THE EFFECTS OF GAMAT ON NOCICEPTIVE PROCESSING IN THE CENTRAL NERVOUS SYSTEM

by

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1. ABSTRACT

Gamat or sea-cucumber is believed to have anti-inflammatory and antinociceptive property. The aim of this study was to investigate the effects of gamat on pain behaviour and c-fos expression in rats injected with formalin. Eight Sprague-Dawley male rats (220-300 gram) were allocated to two different groups. Gamat extracts from Holothuria spp. (4 mg/kg) or distilled water was administered intraperitoneally immediately after intraplantar injection of 0.05 ml formalin (1%). The rats' behaviour was recorded with a digital camcorder for one hour. The tape was viewed and behaviour score was tabulated at every minute and averaged at five minutes interval. The rats were sacrificed two hours after the formalin injection and the brains were removed. The brains were sliced into 20μm sections and examined for c-fos expression. Behaviour data were analysed using SPSS, version 13. Statistical analysis of pain behaviour was analysed using repeated measures analysis of variance (ANOVA) with post hoc Scheffe's test and significance level was taken as 0.05. The pain behaviour in the group receiving gamat was significantly ($P<0.05$ for each) reduced at 5 minutes (4 mg/kg gamat - 0.15 ± 0.3; control - 1.9 ± 0.82) and the differences were maintained until 35 minutes post formalin administration (4 mg/kg gamat – 0 ± 0; control – 1.35 ± 0.93). C-fos expression in the thalamus was also reduced (mean=15.5) in the gamat group compared to the control group (mean=85). The gamat extracts from Holothuria spp has significantly suppressed the pain behaviour and c-fos expressions in formalin injected rat. Results from this investigation throw some light as to the possible use of gamat extract as an analgesic.

1. ABSTRAK

Gamat atau nama lainnya mentimun laut dipercayai mempunyai sifat anti keradangan dan anti nosiseptif. Matlamat utama penyelidikan ini adalah untuk mengkaji kesan gamat ke atas tingkahlaku kesakitan dan ekspresi c-fos ke atas tikus yang disuntik dengan formalin. Lapan ekor tikus Sprague-Dawley jantan dibahagikan kepada dua kumpulan yang berbeza. Ekstrak gamat Holothuria spp. (4mg/kg) ataupun air suling disuntik secara intraperitoneal serta-merta selepas suntikan 0.05 ml formalin secara intraplantar. Tingkahlaku tikus direkodkan dengan perakam digital selama sejam. Rakaman dianalisa dan skor tingkahlaku direkodkan setiap minit dan data pada setiap lima minit dipuratakan. Tikus dikorbankan dua jam selepas suntikan formalin dan otaknya dikeluarkan. Otak tikus tersebut dihiris kepada bahagian-bahagian bersaiz 20µm dan diperiksa untuk ekspresi c-fos. Data tingkahlaku dianalisis menggunakan SPSS versi 13. Analisis statistik tingkahlaku sakit dianalisa menggunakan kaedah ANOVA (repeated measures analysis of variance) dengan ujian Scheffe secara pos-hoc. Nilai adalah signifikan pada $p<0.05$. Tingkahlaku sakit dalam kumpulan yang disuntik dengan gamat ($p<0.05$) berkurangan secara signifikan pada minit ke-5 (4 mg/kg gamat- 0.15 ± 0.3; control - 1.9 ± 0.82) dan kekal sehingga minit ke-35 selepas suntikan formalin (4 mg/kg gamat - 0 ± 0; control - 1.35 ± 0.93). Ekspresi c-fos di talamus juga berkurangan (purata=15.5) dalam kumpulan yang menerima suntikan gamat berbanding kumpulan kawalan (purata=85). Ekstrak gamat Holothuria spp. juga mengurangkan tingkahlaku sakit secara signifikan dalam tikus yang menerima

suntikan formalin. Keputusan dari penyelidikan ini membuka ruang ke arah potensi gamat sebagai analgesik.

2. INTRODUCTION

2.1 Dual transmission of pain signals into the central nervous system (Nociceptive processing in the nervous system)

All pain receptors are free nerve ending. However these endings use two separate pathways for transmitting pain signals into the central nervous system. The two pathways mainly correspond to the two types of pain, a fast-sharp pain pathway and a slow-chronic pain pathway. The fast-sharp pain signals are elicited by either mechanical or thermal pain stimuli; they are transmitted in the peripheral nerves to the spinal cord by small type Aδ fibers at velocities between 6 and 30 m/sec. conversely, the slow-chronic type of pain is mostly elicited by chemical types of pain stimuli but also at times by persisting mechanical or thermal stimuli; this slow-chronic pain is transmitted by type C fibers at velocities between 0.5 and 2m/sec. On entering the spinal cord, the pain signal take two pathways to the brain, through the neospinothalamic tract and the paleospinothalamic tract.

The fast acute Aδ pain fibers transmit mainly mechanical and acute thermal pain. They terminate mainly in Lamina 1 (lamina marginalis) of the dorsal horns, as shown in figure 2.1, and they excite second-order neurons of the neospinothalamic tract. These give rise to long fibers that cross immediately to the opposite site of the cord through the anterior commisure and then pass upward to the brain stem in the anterolateral columns. A few fibers of the neospinothalamic tract terminate in the reticular areas of the brain stem, but

most pass all the way to the thalamus, terminating in the ventrobasal complex along with the dorsal column-medial lemniscal tract for tactile sensations. A few fibers also terminate in the posterior nuclear group of the thalamus. From this thalamic areas, the signals are transmitted to other basal areas of the brain and to the somatosensory cortex.

