# RESULTS

## 1. Light microscope (LM) study

1.1 External morphology of the *C. megacephala* brain under dissecting microscope

The brain of the adult blowfly species, *C. megacephala*, was discovered above the esophagus in the head capsule, connected by a number of fused ganglia. It consisted of three separate lobes: the central median lobe (the midbrain) and two symmetrically lateral optic lobes (Figure 4). The fresh brain was creamy, and wrapped in transparent air sacs. After removing the air sac covering, a large number of nerve fibers were seen. From the anterior aspect, the protruding optic lobes were located in the both sides of the midbrain, which was concealed beneath numerous nerve cords (Figure 4a). A posterior view of the brain (Figure 4b) showed a number of nerve fibers running from several parts of the central regions of the brain, termed 'neuropils', and over the superficial surface of the protocerebrum. These abundant nerve tracts could provide connections between the fly's brain and other parts of the nervous system.

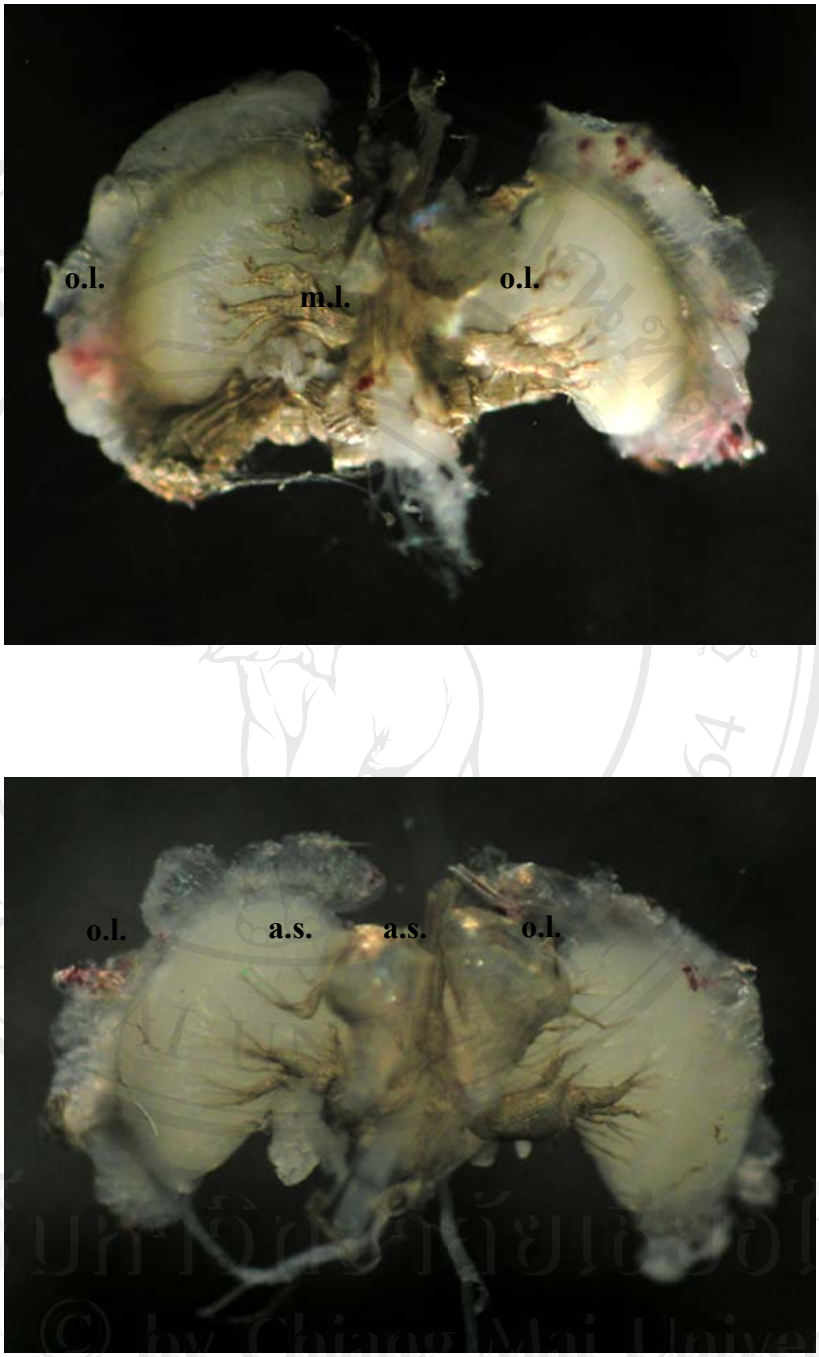





Figure 4 Fresh brain of the 3-day-old *C. megacephala* male in 0.9% saline solution under dissecting microscope at a magnification of 40. **(a)** anterior view **(b)** posterior view. The abbreviations are: o.l. = optic lobe; m.l. = median lobe; a.s. = air sac.

1.2 Histological-anatomical study of a young and old *C. megacephala* brain

1.2.1 Brain Size

The width and length of the brain were not significantly different between young and old flies ($P > 0.05$, Independent-Samples T Test). Mean values of width and length in the young group were 1616.05 and 747.08 µm, respectively, while the mean of old fly brains were 1629.16 µm in width and 758.88 µm in length (Figure 5).

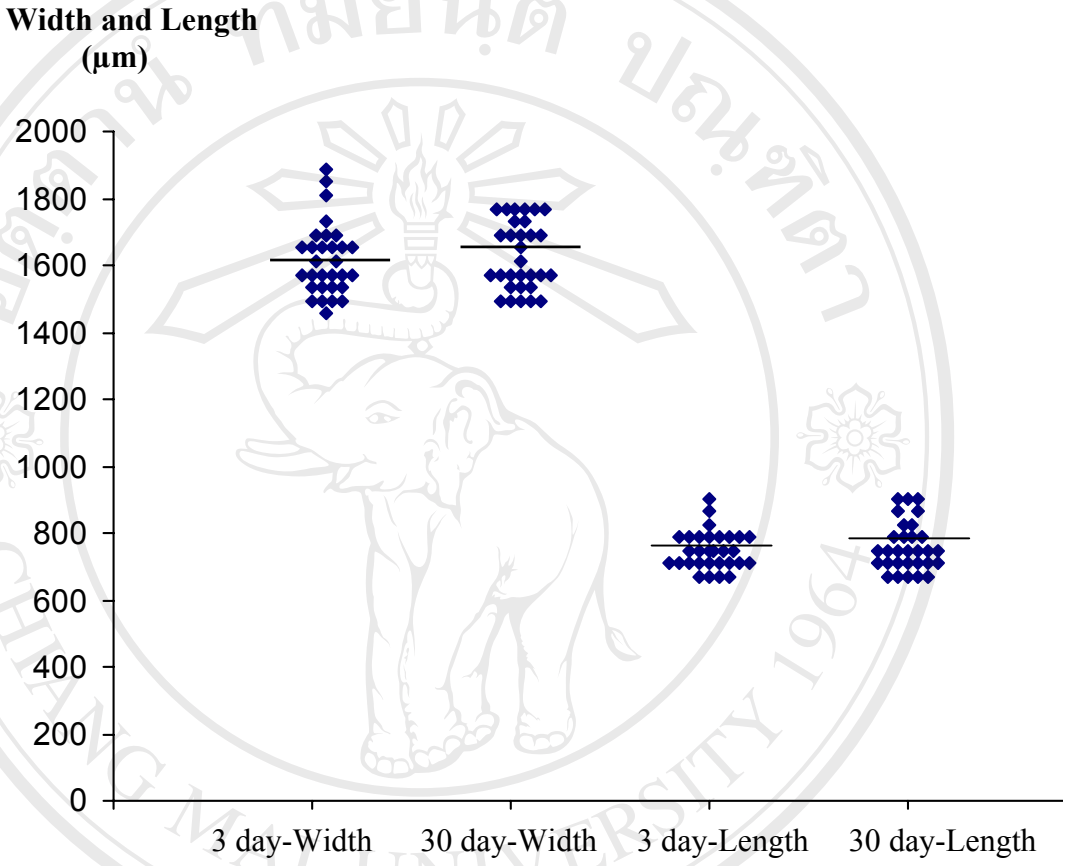



Figure 5 Width and length of the *C. megacephala* brain between 3- and 30-day-old flies (n=30 for each age). Lines in the graph represent the mean value of each group.

1.2.2 Neuroanatomical structure

The blowfly brain compartments were illustrated by hematoxylin-eosin stained sections at various depths cutting to the three major planes (vertical, horizontal and sagittal) under LM at a magnification of 40 and 100. Among these 3 planes, slides from the vertical plane provided the clearest morphology of brain neuropils and the nerve cell body.

Vertical plane

Serially vertical sections of the 3-day-old brain at different depths from anterior to posterior direction were determined under LM, and are demonstrated in Figure 6-14. The brain structures found in each depth were as fellows:

At 20 μm depth: only two optic lobes (o.l.) were displayed. The median lobe (m.l.) could not be seen at this depth (Figure 6).

At 44 μm depth: three lobes of the fly's brain were observed. The protocerebrum located at the dorsal part, including the deutocerebrum and tritocerebrum, were at the middle and ventral part, respectively (Figure7-8).

At 84 μm depth: a quite large optic lobe composed of three neuropils could be seen; the lamina (la), medulla (me) and lobula (l) (Figure 9-10). Neuropil brain structures were formed from nerve axons and their branches, which tangled as a nerve web; enclosed by an outer layer of peripherally dispersed perikarya (nerve cell bodies). Various tracts of fiber could be seen linking the midbrain to the lobula via the optic pedunculus (o.p.).

At 116 μm depth: the midbrain or median lobe, considered to be the most complex structure of the fly brain, was obviously explored (Figure 11). The median protocerebral neuropil was conspicuously grooved in the midline by the

median furrow (m.f.). The central body or central complex (c.c.) was a median mass of neuropil lying in the center of the protocerebrum. It had a great number of connections with different nerve fibers such as those from the perikarya on the borders of the pars intercerebralis or antennal lobes (a.l.) of the deutocerebrum. Nearby, a neuropil of the central complex was the protocerebral bridge (p.b.), which lay across the middle of the brain and posterior to the pars intercerebralis (p.i.). The p.i. in the dorsal median region was situated above both the p.b. and the c.c. with the corpora pedunculata (c.p.) neuropil arranged laterally to it. The c.p. or mushroom bodies were paired neuropilar areas. Basically, each hemisphere consisted of a flattened cap of neuropil; the calyx from which a peduncle (p) ran ventrally before dividing into two lobes, known as alpha ($\alpha$) and beta ($\beta$) lobes. As well as the calyx of c.p., the ocellar center that usually combined with the base of the nerves from the ocelli was not shown in this figure.

At 160 μm depth: other neuropils of the fly's brain, and the accessory lobes (ac.l.), were remarkable (Figure 12). They were placed dorsally posterior to the antennal lobe. In addition, some striking nerve tracts and the noduli (no) were also seen.

At 188 μm depth: rod-liked lateral neuropils of the protocerebral bridge and twin median calyces (m.c.) of the mushroom bodies were apparent. The multi-stranded tract, which ran across the ventral part of the protocerebral neuropil, dorsally to esophageal foramen (e.f.), was the posterior deutocerebral commissure (p.d.c.). Two spotted ventral connectives (v.c.) at the ventral part of the midbrain neuropils were an additional outstanding feature (Figure 13).

At 204 µm depth: the remains of the median and optic lobes were noticeable. The optic lobes were surrounded by plenty of perikarya (Figure 14).

