MATERIALS AND METHODS

1. Fly specimens

1.1 Rearing of *C. megacephala* in the laboratory

The fly rearing procedure was modified from that previously described by Haskell (1990). *C. megacephala* obtained from a laboratory colony that has been maintained for approximately 3 years in the fly rearing room of the Department of Parasitology, Faculty of Medicine, Chiang Mai University. Adult female and male flies (50 of each) were transferred into a 30×30×30 cm rearing cage. They were fed with two kinds of food, (I) a mixture of 10% (w/v) sugar solution and 1.5% multivitamin syrup (Syn-O-Vits), and (II) fresh pork liver as a food source and oviposition site. Twenty grams of fresh pork liver were placed in a glass Petri dish (9 cm in diameter) at the bottom of the cage and changed daily. A plastic cup (4 cm in diameter and 6 cm in height), with a centrally located hole in the lid, was used as a container for supplying the mixture of glucose solution and multivitamin syrup. This mixture was changed every other day. A wick (10 cm in length) was inserted in the hole of the lid as a feeding site. Subsequently, the oviposition site was observed daily for the presence of fly eggs; and if these were any, they were gently transferred with a camel haired paintbrush No. 4, into a 12×15×6 cm transparent rearing box containing 40 g of fresh pork liver. To prevent overcrowding, each box consisted of 30 larvae. The rearing box was transparent, being 12 cm in width, 15 cm in length and 6 cm in height. A rectangular section was cut from the lid of the box, ¾ the size of the total area, and replaced with a fine silkscreen cloth (100 meshes/mm^2) for ventilation, and

prevention of other small insects entering. After placing the larvae into the rearing box, it was closed with the lid and the junction was sealed tightly using adhesive paper tape to prevent the larvae from crawling out. The rearing box was kept under ambient temperature (18-27ºC) and natural conditions in a cabinet in the fly rearing room, Department of Parasitology, Faculty of Medicine, Chiang Mai University (≈ 415 m above sea level). Fresh pork liver was replaced daily until some third instars develop into the pre-pupae, the non-feeding period. The box containing the pupae was still tightly sealed until some adults emerge. Thereafter, the box with some adults was transferred into the rearing cage (30×30×30 cm black net cage), and the lid was taken off to release the adults into the cage. The rearing box was taken out of the cage when all the adults have emerged.