The paleospinothalamic pathway is a much older system and transmits pain mainly from the peripheral slow-chronic type C pain fibers, although it does transmit some signals from type Aδ fibers as well. In this pathway, the peripheral fibers terminate almost entirely in laminae II and III of the dorsal horns, which together are called the substantia gelatinosa as shown by the lateralmost dorsal root fiber in figure 2.1. Most of the signals then pass through one or more additional short fiber neurons within the dorsal horns themselves before entering mainly lamina V, also in the dorsal horn. Here the last neuron in the series gives rise to long axons that mostly join the fibers from the fast pain pathway, passing first through the anterior commissure to the opposite side of the cord, then upward to the brain in the anterolateral pathway. The slow-chronic paleospinothalamic pathway terminates widely in the brain stem, in the large pink-shaded area shown in figure 2.2. Only one tenth to one fourth of the fibers pass all the way to the thalamus. Instead, they terminate principally in one of three areas: (1) the reticular nuclei of the medulla, pons, and mesencephalon; (2) the tectal area of the mesencephalon deep to the superior and inferior colliculi; or (3) the periaqueductal gray region surrounding the aqueduct of Sylvius. These lower regions of the brain appear to be important in the appreciation of the suffering types of pain because animals with their brains

sectioned above the mesencephalon to block any pain signals reaching the cerebrum still evince undeniable evidence of suffering when any part of the body is traumatized. From the brain stem pain areas, multiple short-fiber neurons relay the pain signals upward into the intralaminar and ventrolateral nuclei of the thalamus and other adjacent regions of the basal brain.

(Guyton and Hall, 2000)

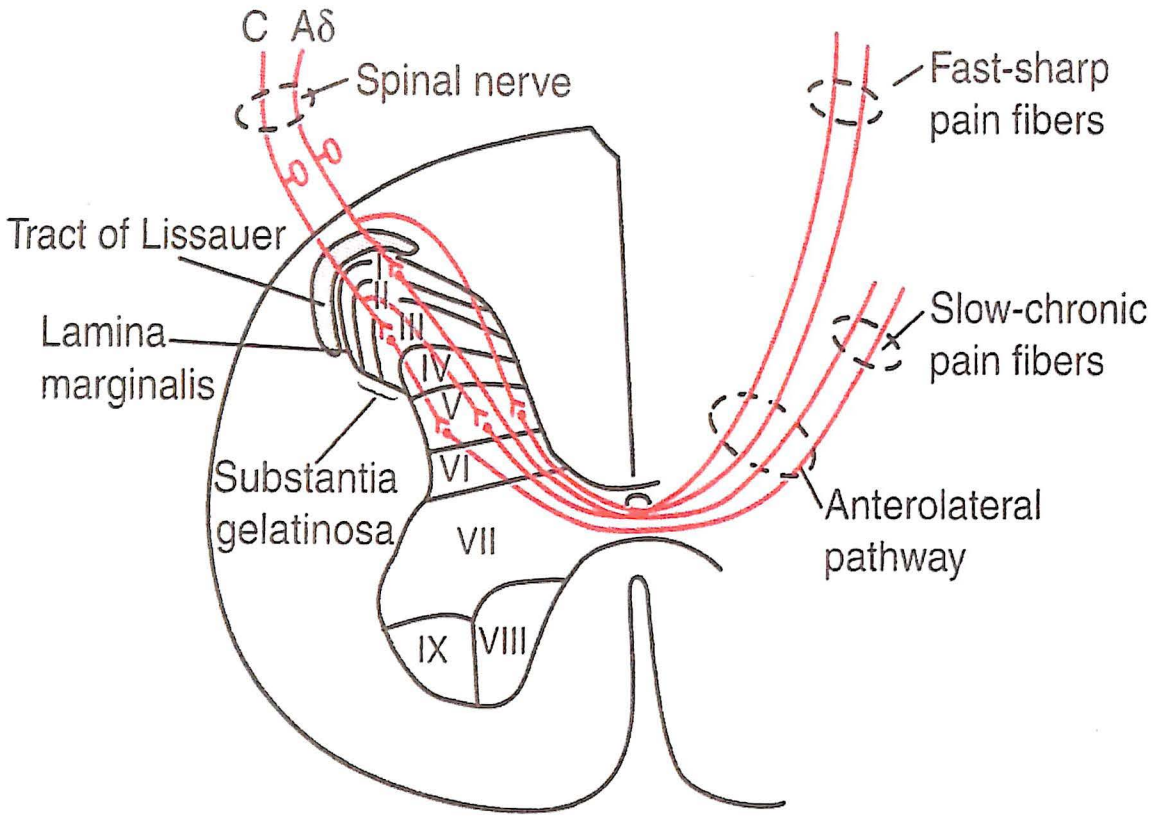

Figure 2.1: Transmission of both fast-sharp and slow-chronic pain signals into and through the spinal cord on their way to the brain. Guyton and Hall (2000).