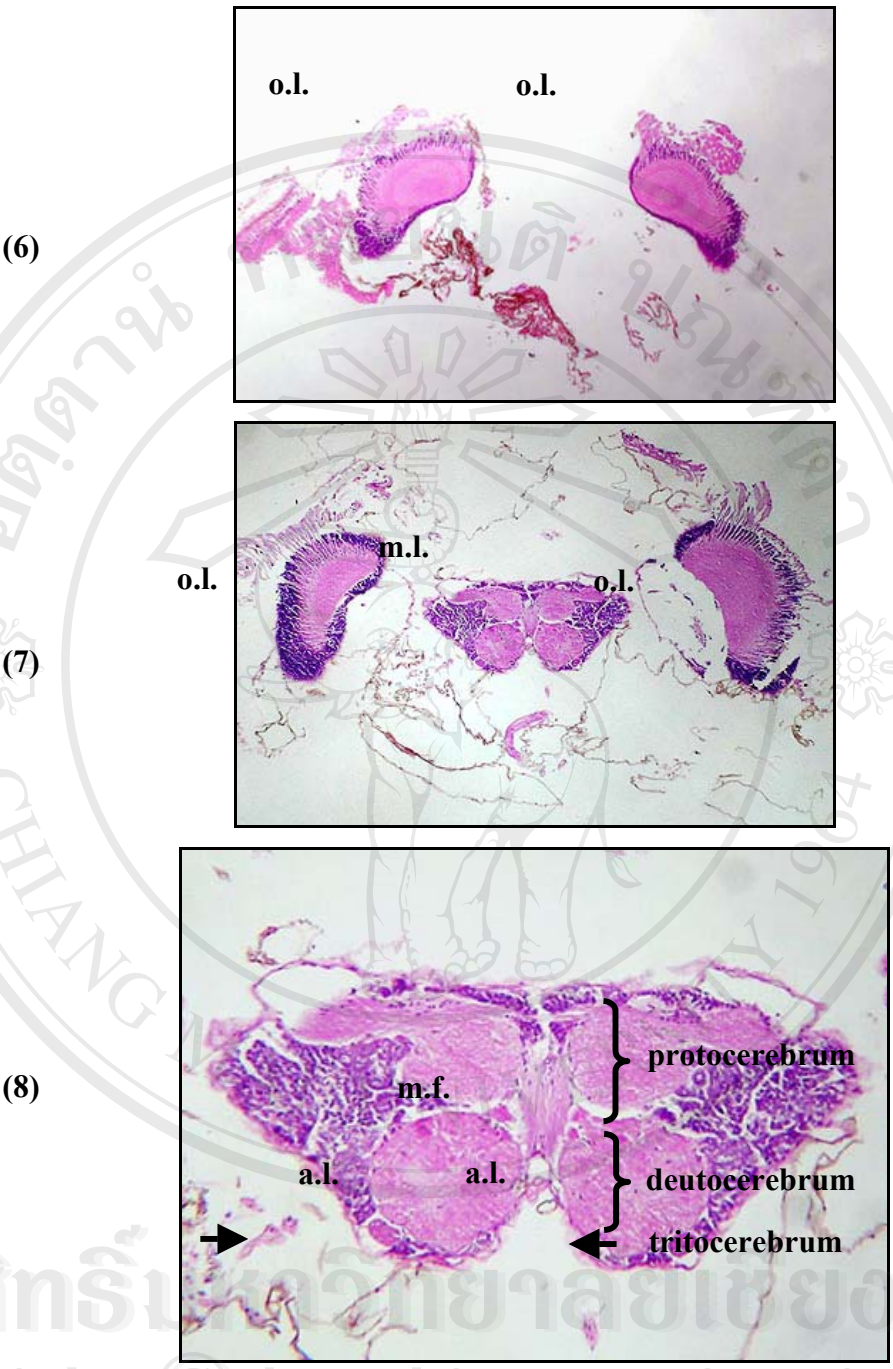



Figure 6-8 Vertical sections of the 3-day-old *C. megacephala* brain at various depths observed under LM at changed magnification. **(6)** 20 µm, 4× **(7)** 44 µm, 4× **(8)** 44 µm, 10× focused on the protocerebrum, deutocerebrum and tritocerebrum (indicate by arrows). The abbreviations are: o.l.= the optic lobe; m.l.= the median lobe; m.f.= the median furrow; a.l.= the antennal lobe.

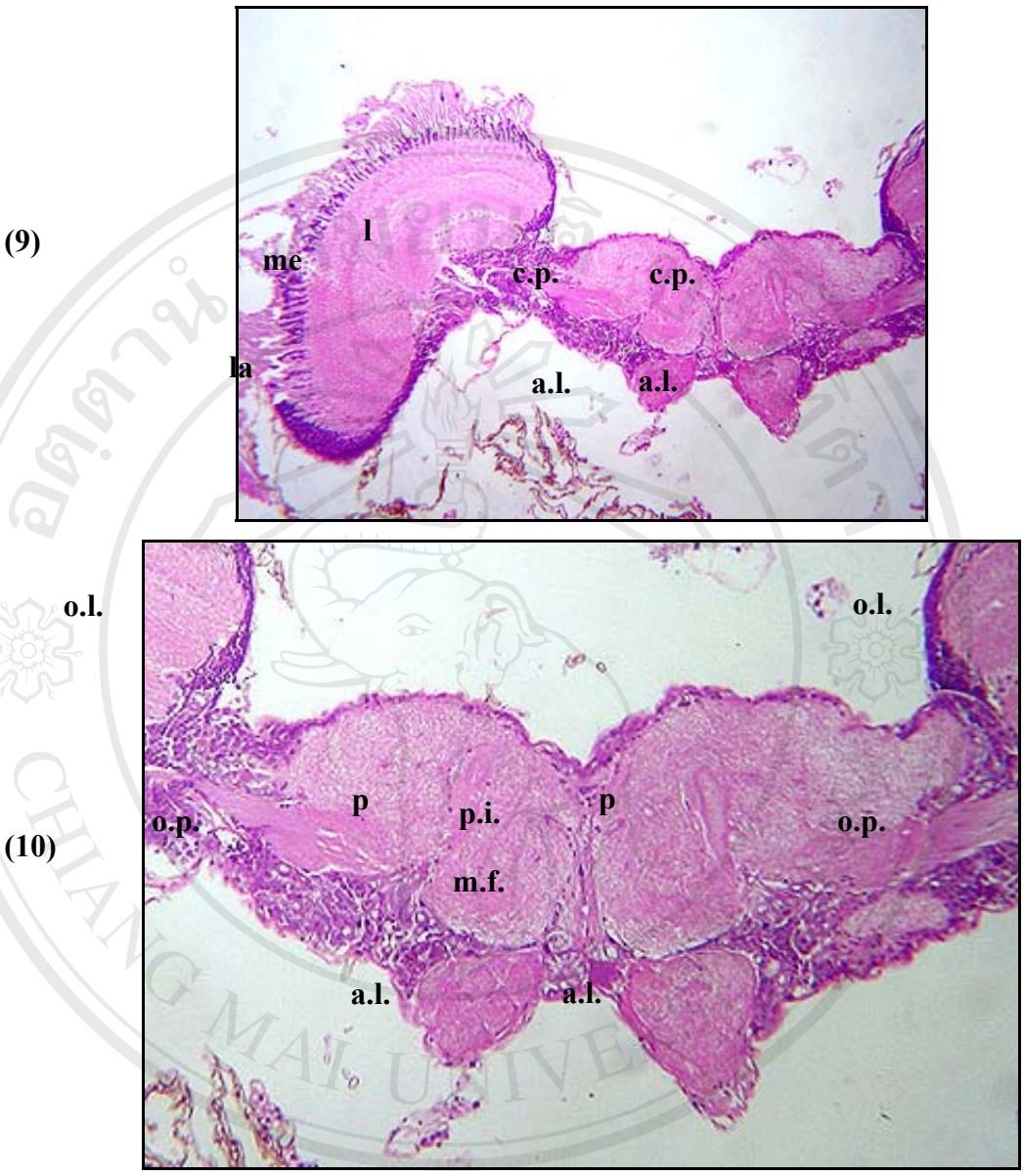



Figure 9-10 An 84 μm vertical section of the 3-day-old *C. megacephala* brain at a magnification of 100. **(9)** Three neuropils of the o.l and the corpora pedunculata of the midbrain **(10)** focused on the corpora pedunculata, with 2 prominent peduncles noted in the center of each neuropil. The abbreviations are: la= the lamina; me= the medulla; l=the lobula; c.p.= the corpora pedunculata; p= the peduncle; p.i.=the pars intercerebralis; o.p.= the optic pedunculus. Other abbreviations, as in Figure 6-8.

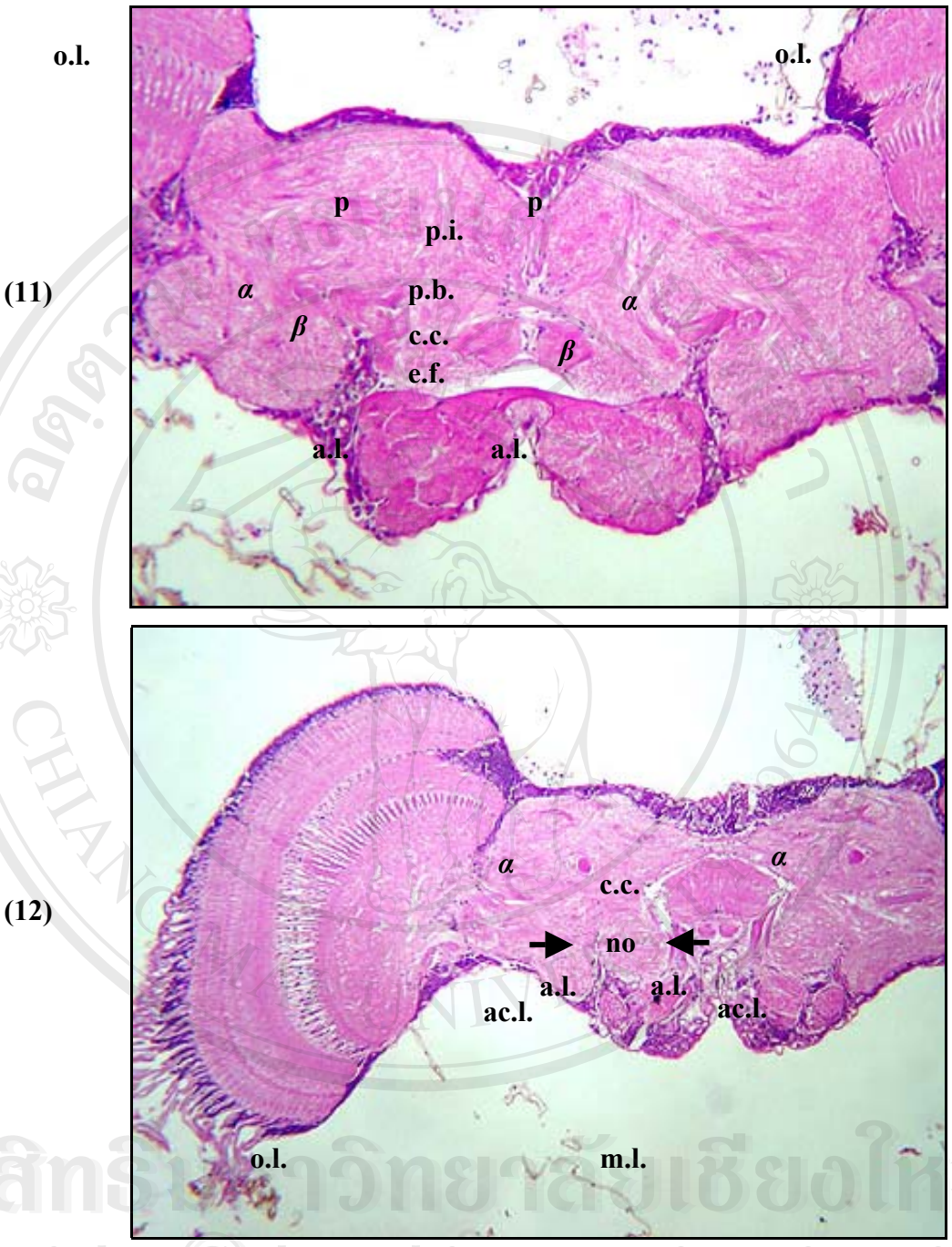



Figure 11-12 Vertical sections of the 3 days old *C. megacephala* brain at a magnification of 100. At depth of **(11)** 116 µm representing the protocerebral neuropils and **(12)** 160 µm showing many nerve tracts (arrows) running from a.l. to c.c. The abbreviations are: p.b.= the protocerebral bridge; c.c.= the central complex; e.f.= the esophageal foramen; *α*= the alpha lobe; *β*= the beta lobe. Other abbreviations, as in Figure 6-10.