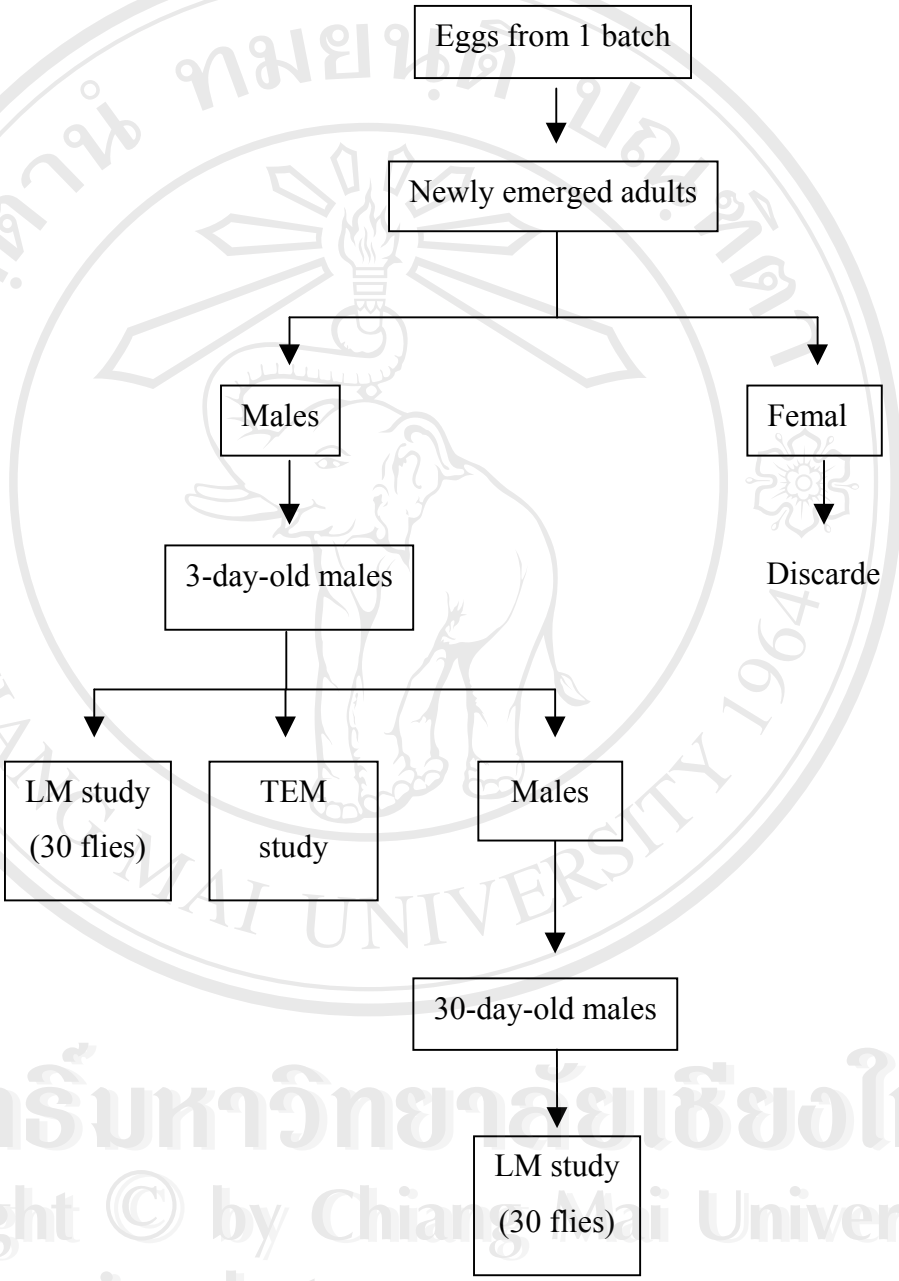
1.2 Brain dissection

Newly emerged flies in the cage were individually collected each day using a 10-ml glass tube and immediately separated into 2 groups - males and females. Males and females were determined by the character of their compound eyes. Females had broad frons, while the male's eyes touched in the middle of the frons. Males were kept in another cage and fed with the mixture of glucose and multivitamin syrup as previously described, while females were discarded.

From previous information on fly age, the median lifespan of adult *C. megacephala* males and females in laboratory condition was 26 and 29 days, respectively (Gabre *et al*., 2005). Hence, the 3-day-old flies were defined as '**young**' while the 30-day-old ones were defined as '**old**' in this investigation. According to Sohal and Sharma (1972), the criteria for selection and observation of the old fly's

activity were the sluggish movement and inability to fly. Sixty young males were randomized from the cage and sacrificed by diethyl ether for 5 mins. Thirty dead flies were immediately dissected to collect the brain, and the specimens were preserved in 10% formalin for light microscopic (LM) study. Another 30 dead flies were decapitated. Their brains were dissected and immediately prepared for the transmission electron microscopic (TEM) study as follows. They were preserved in 2.5% glutaraldehyde mixed in phosphate buffer (PB) at pH 7.4 at 4ºC for 24 hrs. The remainders of male were maintained in the rearing cage in the same manner as previously described. When they reached 30 days old, 60 males were sacrificed and prepared for the LM study using the same method for the young ones mentioned above.

The specimen preparation can be summarized in the flow chart as follows:



2. Light microscope (LM) study

Hematoxylin-eosin staining was used to examine the cells of the fly's brain. Thirty adult males were dissected to obtain the brain under dissecting microscope. These small specimens were wrapped by sheer paper and then placed in a plastic embedding cassette to prevent floatation. Subsequently, the embedding cassette was shaken overnight in a 10% formalin jar on an orbital shaker, followed by dehydration in an ascending gradient of ethyl alcohol concentrations (80%, 95% for 2 changes, absolute ethyl alcohol for 3 changes; 30 mins for each step). Xylene was used to clear 2 changes, 30 mins each. Then, the brain specimens were transported to the micropathology I laboratory room, Department of Pathology, Faculty of Medicine, Chiang Mai University, to embed them in paraffin wax by a tissue embedding center (Shandon®). Using forceps warmed with a Bunsen burner to prevent the paraffin from collecting on them, discarded a lid of plastic cassette that contained the impregnated brain from the paraffin holding area. A bottom of the plastic cassette was retained for embedding purpose. Initially, the mold placed on the hot plate was partially filled with molten paraffin. Re-warm forceps were applied to remove the brain from the cassette and position it at the bottom of the mold. During the wax quickly formed a thin solid layer, gentle pressing the surface of the specimen was conducted for properly orientation. After that, a bottom of the plastic cassette was placed firmly on the top of the embedding mold. The combined mold and plastic cassette was filled up with molten paraffin and immediately cooled by placing the mold on the cold plate of the embedding console. The paraffin was solidified in ≈15 mins; the mold was then separated from the embedding cassette. The brain and

solidified wax which attached to the embedding cassette formed a ready paraffin block for sectioning.