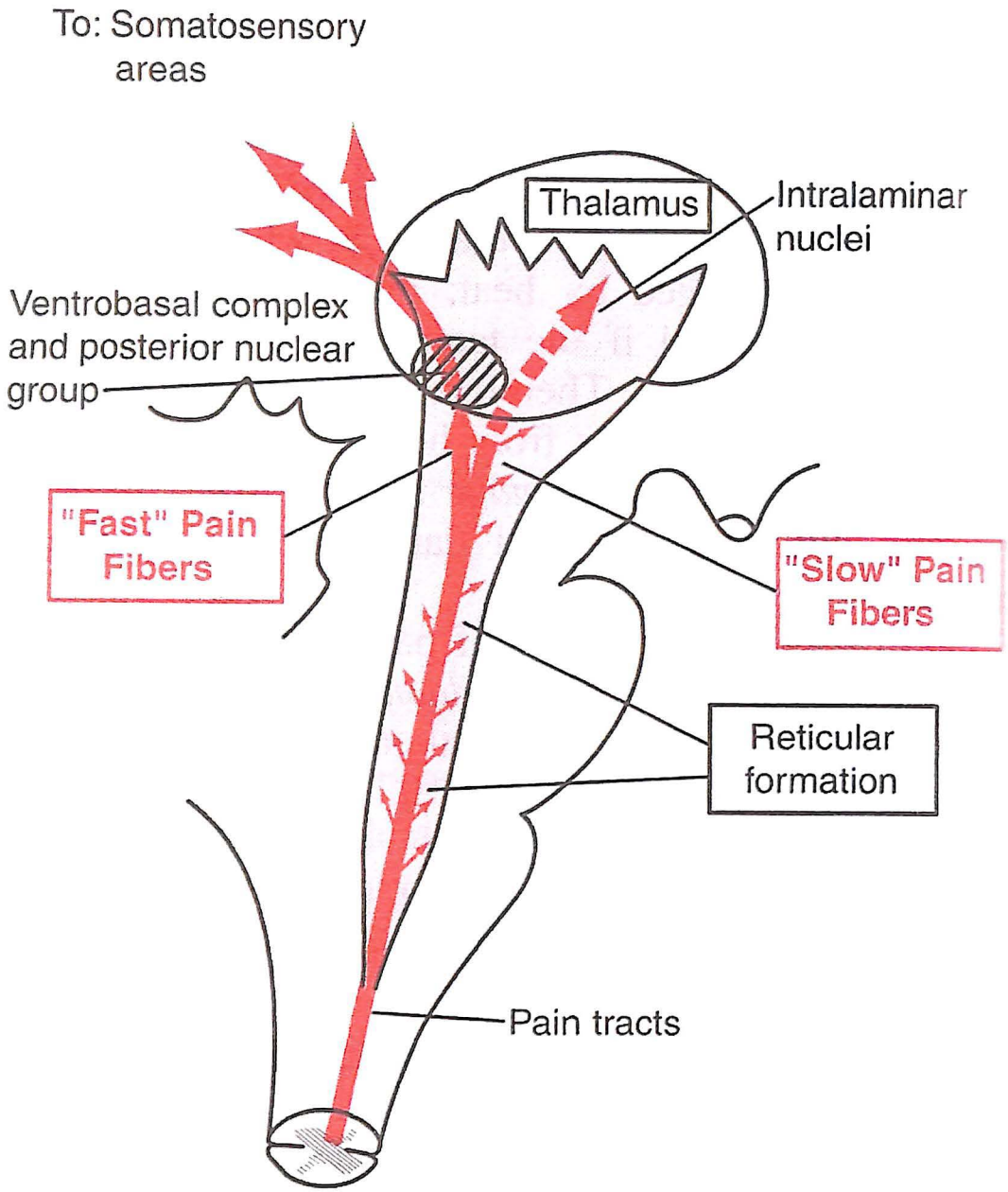

Figure 2.2: Transmission of pain signals into the brain stem, thalamus, and cerebral cortex by way of the fast pricking pain pathway and the slow burning pathway. Guyton and Hall (2000).

2.2 C-fos and pain

An immediate early gene, c-fos and its protein product are expressed in the neurons in response to noxious stimulation (Harris, 1998). C-fos induces conversion of information encoded in the c-fos gene to messenger RNA (mRNA) rapidly (within minutes after a particular stimuli) and transiently. Further, c-fos is widely used as a tool in pain research and is a marker for neuroplasticity (Dubner & Ren, 2004; Kosai *et al.*, 2001). Neuroplasticity is initiated by afferent input generated by intense noxious stimuli that trigger an increased excitability of nociceptive neurons in the central nervous system (Ji & Woolf, 2001). The hyperexcitability may develop as a result of tissue injury-induced inflammation and nerve injury-induced neuropathic pain (Drew *et al.*, 2000; Sung & Ambron, 2004). Neuroplasticity is expressed clinically as pathological pain e.g. hyperalgesia and allodynia. It is important to prevent neuroplasticity from occurring as 'pathological pain' is more difficult to treat (Woolf, 1994; Wollf & Chong, 1993).

The spinal cord plays a major role in the integration and modulation of nociceptive inputs prior to messages being sent to higher centres (Dickenson *et al.*, 1997) and most of these neurons project and terminate in the thalamus. There are evidences for the important role of the thalamus in pathological pain responses (Abdul Aziz *et al.*, 2005; Guilbaud *et al.*, 1986; 1987). Ventrobasal thalamic neurons exhibit lowered thresholds and enhanced peripherally-evoked responses following hindpaw inflammation (Guilbaud *et al.*, 1986; 1987) and nerve injury (Guilbaud *et al.*, 1990). Moreover, thalamic N-methyl-D-aspartate (NMDA) receptors contribute to the development and maintenance of

hyperalgesia in the carrageenan model of inflammatory pain (Kolhekar *et al.*, 1997).

Studies have shown that c-fos is expressed in the central nervous system in this acute pain model (Buritova *et al.*, 1998; Nackley *et al.*, 2003). Therefore, c-fos is a useful marker for the testing of drug efficacy at the central nervous system level. Inhibition of c-fos expression in the central nervous system has been shown by modern medication e.g. morphine and ketamine (Catheline *et al.*, 1999; Zhang *et al.*, 2004). Morphine suppressed noxious stimulus-evoked fos protein-like immunoreactivity in the rat spinal cord (Presley et al. 1990) whether administered supraspinally (Gogas *et al.* 1996) or systemically (Abbadie & Besson, 1993a). A study that uses intrathecally administered μ opioid receptor agonist on formalin-evoked nociception showed complete prevention of expression of fos-like immunoreactivity in all regions of the spinal cord (Hammond *et al.*, 1998).