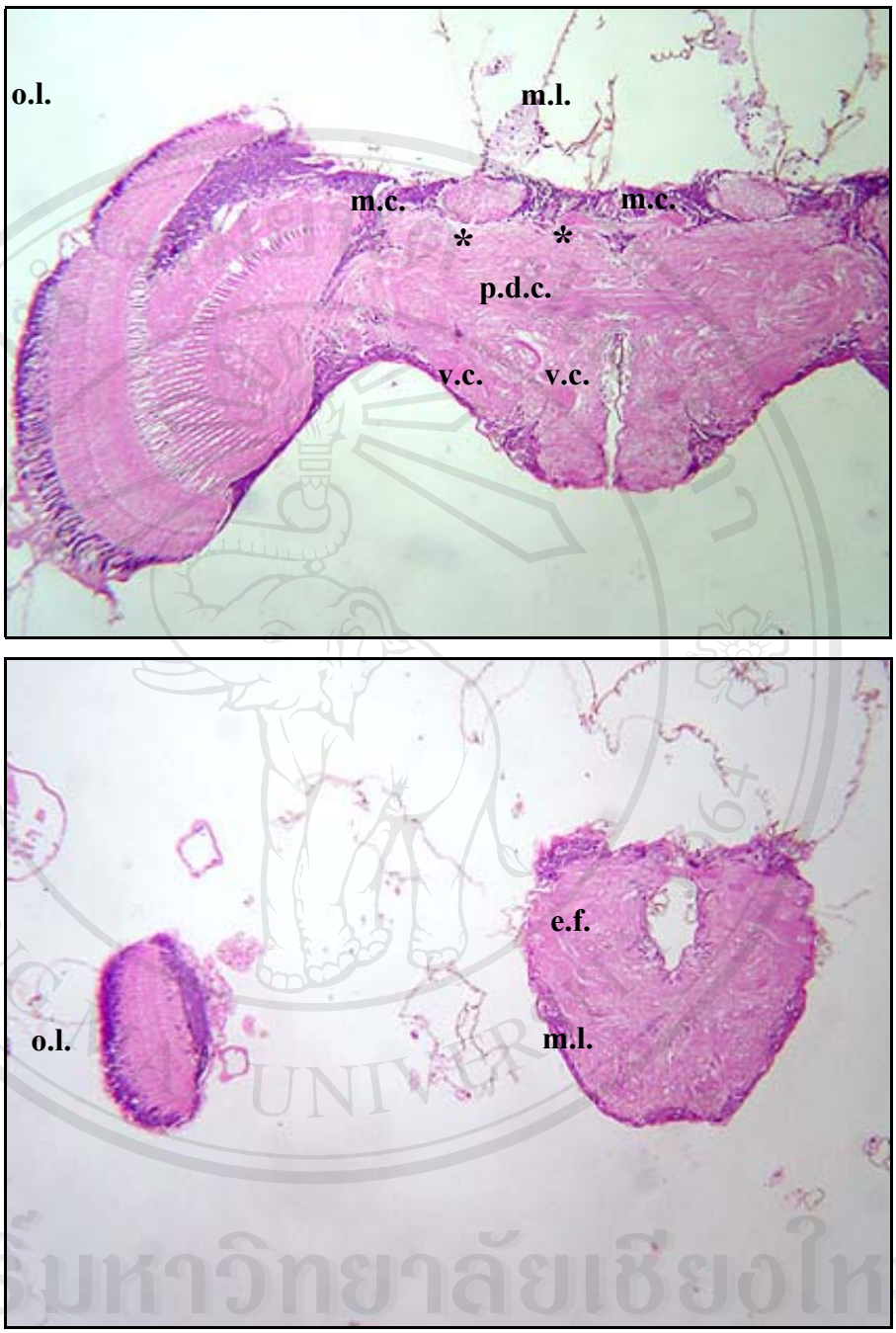



Figure 13-14 Vertical sections of the 3 days old *C. megacephala* brain at a magnification of 100. At depth of **(13)** 188 µm, indicating the lateral neuropils (asterisks) of the protocerebral bridge and **(14)** 204 µm, showing the remains of the brain. The abbreviations are: m.c.= the median calyx; p.d.c.= the posterior deutocerebral commissure; v.c.= the ventral connective. Other abbreviations, as in Figure 6-12.

Horizontal plane

The horizontal plane of the fly's brain was inferior to the vertical plane, since a pile of perikarya in a thick and dense layer covering the brain was not discriminated. The LM photographs of serially horizontal sections of the 3-day-old *C. megacephala* brain at various depths in dorsal- ventral direction are demonstrated in Figure 15-24.

At 32 μm depth: only joined neuropils of the optic lobe were observed at the outward sectioning (Figure 15).

At 88 μm depth: distinctly separated neuropils of the optic lobe were displayed. The striated optic chiasma was also visible (Figure 16).

At 108 μm depth: the optic lobe in horizontal sections comprised the medulla and twin lobula neuropils, but the lamina was definitely absent (Figure 17). Closer examination (Figure 18) revealed the lobula neuropils, which consisted of an anterior lobula complex and a posterior lobula plate. The median lobe in the dorsal part was seen. The median calyces of the mushroom bodies came into view gradually.

At 132 μm depth: more median calyces of the mushroom bodies in this section appeared than at the former depth (108 μm depth); besides, the lateral calyces were distinguished (Figure 19).

At 184 μm depth: the central complex was situated posterior to the beta lobes of the mushroom bodies. A dimmed peduncle was noted (Figure 20).

At 224 μm depth: an obviously median lobe in the middle of horizontal sections was studied (Figure 21). The central complex, a fan-shaped structure, was anterior to the protocerebral bridge. The mushroom bodies, which occupied a

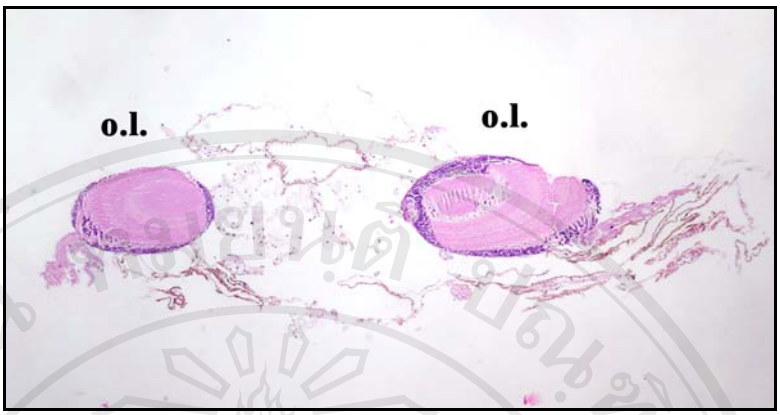
majority of the protocerebral neuropil, exhibited several main components, i.e. two oblique peduncles, two $\beta$ lobes and a well-defined $\alpha$ lobe.

At 312 µm depth: the great anterior commissure of the protocerebrum passed over the central complex (Figure 22). The accessory lobes, antennal lobes and noduli were seen in the anterior ventral part of the brain.

At 356 µm depth: two circular ventral connectives were observed in Figure 23.

At 376 µm depth: the most ventral part of the brain that terminated by the collateral labial nerve roots was notable (Figure 24).

**(15)**




**(16)**


**(17)**

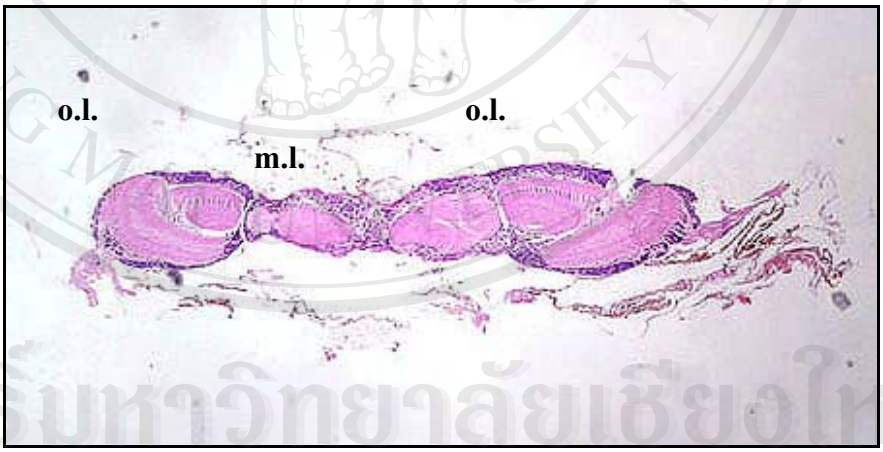



Figure 15-17 Horizontal sections of the 3-day-old *C. megacephala* brain at a magnification of 40 and different depths: **(15)** 32 μm **(16)** 88 μm **(17)** 108 μm. The asterisk indicates the optic chiasma. Other abbreviations, as in Figure 6-14.