Serially horizontal, sagittal and vertical sections of the brains were cut at a thickness of 4 µm using a microtome (Leica®), and then fixed on the glass microscopic slides using gelatin adhesive. Subsequently, the slides were dried in an incubator set at 60°C for 30 mins and allowed to cool before staining. The brain sections were stained with Harris' hematoxylin-eosin stain (Sribamroongsanti, 2001) as follows:

- deparaffinized slide with xylene, 3 changes, 2 mins each
- rehydrated by placing in absolute, 95% and 80% ethanol for 2 mins in each concentration and rehydrated 2 times in each concentration
- placed in distilled water
- stained in freshly filtered Harris' hematoxylin for 10 mins
- washed in running tap water for 2 mins
- differentiated in 1% acid alcohol, 1-2 dips
- washed briefly in running tap water
- placed in saturated lithium carbonate solution until the section was bright blue (≈5-10 secs)
- washed thoroughly in running tap water, 10 mins
- placed in 80% ethanol for 2 mins
- counterstained in eosin-phloxine solution for 2 mins
- dehydrated with 2 changes of 95% and absolute ethanol, 2 mins for each step
- cleared through xylene, 3 changes, 2 mins each

- mounted with mounting medium, Permount®.

The brain structure in vertical, sagittal and horizontal planes and the observation of cellular changes in each brain specimen was performed using a light microscope (LM) and then photographed using an Olympus C4040Z digital camera. The criteria for cellular changes were modified from Min (2001). The histological patterns of brain degeneration were (i) widespread vacuolization or occurrence in any brain regions such as the lamina, medulla or central complex, (ii) the loss of neurons in each area of the fly brain.

3. Transmission electron microscope (TEM) study

The ultrastructure of the fly brain was observed using TEM study, as suggested by Bozzola and Russell (1999). Thirty young and 30 old males were sacrificed by diethyl ether as previously described. Only head specimens were immediately placed in a Petri dish (9 cm in diameter) with 0.9% saline solution and dissected under dissecting microscope to obtain the brain. The brain specimens were immediately fixed in 2.5% glutaraldehyde mixed in phosphate buffer (PB) at pH 7.4 at 4ºC for 24 hrs. After primary fixation, samples were washed 2 times with PB (10 mins for each time). The rinsed brains were chemically maintained in 1% osmium tetroxide (OsO_4) at room temperature for 3 hrs, then rinsed 2 times with PB and dehydrated with ethyl alcohol. To replace water with alcohol, gradually different concentration of 30%, 50%, 70%, 80% and 95% alcohol were applied chronologically; 6 hrs for each step. The brains were transferred in absolute alcohol for 6 hrs, and transferred to another jar with absolute alcohol for 6 hrs. The specimens, which have already been dehydrated twice in absolute alcohol, were dehydrated further in acetone in the same manner as absolute alcohol. The complete dehydrated samples were infiltrated with resin to obtain resin blocks. To replace acetone with epoxy resin, progressively different acetone: epoxy resin ratios were applied respectively as follows:

3 acetone: 1 epoxy resin	3 hrs
1 acetone: 1 epoxy resin	3 hrs
1 acetone: 3 epoxy resin	3 hrs
pure epoxy resin I	3 hrs
pure epoxy resin II	3 hrs

The complete infiltrated specimens were positionally embedded in an embedding mold and dried in an incubator at 70ºC for 24 hrs. Thick sections were cut from the resin block using a glass knife ultramicrotome. The 0.5 μm section was placed on a glass microscopic slide. Then, the slide was placed on a hot plate, stained with Toluidine blue O for ≈5 mins. The slide was rinsed with distilled water and observed further under light microscope. After selection of the areas required, ultrathin sections (90 nm) were cut from the resin block, with serial sections being collected from copper slot grids. Sections on the grid were stained with uranyl acetate and lead citrate in a dark Petri dish at duration of 10 mins for each staining. Consequently, sections on the grid were dipped in 1N NaOH for 2 mins, in order to scavenge any carbon dioxide that may cause precipitation of the lead stain, and then rinsed with distilled water for 1 min. Before pathological observation under JEOL-2010 TEM, excess distilled water was absorbed from sections on the grid using filter paper. Then, the sections on the grid were coated with gold (Au) for 5 mins. As suggested by Sohal and Sharma (1972), the specific targets of deterioration changes for brain degeneration were focused on the loss of ribosomes, focal cytoplasmic degeneration, and accumulation of dense residual bodies.