2.3 Formalin test

The formalin test is used in this study on rats. It involves the injection of dilute formalin either in one of the hindpaws (Abbadie *et al.*, 1997) or one of the forepaws (Dubuisson & Dennis, 1997). The formalin test is now used frequently as a nociceptive assay for modelling persistent pain in laboratory animals (Tjolsen et al., 1992). Although originally described as an animal model of tonic inflammatory pain (Dubuisson & Dennis, 1977), the formalin test has also received much attention recently as a proposed model of persistent pain which

depends on sensitization in spinal cord dorsal horn (Coderre *et al.*,1993) and brain (Vaccarino *et al.*, 1989, 1992; Coderre *et al.*, 1993). In particular, it has been demonstrated that nociceptive responses in the late phase of the formalin test are, in part, dependent on nociceptive inputs which impinge on the central nervous system during the early phase (Dickenson & Sullivan, 1987; Vaccarino *et al.*, 1989, 1992; Coderre *et al.*, 1990). While persistent nociceptive responses to formalin may, in part, be dependent on central sensitization, there is also, most likely, a significant role of peripheral inflammation (Winter *et al.*, 1962; Yashpal *et al.*, 1996). However, the relationship between the nociceptive and the inflammatory responses to formalin is not well understood.

In most study, the hindpaws is usually chosen because they are rarely used during normal grooming behavior, hence they may be more nociceptive-specific than forepaw licking (Coderre *et al.*, 1993). Formalin test also provides an objective rating of the behavioral responses of the animal to formalin, permitting a quantitative assessment of pain intensity in these animals (Coderre *et al.*, 1993). First described in detail by Dubuisson & Dennis (1997), the quantification was based on the total time spent in 4 behavioral categories. A numerical value (or weighted score) was assigned to each category so that behaviors believed to reflect higher levels of pain intensity were given higher weighted scores (Coderre *et al.*, 1993). In this study, one of the hindpaws (right side) is injected and the behavioral categories and their weights were as follows.

0 –The hindpaws are place don the floor, and weight is evenly distributed. During locomotion, there is no discernible favouring of the injected

paw.

1 – The injected paw rests lightly on the floor or on another part of the animal's body, and little or no weight is placed upon it. During locomotion, there is an obvious limp.

2 – The injected paw is elevated, and not in contact with any surface. The uninjected paw is placed firmly on the floor. Attempts to sleep by curling up with only the injected paw off the floor, even when it is tucked under the body, are given this rating.

3 – The injected paw is licked, bitten, or shaken, while the uninjected paw is not.

(Dubuisson & Dennis, 1997)

A weighted average pain intensity score ranging from 0 to 3 is then calculated by multiplying the time spent in each category weight, summing these products and dividing by the total time in a given time interval (Coderre *et al.*, 1993). During the formalin test, two distinct phases of nociceptive behavior in the rat occur. The intraplantar injection of formalin can induce both peripheral and central biphasic responses (Dickenson & Sullivan, 1987). The first phase starts immediately after injection of formalin and lasts for 3-5 minutes (Tjolsen *et al.*, 1992). It is probably due to direct chemical stimulation of nociceptors (Dubuisson & Dennis (1997) and predominantly evokes activity in C fibres, not in Aδ afferents (Tjolsen *et al.*, 1992). Cells which respond only to innocuous inputs are hardly influenced by formalin (Dickenson & Sullivan, 1987). It has been shown that substance P and bradykinin are involved in the first phase (Shibata *et al.*, 1989). The second phase starts approximately 15-20 minutes

after formalin injection and lasts for 20-40 minutes (Tjolsen *et al.*, 1992). Histamine, serotonin, prostaglandin and bradykinin are involved, suggesting involvement of peripheral inflammatory processes (Shibata *et el.*, 1989). Other evidence shows that spinal processes are also of importance for the development of the second phase. It seems that processes in the spinal cord induced by the first phase, are necessary for the full manifestation of the second phase (Tjolsen *et al.*, 1992).

Early reports suggested that the early phase of the formalin response is due to the direct stimulation of nociceptors, while the late phase is directly due to an ensuing inflammatory response (Dubuisson & Dennis, 1977). Indeed, steroids and non-steroidal anti-inflammatory drugs (NSAIDs) reduce nociception in the late phase, but have little or no effect on the early phase responses to formalin (Hunskaar *et al.*, 1986; Hunskaar & Hole, 1987; Shibata *et al.*, 1989). A significant contribution of peripheral inflammation to the formalin response is further supported by the fact that injection of between 4 and 5% formalin into a rat's hind paw increases paw volume by 30-35% within 1 hour after the injection (Brown *et al.*, 1968; Wheeler-Aceto et al., 1991). It is important to note, however, that formalin-induced oedema does not reach its peak until 4-5 hours after injection (Brown *et al.*, 1968; Wheeler-Aceto et al., 1990), while its peak nociceptive response is exhibited between 20 and 35 min post-injection (Dubuisson & Dennis, 1977; Wheeler-Aceto *et al.*, 1991).

2.4 Gamat

A well known traditional medication, gamat or sea-cucumber (figure 2.3) is believed to have anti-inflammatory and antinociceptive property. In Malaysia, gamat are well known as a traditional remedy for healing of various internal and external wounds. Reports claimed that gamat have medicinal values in wound healing during the postpartum period (Chan & Liew, 1986; Zainudin *et al.*, 1986). Chinese also consume the sea cucumber because they believe that it has the ability to cure diseases such as high blood pressure, asthma, internal bleeding, body weakness, impotence and also healing wounds after giving birth or having an operation (Tarlochan 1980; Chan & Liew 1986). The ability of the Stichopus extract to promote tissue healing was reported by Taiyeb-Ali *et al.* (2003). This is further substantiated by the study of Fredalina *et al.* (1999) that demonstrated the high content of eicosapentanoic acid (EPA) in the Stichopus extract which was associated with the ability to initiate tissue healing. Another study also demonstrated that holothuria could heal wounds faster than controlled petroleum jelly (Ridzwan 1990 *et al.*).

Animal studies have also reported the antinociceptive property of the gamat extract using the abdominal contraction test (Ridzwan *et al.*, 2003) and tail flick test (Zury Azreen, 2004). This may suggest that some species of gamat may have analgesic property. However, up to date, very little is known regarding the effects of gamat on c-fos expression in the central nervous system. The aim of the present study is to investigate the effects of gamat on pain behaviour and c-fos expression in the thalamus using the acute pain model. Results from this

3. OBJECTIVE OF THE STUDY

3.1 Objectives

The study objectives are listed as follows:

1. To determine the effects of gamat on pain behaviour in formalin injected rats
2. To investigate the effects of gamat on c-fos expression when given after the inflammation.

