPSS. After normal distribution was verified, F-test in Excel was employed to compare the variances between 2 groups.
Depending on the variances, either Student's t-test with equal-variance or unequal-variance mode was used. Where the distribution was not normal, the data were imported to SPSS and a Mann-Whitney U test was used instead of a t-test.

3.2 Comparison between 3 independent groups

The data were checked for normal distribution and homogeneity of variances. One-way analysis of variance (ANOVA) was employed to compare the means of more than two groups with normal distribution and similar variances using SPSS. However, if the measurements were extremely skewed from normal

distribution and/or had a markedly significant difference in variances, a Kruskal-Wallis test (non-parametric test for ANOVA) was used. Where statistically significant differences were observed in ANOVA or Kruskal-Wallis test, pair-wise or post-hoc comparisons were achieved by using Tukey's HSD or Mann-Whitney U test, respectively.

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