4. Nerve cell counts

A determination of nerve cell distribution applied from Sohal's technique (Sohal, 1972). Population densities of the nerve cells were examined in the serially hematoxylin-eosin vertical plane of the brains which were classified into four levels: (1) 50-90 μm, (2) 91-130 μm, (3) 131-170 μm and (4) 171-210 μm of sectioning depth (Figure 2). Thirty 3- and 30-day-old fly brains were examined using a calibrated ocular grid at a magnification of 1,000. Glial cells were excluded from the count. The number of neurons filled the given grid areas at various depths were counted in 8 selected regions of the brain (Figure 3) as follows:

Location 1: the pars intercerebralis neuropilar region, near the calyx of the corpus pedunculatum

Location 2: laterally outside rind of the optic lobe

Location 3: ventrally outside rind of the optic lobe

Location 4: laterally outside rind of the accessory lobe

Location 5: laterally outside rind of the antennal lobe

Location 6: dorsally outside rind of the protocerebrum, above the calyx of the corpus pedunculatum

Location 7: ventral region of the protocerebrum, below the central complex

Location 8: fusing area at central region of the optic lobe and the median lobe

The amount of neurons in each area of the fly's brain was analyzed. The mean and standard deviation was computed for each age group. The Students' *t* test or Mann-Whitney *U* test was used to determine significance of the difference between each area of young and old flies at the critical level 0.05 by SPSS version 12.0.

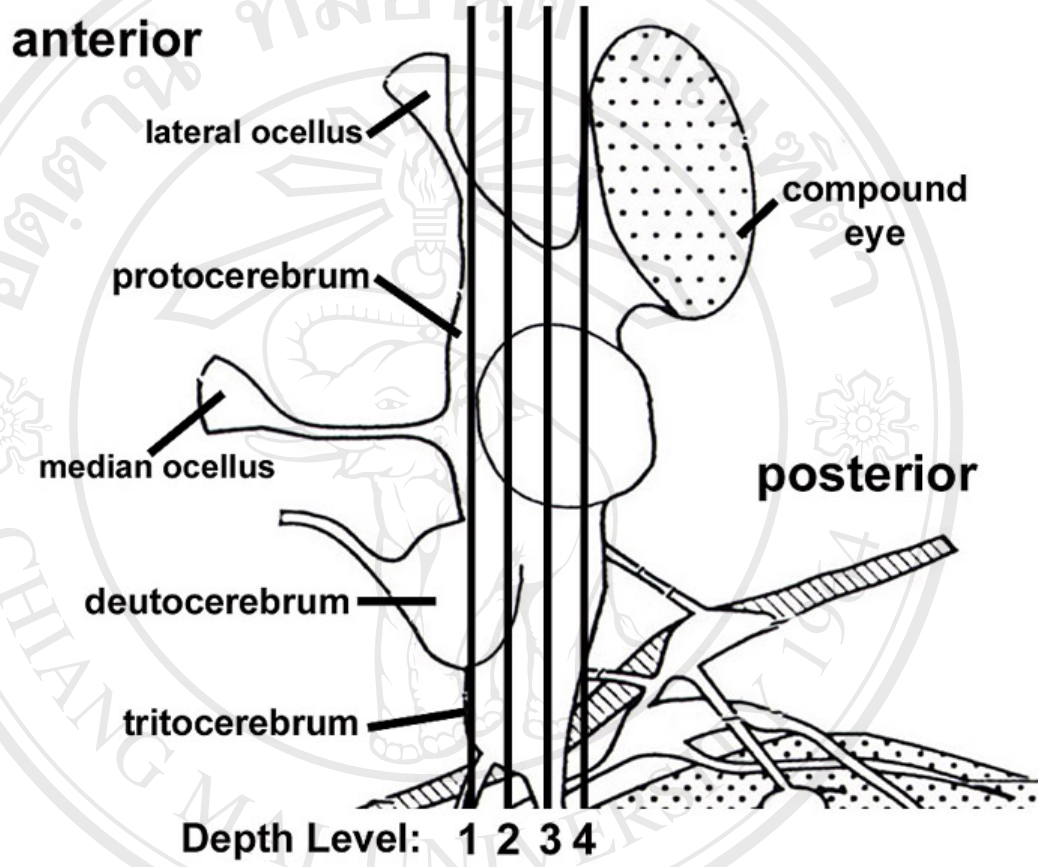


Figure 2 Lateral view diagram of the fly's brain, showing selected 4 depth levels. (adapted from Chapman, 1998; p.552).