3.2 Hypothesis

The analgesic property of gamat is realized through its capability of inhibiting the pain behavior and c-fos expression in the acute inflammatory pain model.

4. METHODS

4.1 Animals

Eight Sprague-Dawley male rats (220-300 gram) (figure 4.1) were allocated to two different groups (figure 4.8). Gamat extracts (figure 4.2) from Holothuria spp. (4 mg/kg) or distilled water was administered intraperitoneally immediately after intraplantar injection of 0.05 ml formalin (1%). The rats' behaviour was recorded with a digital camcorder for one hour (figure 4.3) and they were sacrificed (figure 4.5) two hours following the intraplantar formalin injection. The rats' brains were removed and fixed in paraformaldehyde solution. The brains were sectioned the following day using a cryostat (figure 4.7) and immunohistochemistry tests were performed to detect the presence of c-fos and fos positive cells were counted (figure 4.11) in the region of the thalamus (figure 4.4) with an image analyzer (figure 4.6).

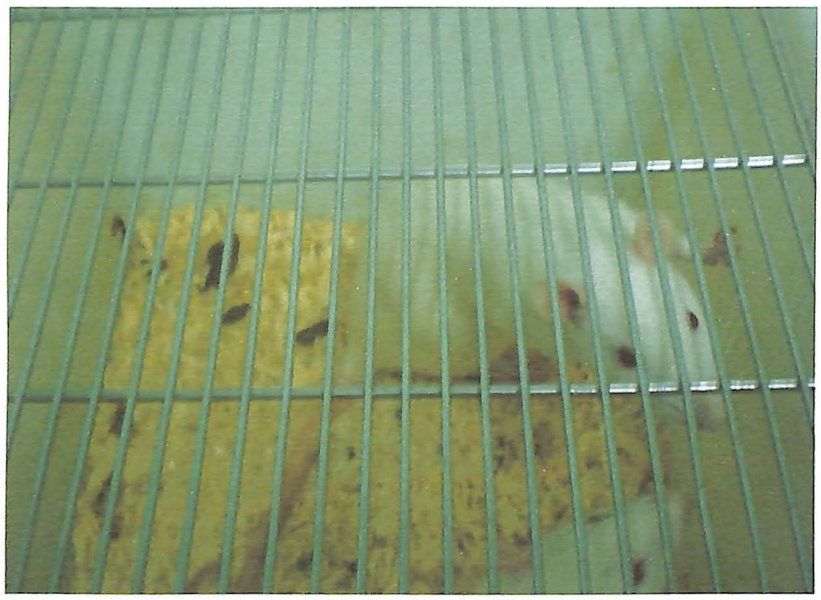

Figure 4.1: Sprague-Dawley male rats

Figure 4.2: Gamat extract (refer to appendix 1)