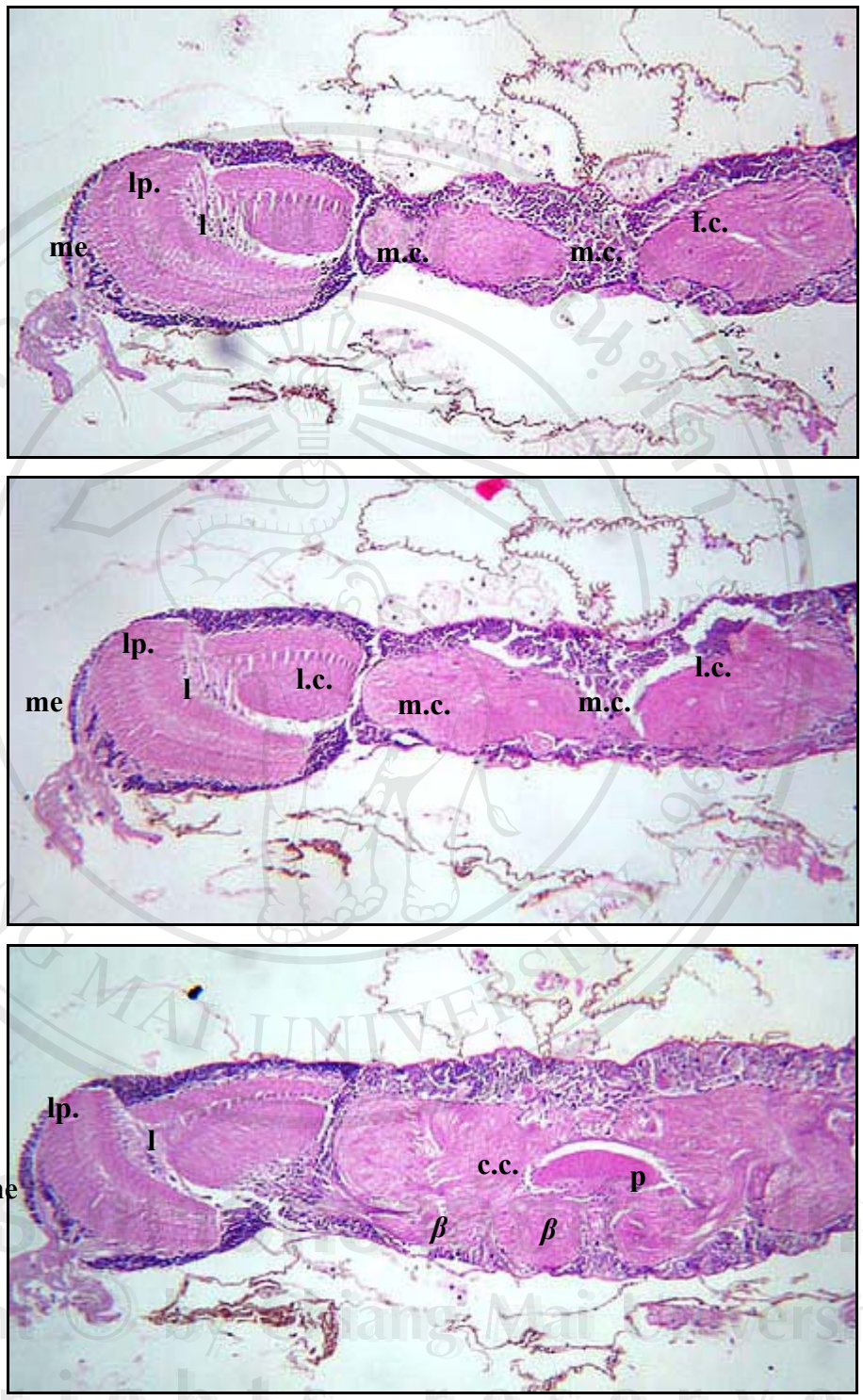



Figure 18-20 Horizontal sections of the 3-day-old *C. megacephala* brain at a magnification of 100 and different depths: **(18)** 108 µm **(19)** 132 µm **(20)** 184 µm. The abbreviations are: lp= the lobula plate; l.c.= the lateral calyx. Other abbreviations, as in Figure 6-14

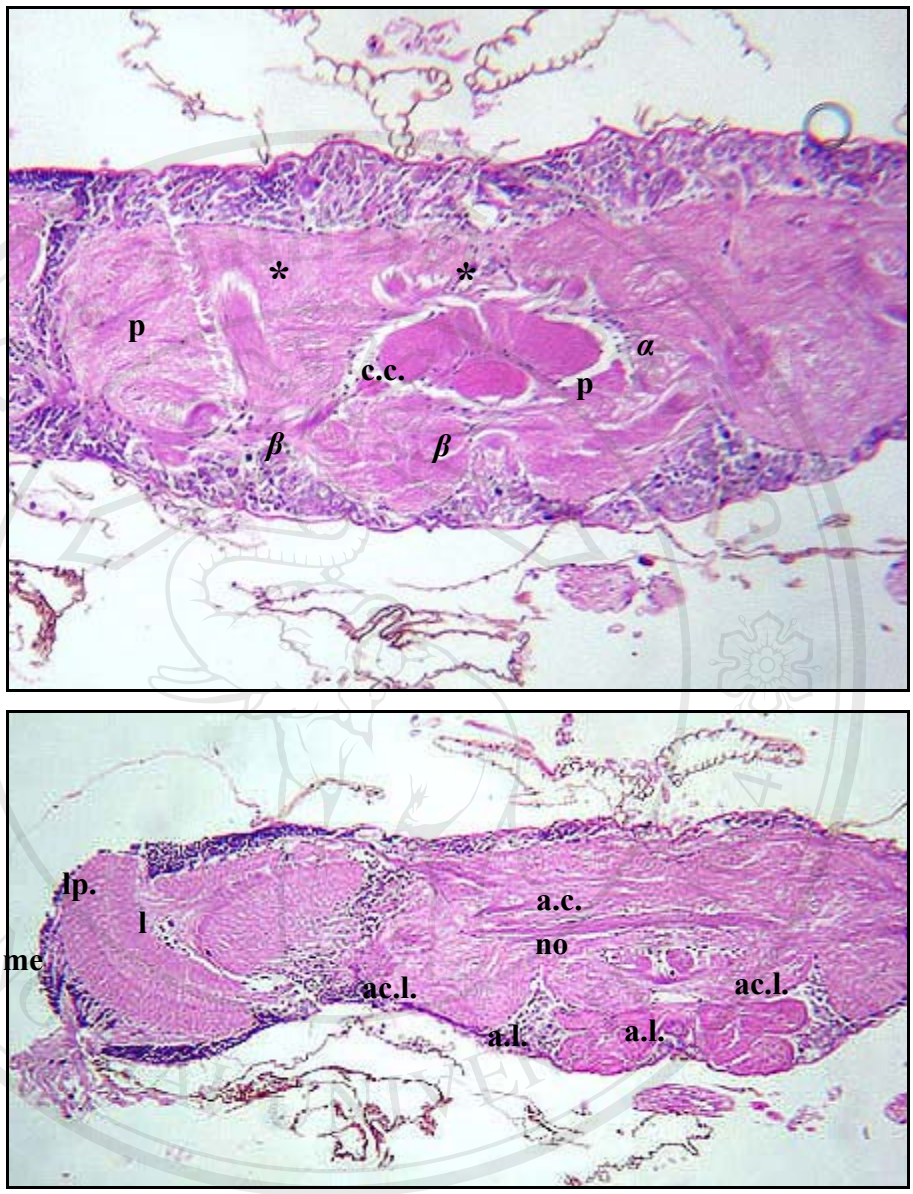



Figure 21-22 Horizontal sections of the 3-day-old *C. megacephala* brain at a magnification of 100 and depth of **(21)** 224 µm, representing two strikingly oblique peduncles (p) and two rod-shaped lateral neuropils of the protocerebral bridge (asterisks), **(22)** 312 µm. The abbreviations are: a.c.= the great anterior commissure. Other abbreviations, as in Figure 6-14.

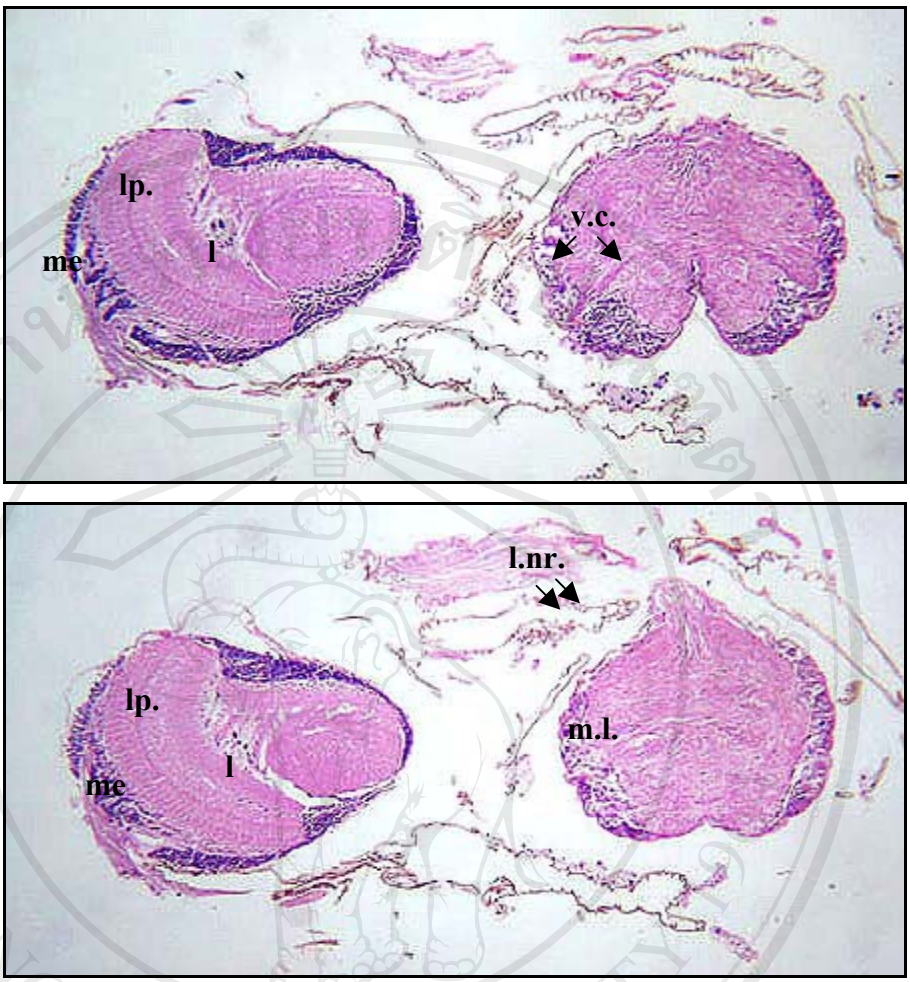
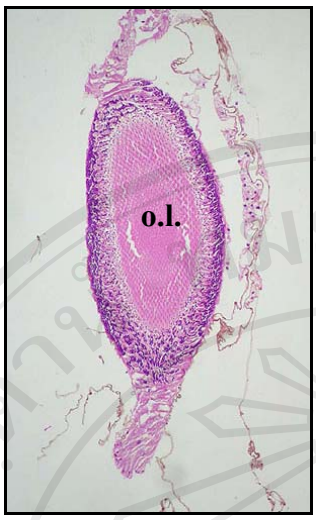


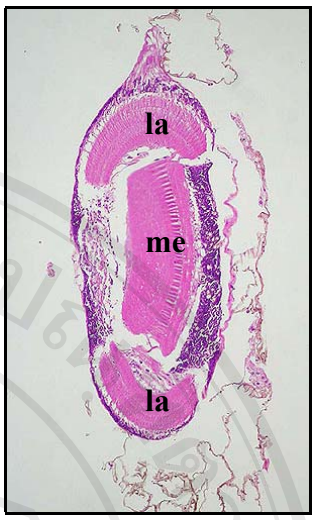

Figure 23-24 Horizontal sections of the 3-day-old *C. megacephala* brain at a magnification of 100 and depth of **(23)** 356 µm, **(24)** 376 µm. The abbreviations are: a.c.= the great anterior commissure; l.nr.= the labial nerve root. Other abbreviations, as in Figure 6-14.

Sagittal plane

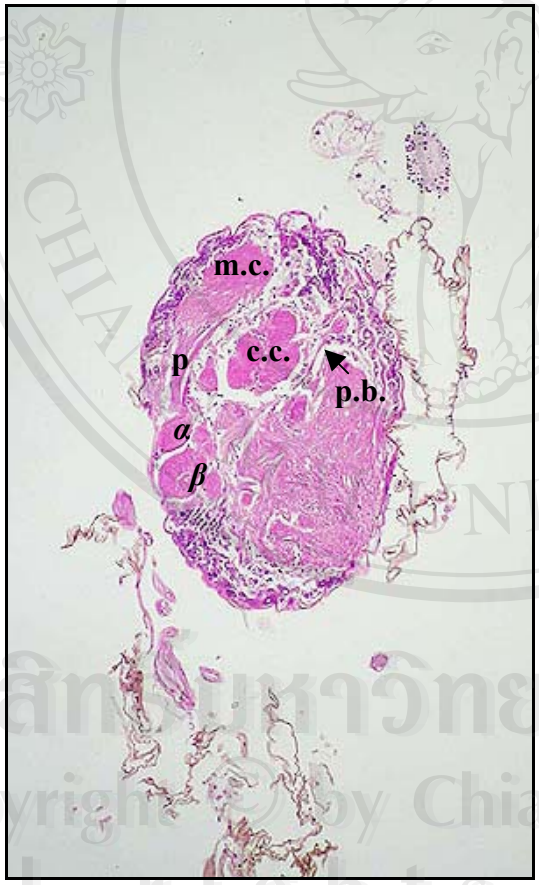
The sagittal sections of the 3 day old *C. megacephala* brain were photographed at different depths under LM before being presented and briefly explained. The 56 μm depth in Figure 25 displayed the optic lobe neuropil, whereas the subsequent 192 μm depth (Figure 26) indicated the central medulla neuropil and two fractional lamina neuropils in both dorsal and ventral parts of the brain. At the deeper region of 524 μm depth, the rod-shaped lateral neuropils of the protocerebral bridge sloped posteriorly across the brain area. The constant central complex as well as the mushroom bodies was easily distinguished. The $\alpha$ lobe extended frontally and the $\beta$ lobe ventrally (Figure 27). These two lobes were connected within the protocerebral neuropil by two short stalks, the peduncles, to the median calyx and lateral calyx, as seen at 704 μm depth, Figure 28. Inclusive information regarding the features of homologous brain neuropils at different depths was mentioned in both vertical and horizontal planes.