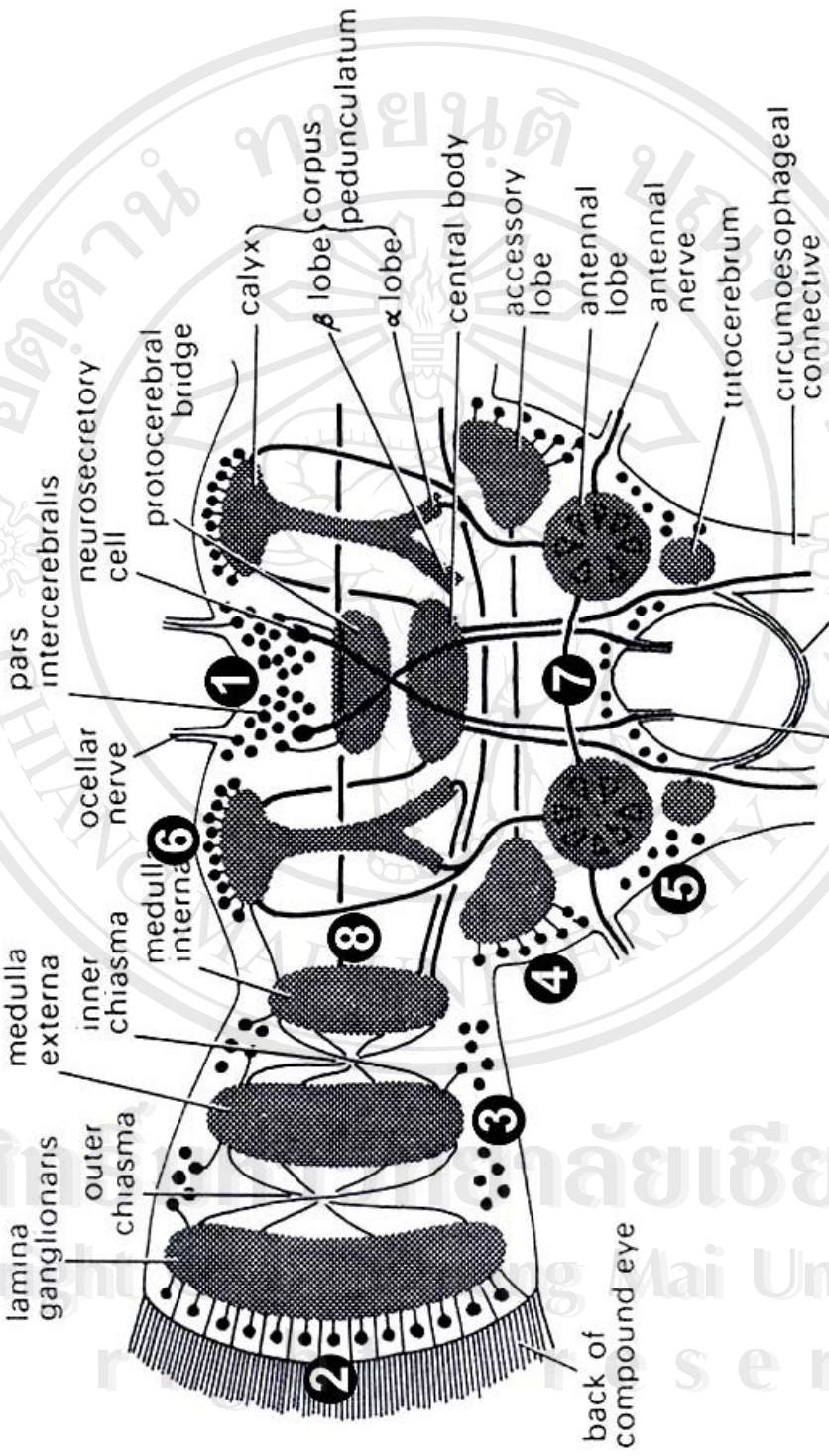


Figure 3 Vertical plane diagram of the fly brain, showing 8 selected areas for nerve cell count.
(adapted from Chapman, 1998; p.553).