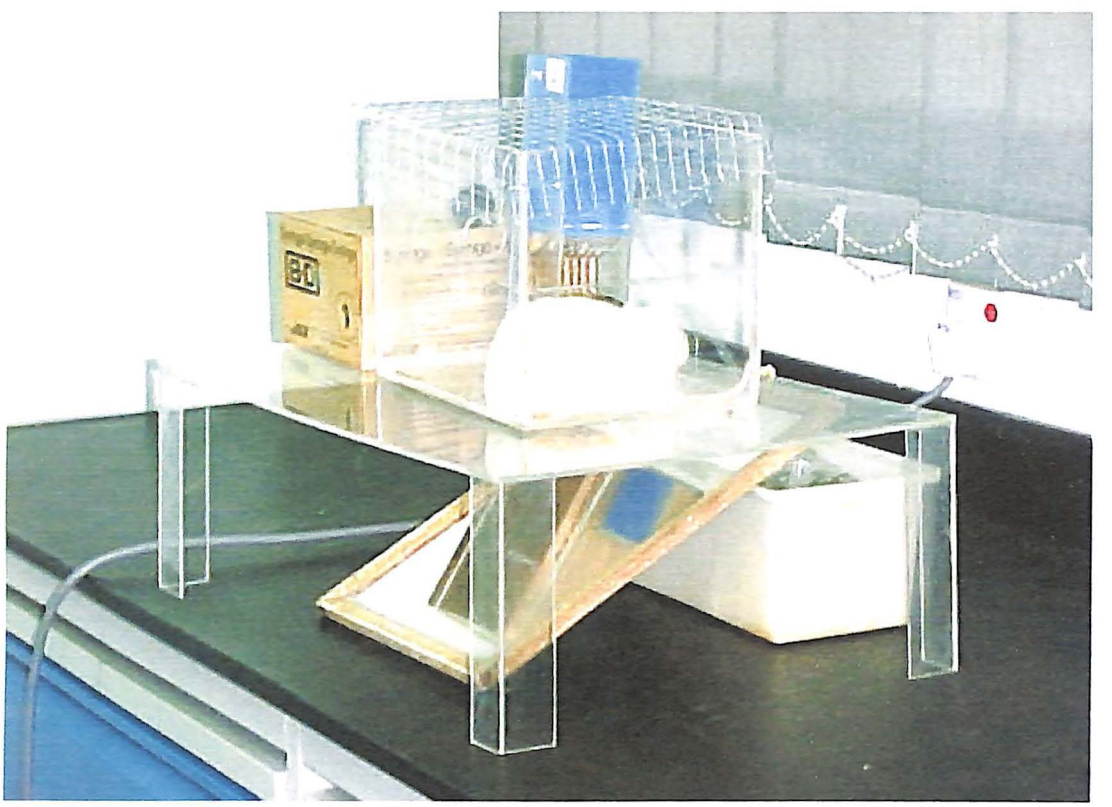

Figure 4.3: The rats' behaviour was recorded with a digital camcorder

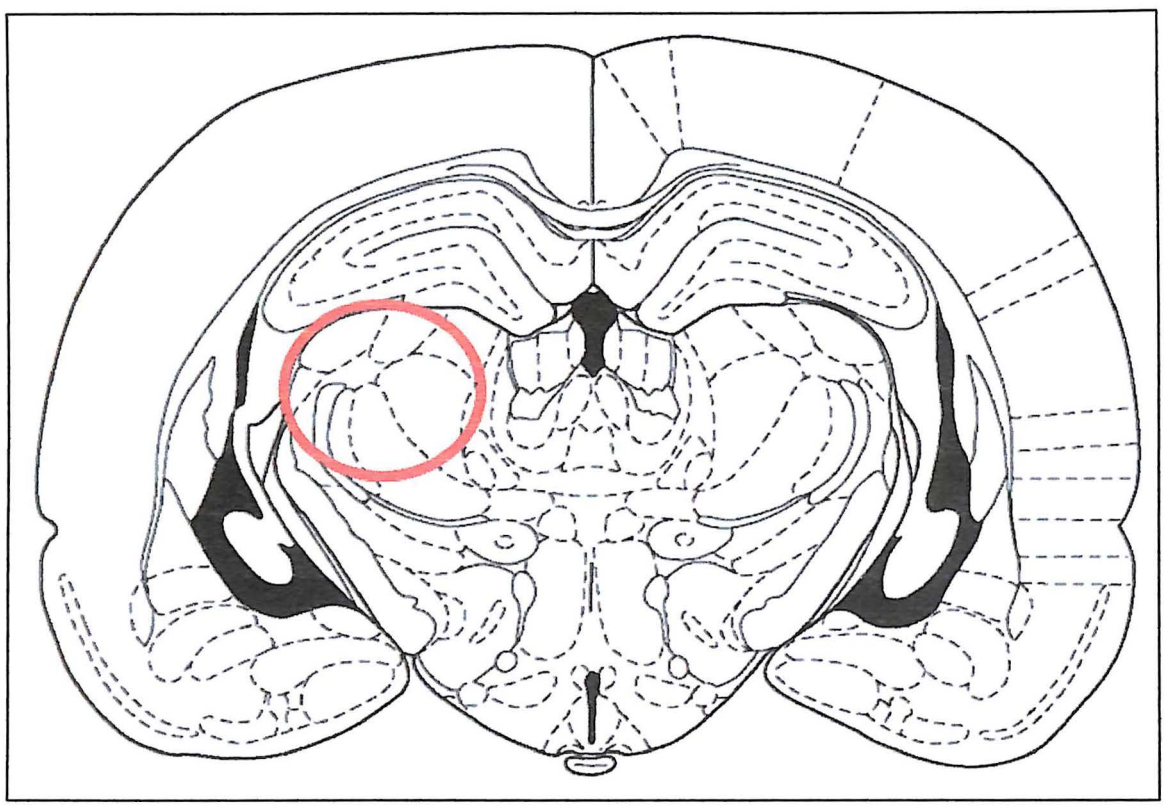

Figure 4.4: An illustration of a cross section of the rat's brain taken from Paxinos and Watson (1997). The area in the circle corresponds to the thalamus where the c-fos was counted in this study.