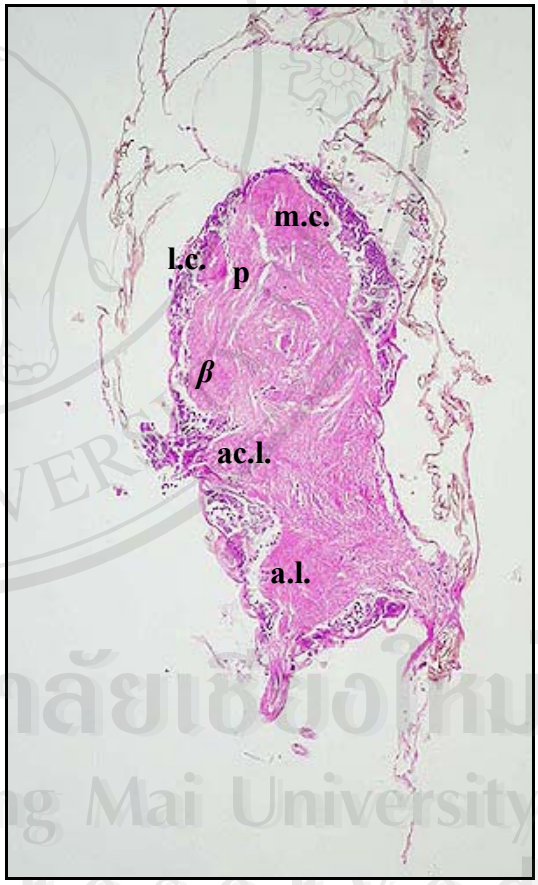

(25)




(26)




(27)




(28)



Figure 25-28 Sagittal sections of the 3-day-old *C. megacephala* brain at a magnification of 100 and different depths of **(25)** 56 µm **(26)** 192 µm **(27)** 524 µm **(28)** 704 µm. Other abbreviations, as in Figure 6-24.

1.2.3 Pathological changes in the 30-day-old *C. megacephala* brain

All three planes of the *C. megacephala* brain section were examined at young and old ages by hematoxylin-eosin staining under LM, as seen in Figure 29-38. In young flies, a few abnormalities were detected at various depth sections and the neuropilar mass of their brain. Pathological changes were totally (100%) found in the old flies, whereas different patterns of age-dependent brain degeneration were also determined. Thoroughly loose neuropils of the whole brain, with extremely stretched optic chiasmas of the optic lobe, were observed. The major midbrain neuropils such as the protocerebral bridge or calyx of the mushroom bodies could not be distinguished; besides, they seemingly declined in content. A number of cavities apart from the brain outline were occasionally visible (Figure 29-31). The most outstanding feature of brain degeneration was the spread of vacuolization in aged flies, which occurred in all three section planes (Figure 32-38). Deteriorated neuropils appeared, particularly in the lamina region (Figure 32-33), medulla region (Figure 36), and lobula region (Figure 37), and they were also evident in the median lobe (Figure 34 and 38).

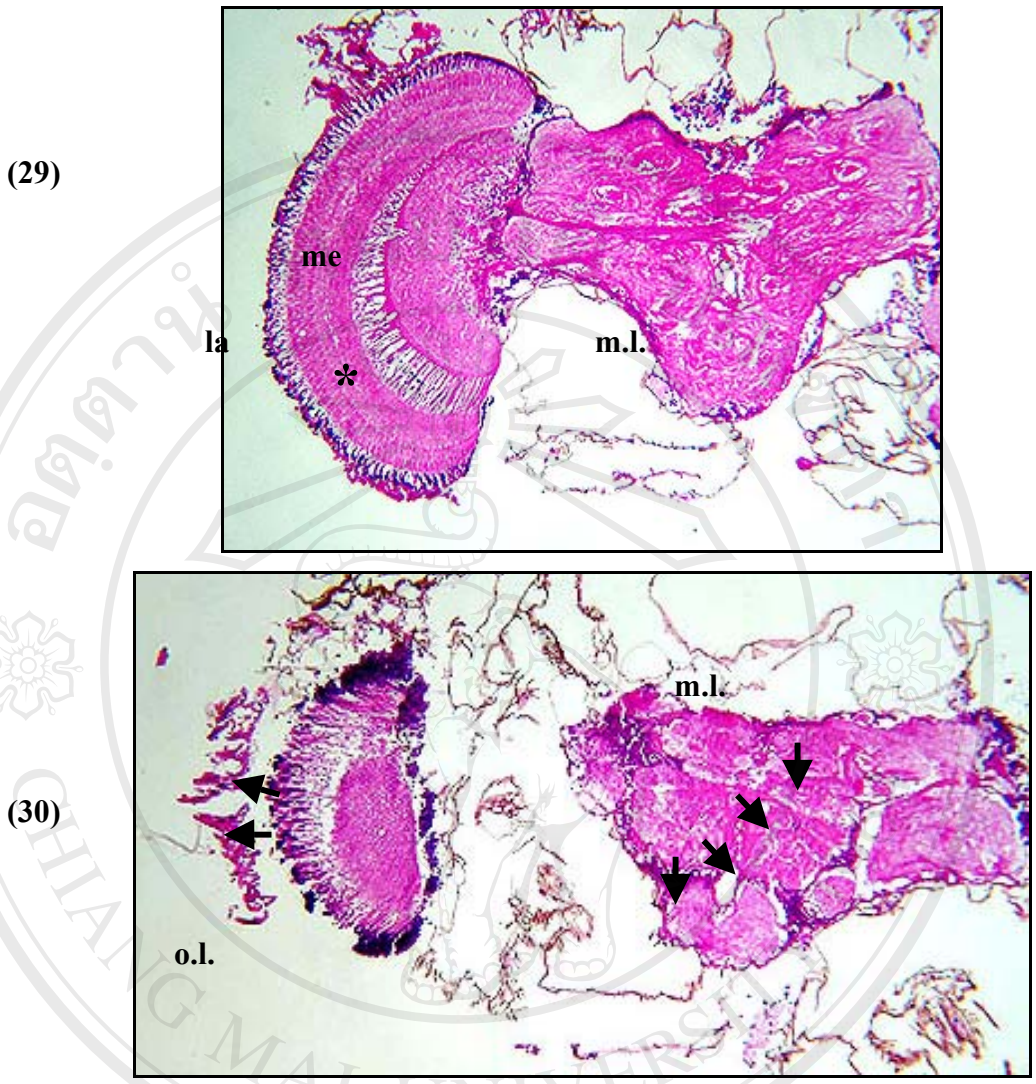
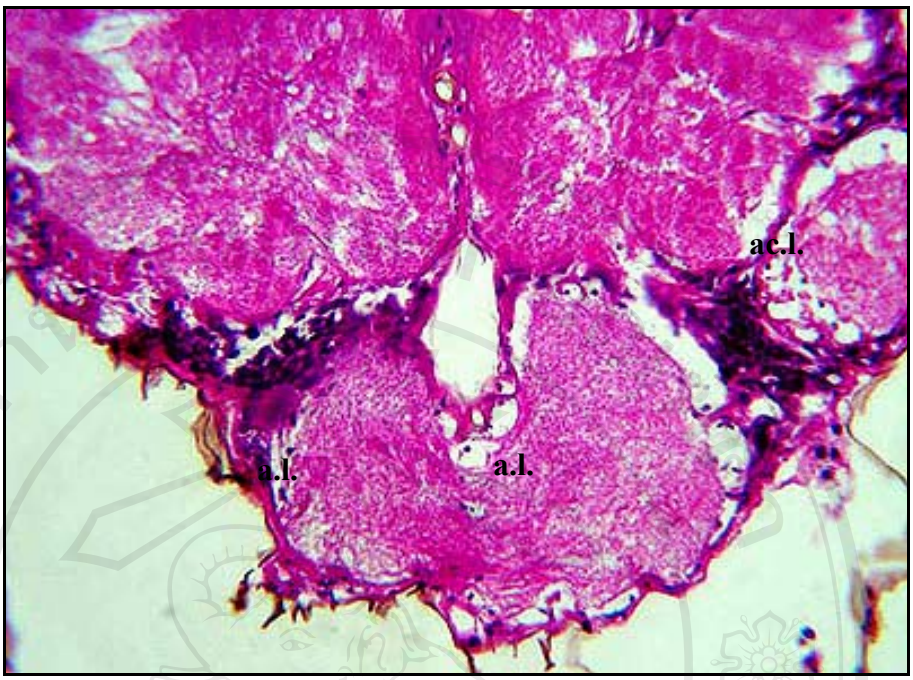



Figure 29-30 Pathological characteristics in the 30-day-old *C. megacephala* brain under LM at a magnification of 100. The loose brain neuropils widely occurred. On the vertical plane at **(29)** 184 µm depth, the stretched optic chiasma was noted (asterisk). **(30)** At 212 µm depth, distinct cavities were indicated by arrows. Other abbreviations, as in Figure 6-14.