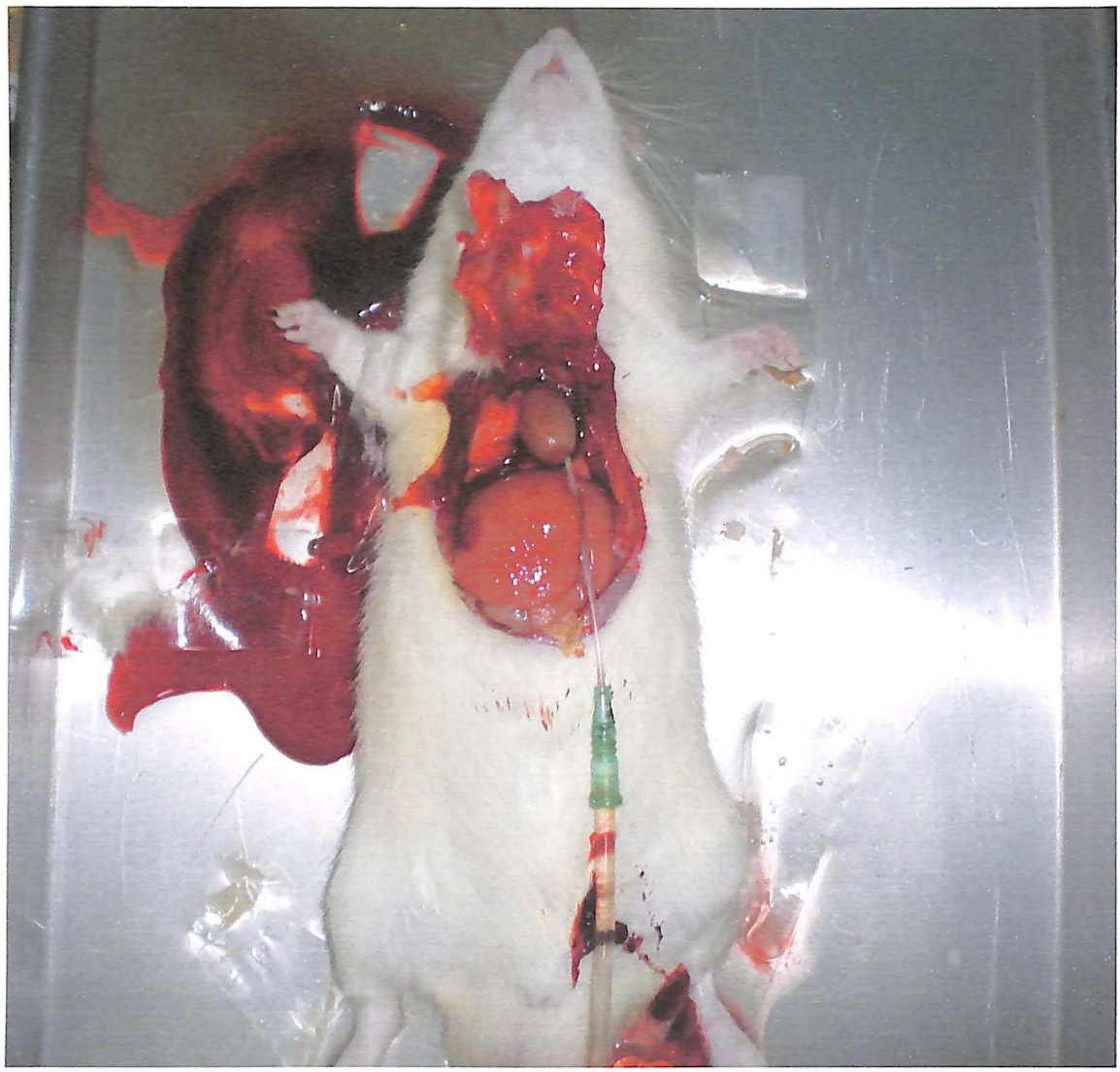

Figure 4.5: Cardiac perfusion on rat

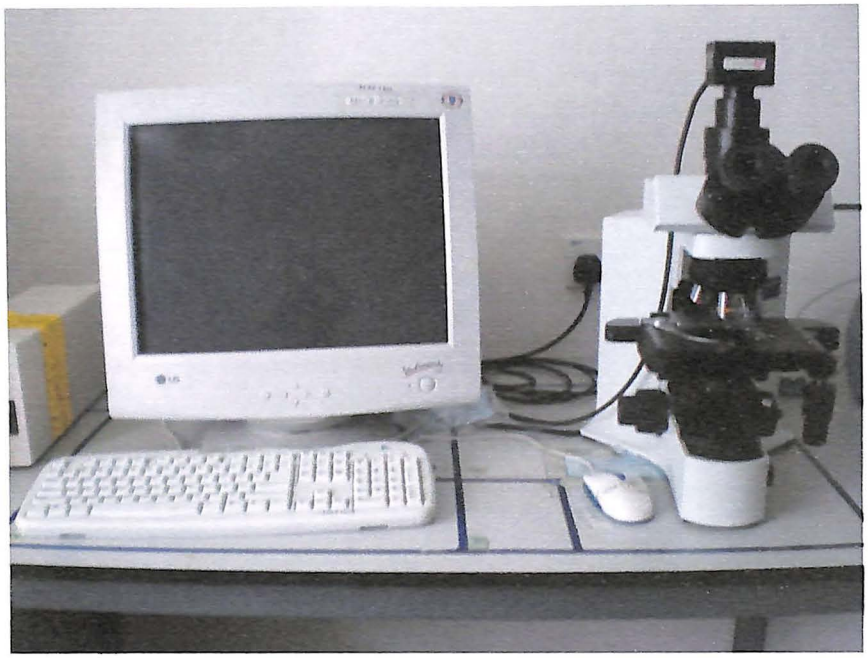

Figure 4.6: Image analyzer

Figure 4.7: Cryostat

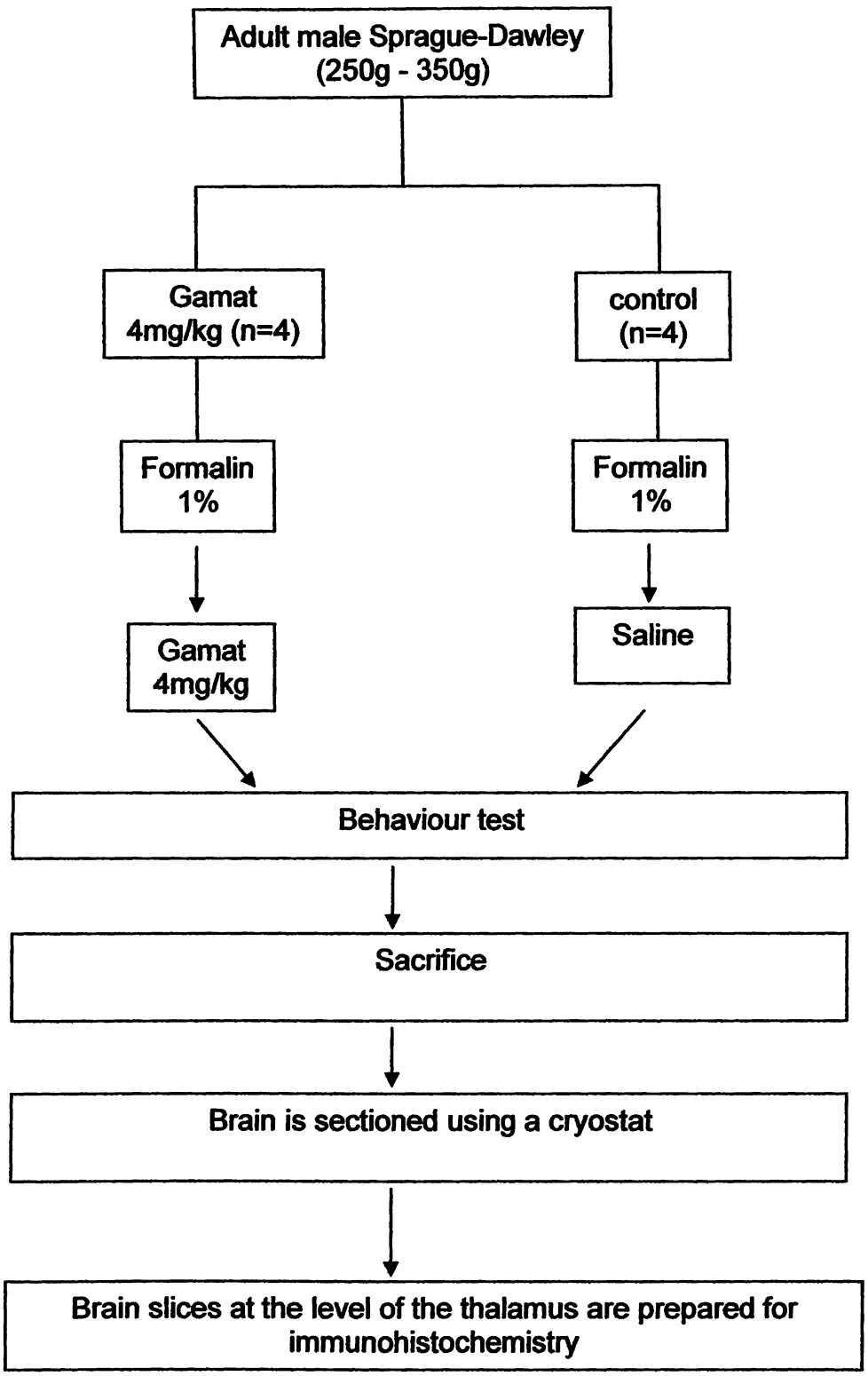

Figure 4.8: Flowchart of experiment in this study

4.2 Immunohistochemistry

Immunohistochemistry kit (figure 4.9) was used in this step. Sections were stained with a three-step peroxidase avidin-botin complex (ABC) method (purified primary antibody, biotinylated secondary antibody, and ABC with DAB) for immunohistochemical localization of Fos protein (Huang & Simpson, 1999) (figure 4.10). For each step, removal of a reagent by aspiration using a pipette and then replacing it with another reagent is called a rinse. After rinsing twice with Tris-buffered saline (TBS) at 5 minutes each, the sections were incubated with primary rabbit polyclonal anti-Fos antiserum directed against residues 4-17 of the N-terminal region of Fos peptide (Ab-5, Oncogene) diluted 1:20 000 with TBS containing 2% normal goat serum (NGS) and 0.2% Triton X-100 for 48 hours at 4°C with constant gentle agitation. After rinsing with three changes of Tris-triton (Gilron *et al.