**(31)**




**(32)**

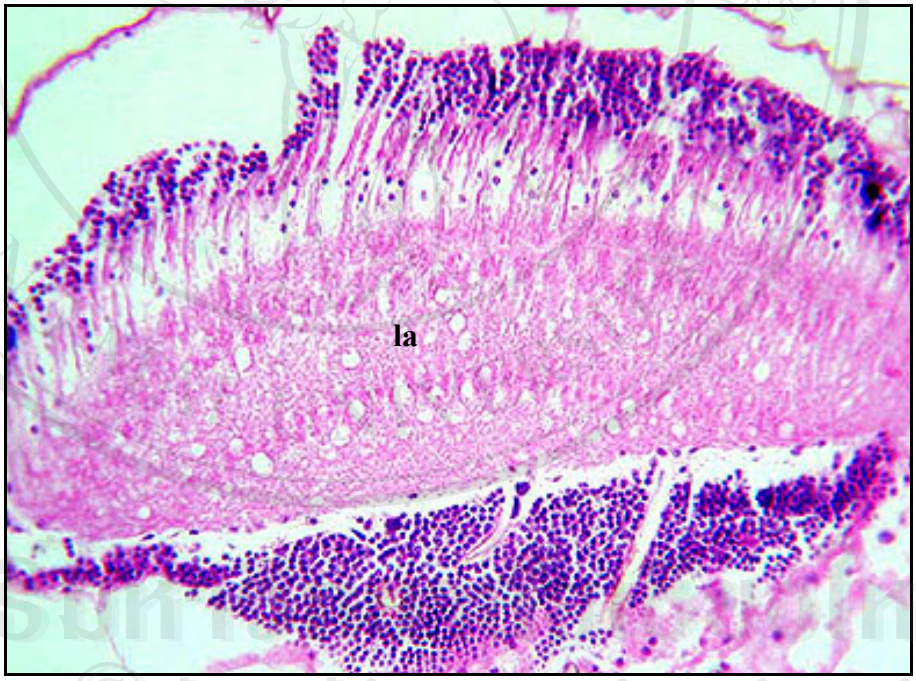
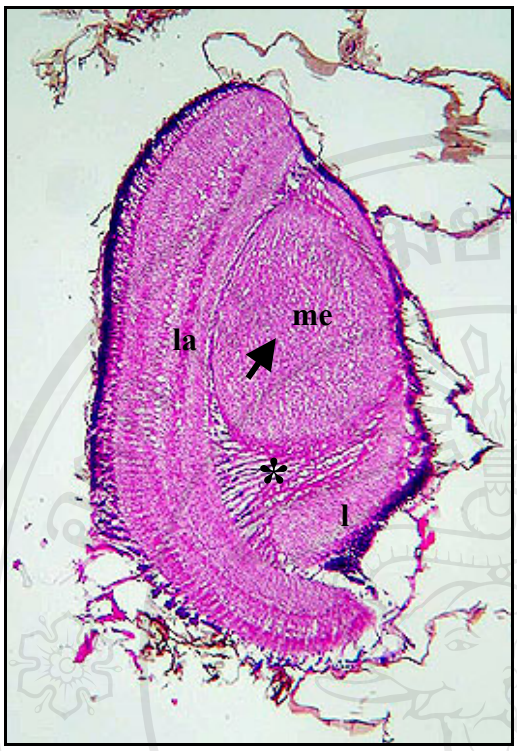
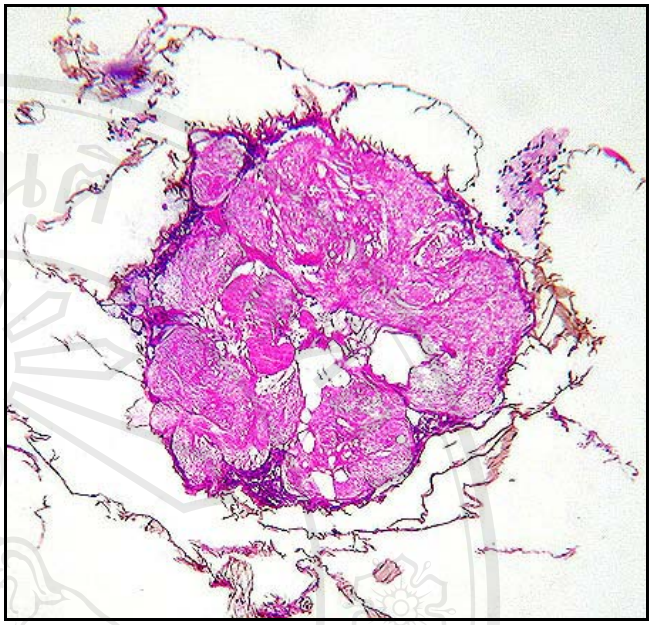



Figure 31-32 Pathological characteristics in the 30-day-old *C. megacephala* brain in the vertical plane under LM at a magnification of 400. The distinct vacuolization widely occurred at **(31)** 212 µm depth; focused on the degenerated antennal lobes, and **(32)** at 208 µm depth; scattered vacuolization in the lamina region was noted. Other abbreviations, as in Figure 6-14.

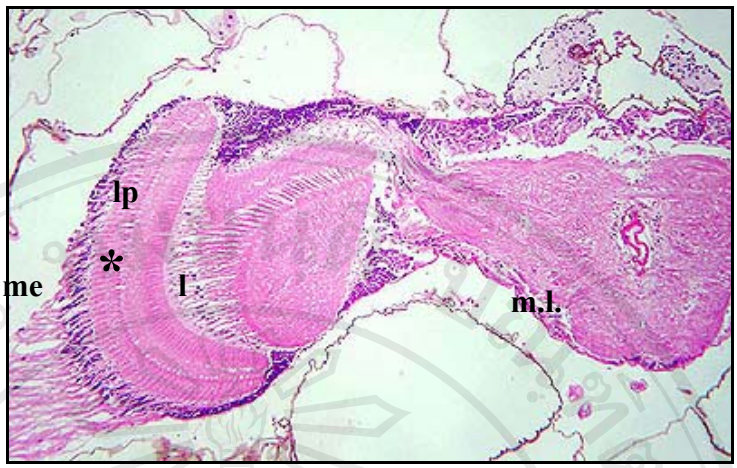


**(33)** **(34)**

Figure 33-34 Pathological characteristics observed in the 30-day-old *C. megacephala* brain in the sagittal plane. Using a different magnification of 100: **(33)** at 104 µm depth; the stretched optic chiasma was noted (asterisk), **(34)** at 472 µm depth; the deteriorated central complex (arrow) and a few large vacuoles inside the midbrain were indicated. Other abbreviations, as in Figure 6-14.

**(35)**




**(36)**

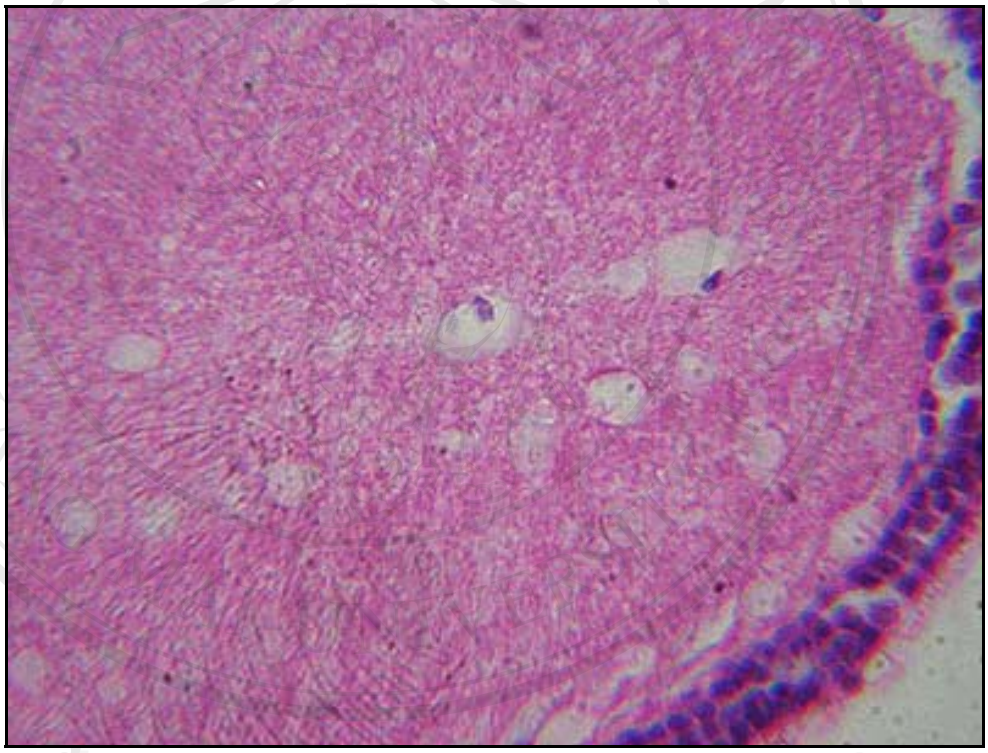
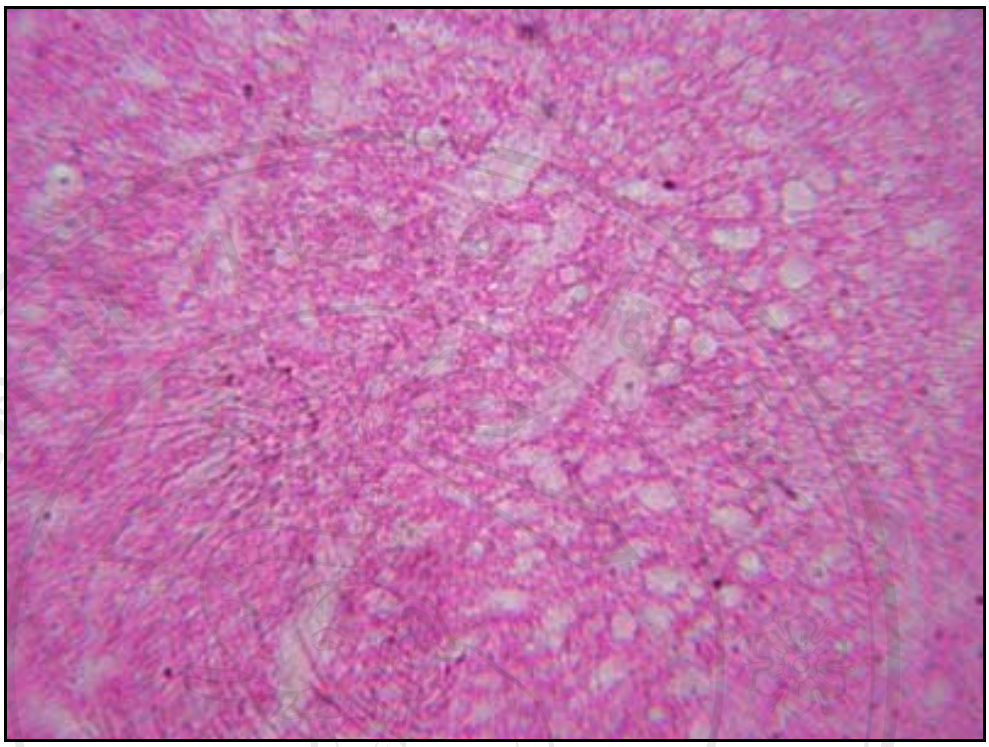


Figure 35-36 Pathological characteristics in the 30-day-old *C. megacephala* brain, observed at 220 µm depth in the horizontal section using a changed magnification of **(35)** 100; the stretched optic chiasma was noted (asterisk) in the optic lobe, **(36)** and at 1,000; a number of vacuoles in the medulla region was seen. Other abbreviations, as in Figure 6-14.

**(37)**



**(38)**

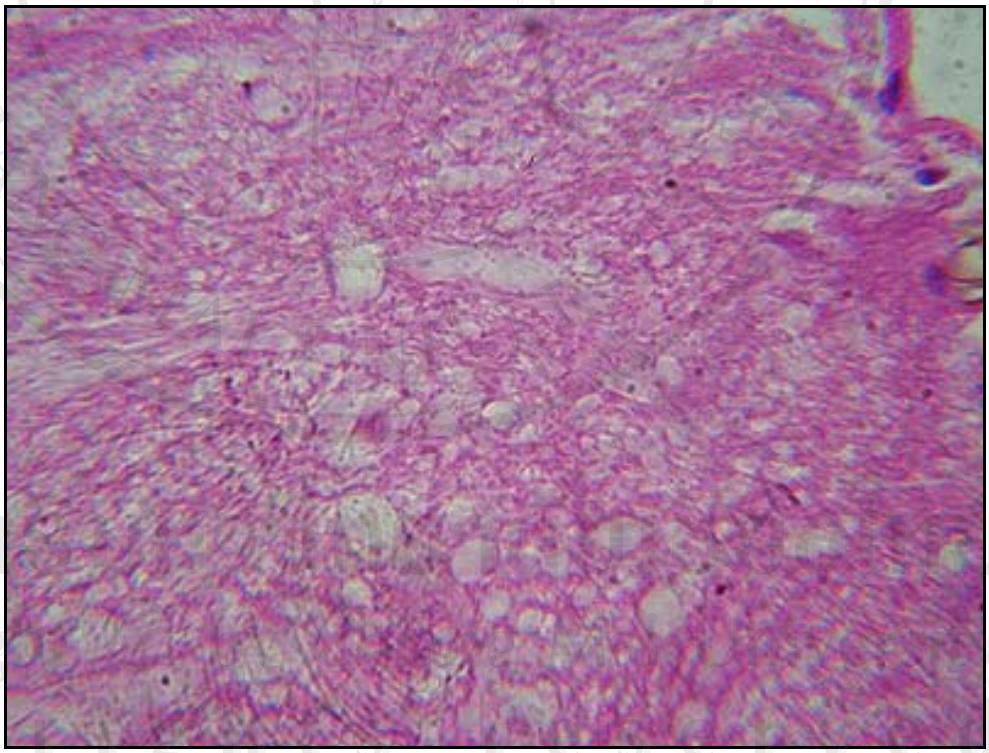




Figure 37-38 Distributed vacuolization in the 30-day-old *C. megacephala* brain focusing at 220 µm depth in the horizontal section at a magnification of 1,000 on: **(37)** the lobula neuropil and **(38)** the midbrain neuropil.