*, 1999) at 10 minutes each, the sections were incubated with biotinylated secondary antibody (goat anti-rabbit antiserum; Vectastain, Vector Laboratories) diluted 1:200 in TBS containing 2% normal goat serum (NGS) and 0.2% Triton X-100 for 1 hour at room temperature. Sections were then rinsed with three changes of Tris-triton at 10 minutes each, and then reacted with an avidin-biotin complex (Elite ABC, Vectastain) diluted 1:50 in TBS for 1 hour at room temperature. They were then rinsed in Tris-triton at three times 10 minutes and reacted with diaminobenzidine solution (0.5mg per ml of TBS) for 10 minutes. 0.2% hydrogen peroxide (H_2O_2) was added to each well and a brown discolouration was observed after 15 minutes. The reaction was stopped by rinsing the sections with three changes of TBS at 10 minutes each. After a quick rinse in deionized water, all sections were then mounted on gelatin-subbed slides and air-dried overnight. Slides were then dehydrated with

absolute ethanol for 15 minutes, mounted with Styrolyte Mounting Medium and protected with a coverslip.

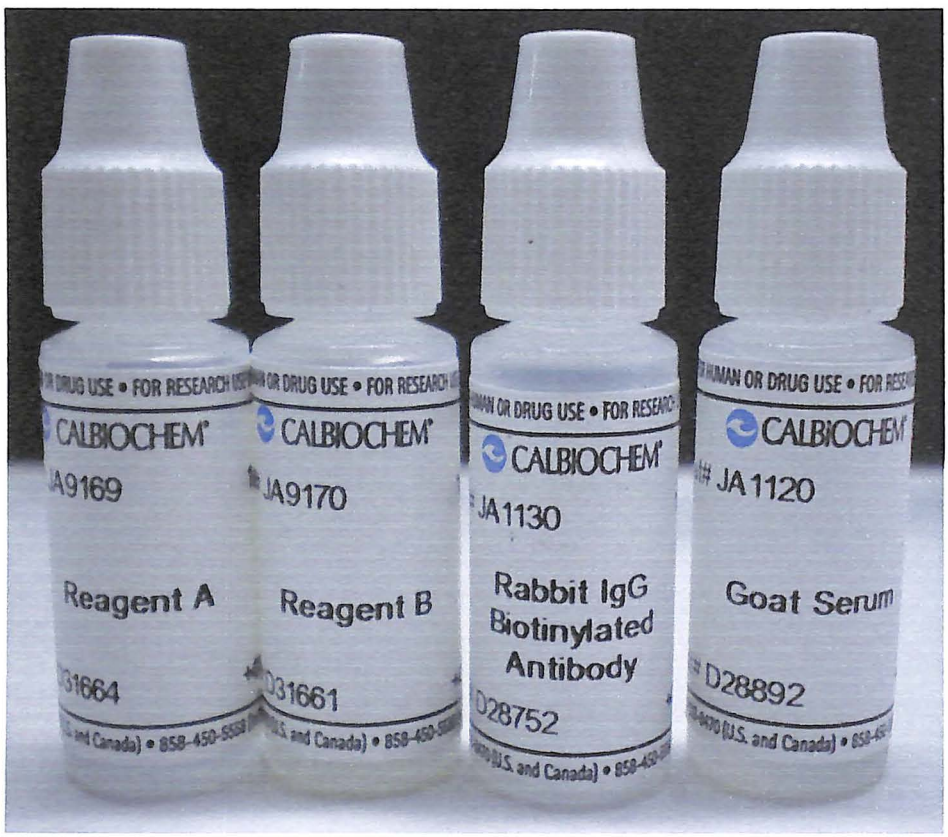

Figure 4.9: Immunohistochemistry kit used