1.2.4 Nerve cell structure

The nerve cell bodies were situated in the peripheral region of the brain neuropils. Those perikarya showed a range of diameters and different cell characteristics, which could be classified. Estimation of the perikarya diameter for each type is shown in Figure 39. The Students'*t* test revealed a significant size difference (*P*=0.000) for all neuron types and the glial cell. Apart from glial cell, three types of neuron were found in the *C. megacephala* brain. Type 1 neurons had a small spherical cell body. The cell body was ≈4-6 μm in diameter and occupied mostly by a dense round nucleus (Figure 40). Besides the same size in diameter, the cell body of the Type 2 neuron differed from Type 1 in the density of the nucleus. The major characteristic of this type was a loose round nucleus with some dense granules distributed inside, which occupied a large proportion of the cell body (Figure 41). The Type 3 neuron (Figure 42) was easily categorized, since it was the biggest one (≈7-10 μm in diameter) and had a vacuolated nucleus that possessed the whole cell body. Another distinct cell found in the fly brain was the glial cell. It was 15-20 μm in diameter with a giant oval appearance. The cytoplasm was sponging, while a single spherical or slightly elongated nucleus measuring approximately 9-10 μm in diameter (data not shown) had a considerably centric dense round nucleolus (Figure 43). The glial cell, and Type 1 and Type 2 neurons were usually seen in all locations of the brain. The Type 3 neuron was found at location 6 and 8, especially in the sectioning depth level 2 and 3 (≈90-170 μm) of the vertical plane. The distinguishing appearance of the glial cell and three types of neuron is shown in Figure 44-46.

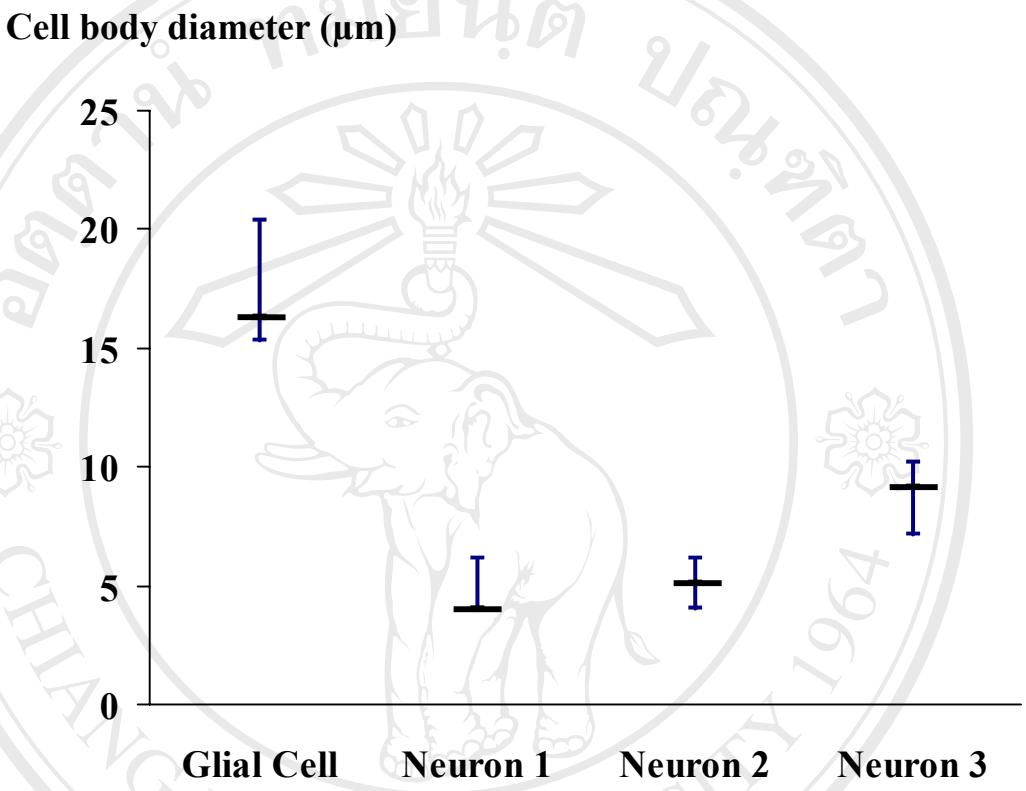
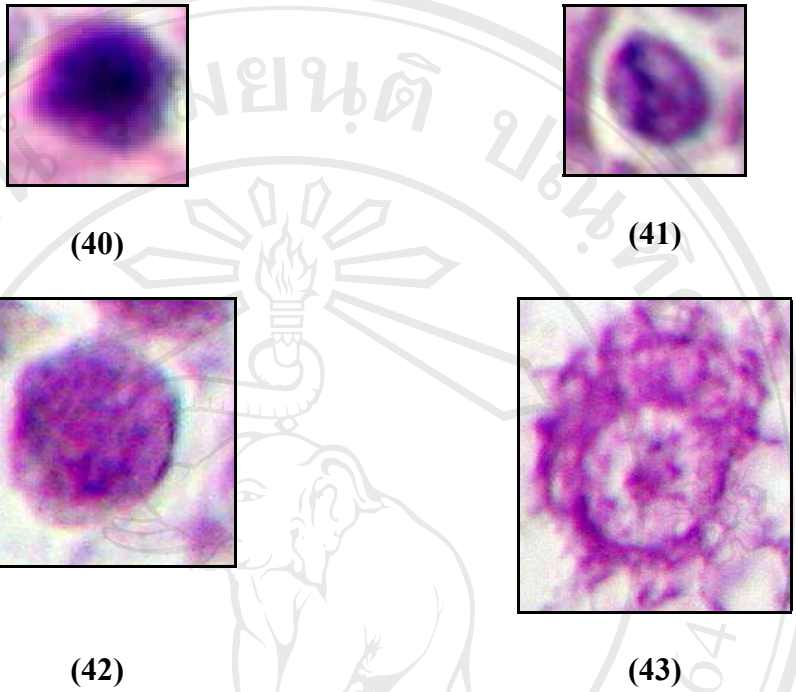



Figure 39 Cell body diameter (µm) of all 3 neuron types and the glial cell (n = 100 for each group). The horizontal middle thick line represents the median value, while the horizontal minor thin lines indicate the range of each group.



**(40)** **(41)**

**(42)** **(43)**

Figure 40-43 Glial cell and the 3 types of perikarya found in the *C. megacephala* brain at a magnification of 1,000. **(40)** Type 1 neuron **(41)** Type 2 neuron **(42)** Type 3 neuron **(43)** glial cell.

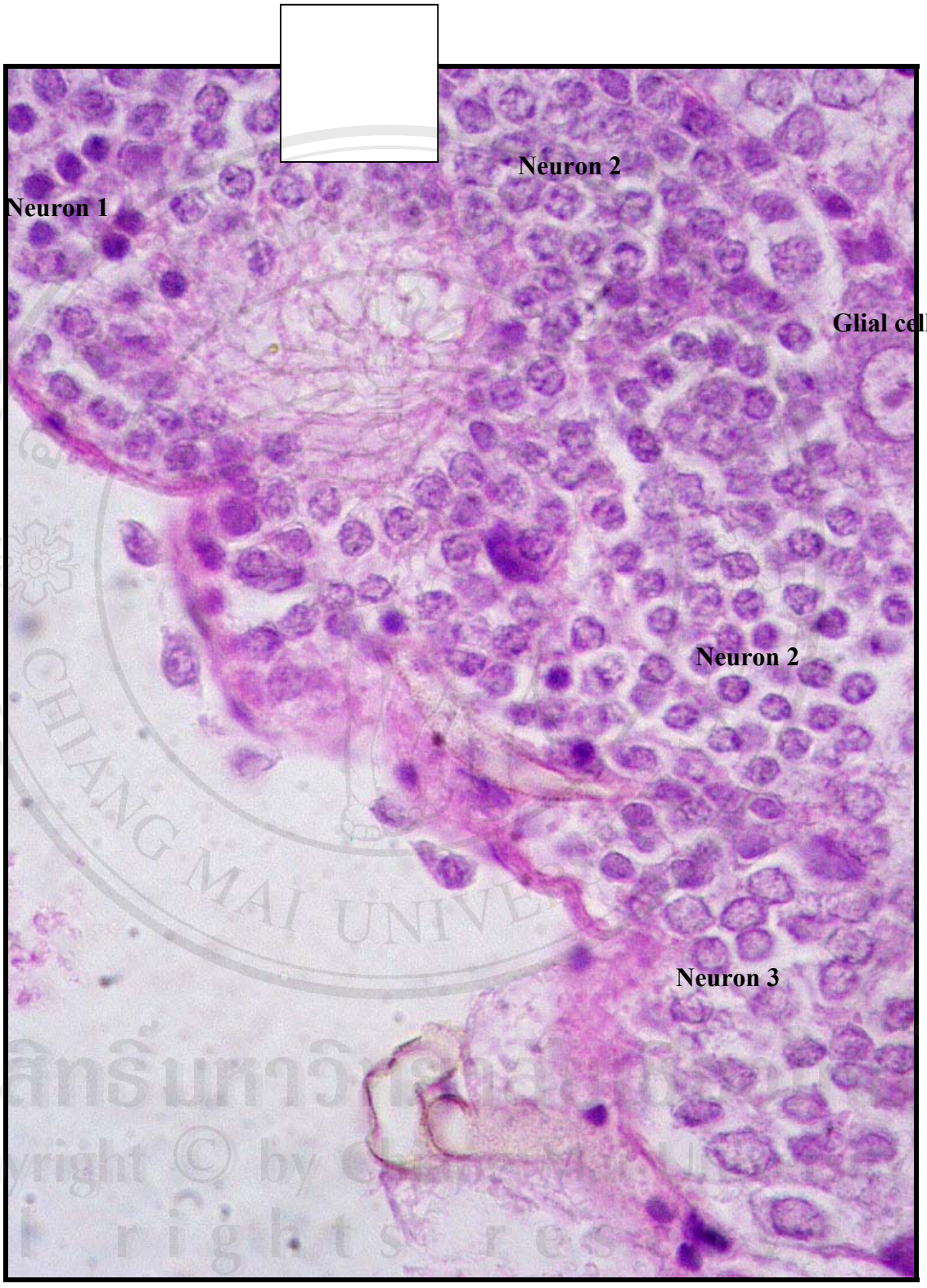
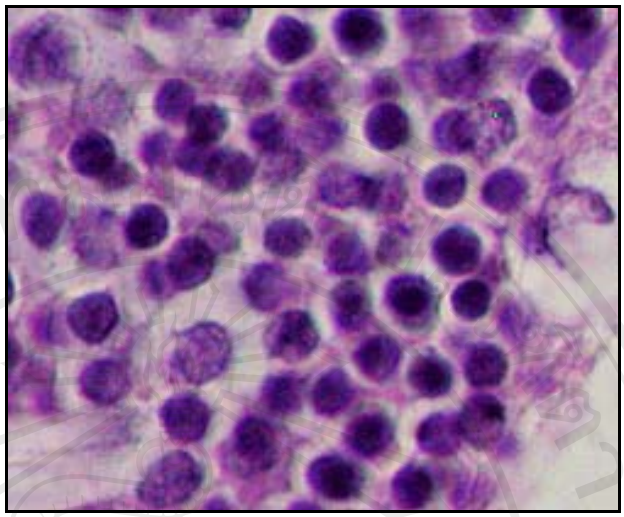



Figure 44 Glial cell and the 3 types of perikarya found at location 6 of the *C. megacephala* brain under LM at a magnification of 1,000.

**(45)**



**(46)**

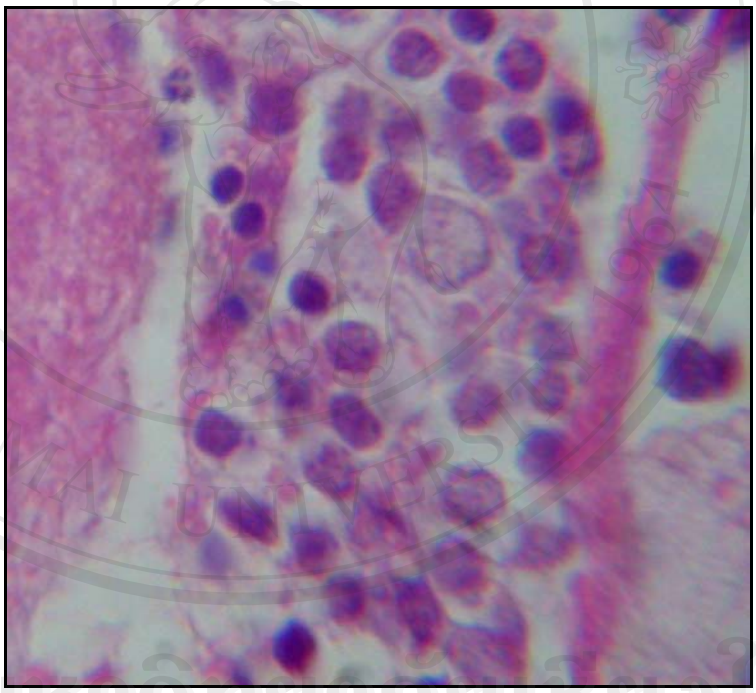




Figure 45-46 Different perikarya in the *C. megacephala* brain under LM at a magnification of 1,000. **(45)** Type 1 and Type 2 neuron at location 2 **(46)** Type 1, Type 2 and Type 3 neuron at location 8.

**2. Transmission electron microscope (TEM) study**

The brain of the young blowfly species, *C. megacephala* was observed precisely using both JEM 2010 (JEOL®) and EM10 (ZEISS®) TEM. The fine structure of the fly's neuron was typical. It was monopolar, as there was no dendrite arising from the nerve cell bodies. The neuronal perikarya were utterly surrounded by glial cells, which often sent their cytoplasmic processes into the peripheral cytoplasm of the large nerve cell bodies. They contained considerable arrays of rough endoplasmic reticulum, some Golgi complexes, numerous mitochondria and a number of dense bodies in their cytoplasm.

Corresponding to the LM observation, at least three types of neuron were recognized and designed as Type 1, Type 2 and Type 3. The differences between cell types in this study, which was based on variations in size and nucleus structure, were discussed. The Type 1 neuron was relatively small and generally polygonal in shape. The nucleus of this type applied to a large proportion of the cell body. Predominant chromatin showed several randomly scattered dense patches (Figure 47). The Type 2 neuron was the smallest one. A round nucleus had the chromatin raised in both dense and diffused form (Figure 48). In contrast to the Type 1 and Type 2 neuron, Type 3 was developed better, with the large oval nucleus having chromatin primarily diffused with rare dense patches (Figure 49).

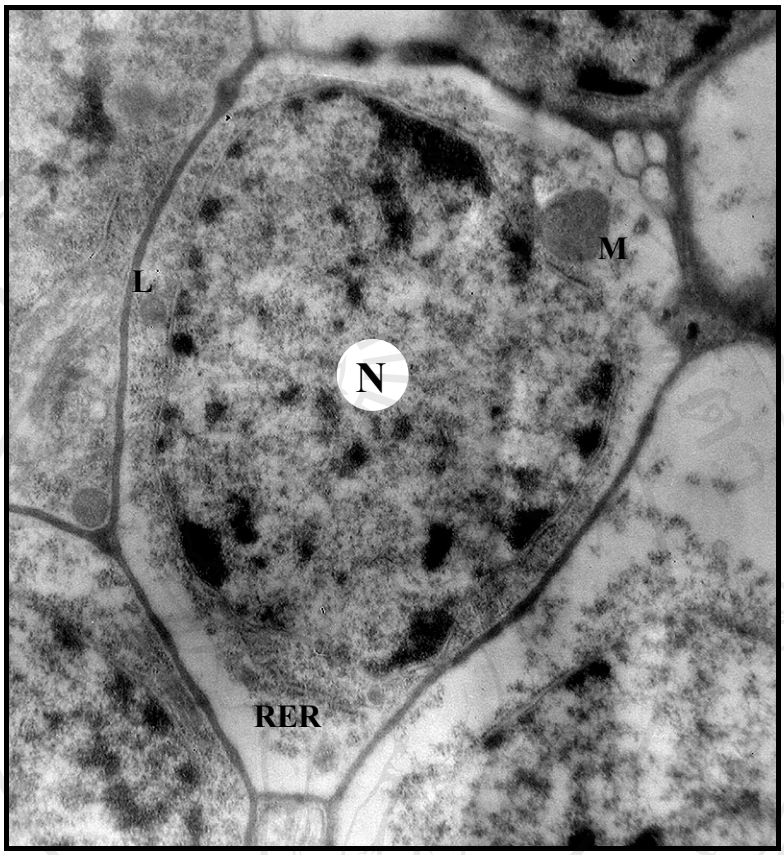



Figure 47 TEM micrograph (ZEISS®) of the Type 1 neuron at a magnification of 12,000. N= nucleus; M= mitochondria; RER= rough endoplasmic reticulum; L= lipid droplet.

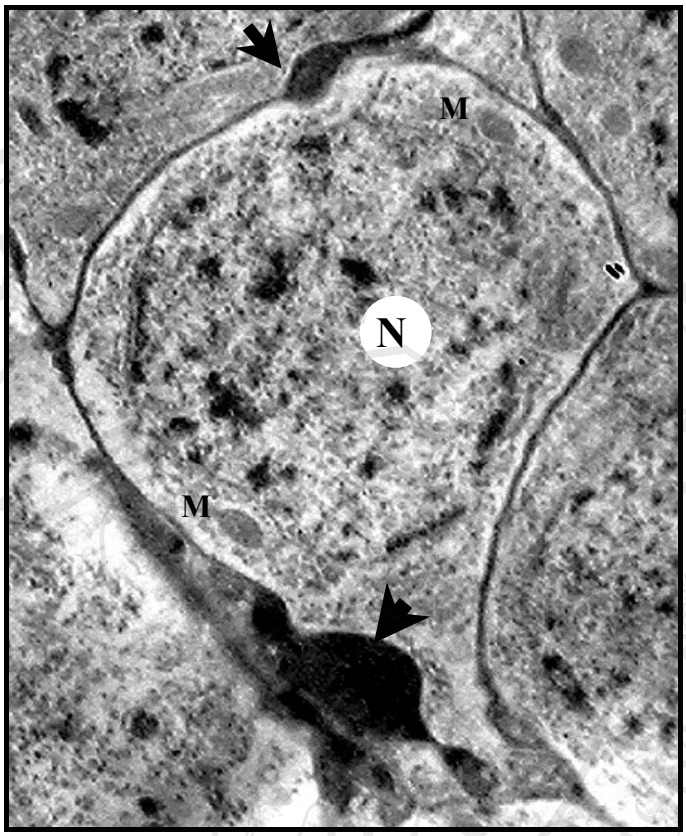



Figure 48 TEM micrograph (ZEISS®) of Type 2 neuron at a magnification of 11650. N= nucleus; M= mitochondria. Noted the electron dense material (arrows) lying in the distended extra cellular space.

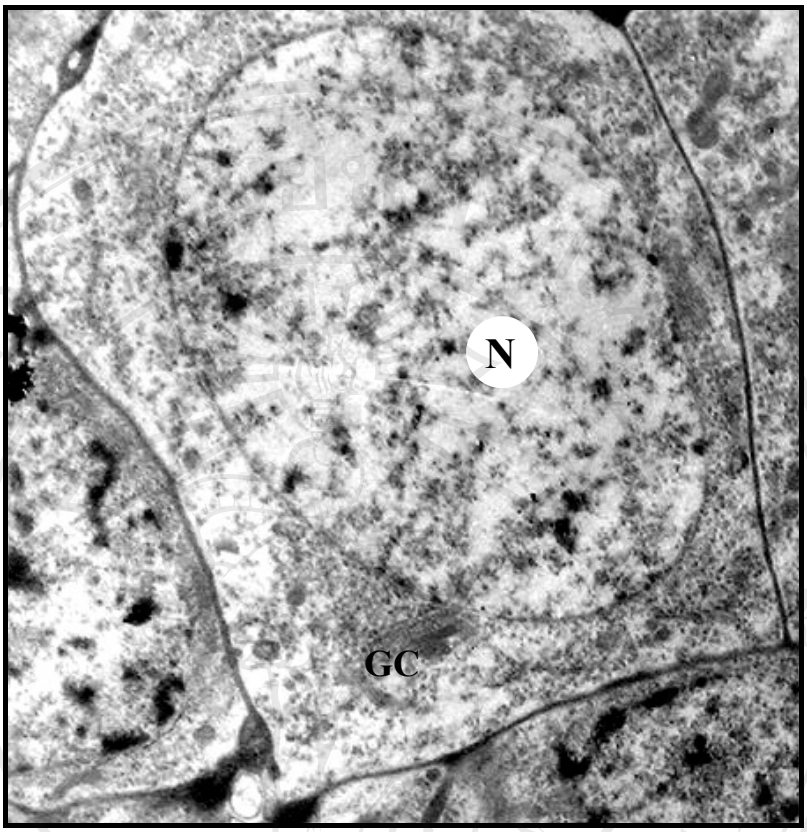



Figure 49 TEM micrograph (ZEISS®) of the Type 3 neuron at a magnification of 13,750. N= nucleus; GC= Golgi complex.

For elucidating details of the intracellular structure of the fly's neuron, sections through the optic lobe were determined, as seen in Figure 50-54. Golgi complexes were composed of typically stacked saccules with associated vacuoles and vesicles, while a distended rough endoplasmic reticulum was exhibited in the single isolated profiles, and not the convoluted profiles seen in vertebrate neurons (Figure 50). Even though the mitochondria often showed structural variations in different intercellular regions (Figure 51), all of them consisted of closely spaced cristae. Glial cells that lay around the axons were also visible. The axon process was likely to lack any organelle, save for mitochondria, and neurotubules (Figure 52). The glial cell cytoplasm tended to be more electron opaque than that of the neurons; hence, the glial cells were often immediately distinguishable from the nervous tissues they ensheathed. Endoplasmic reticulum and mitochondria were observed around the nucleus in the glial cell body (Figure 53). Other outstanding features of the fly's brain seen in the TEM section were a large quantity of tracheoles, which carried oxygen to the brain (Figure 54).

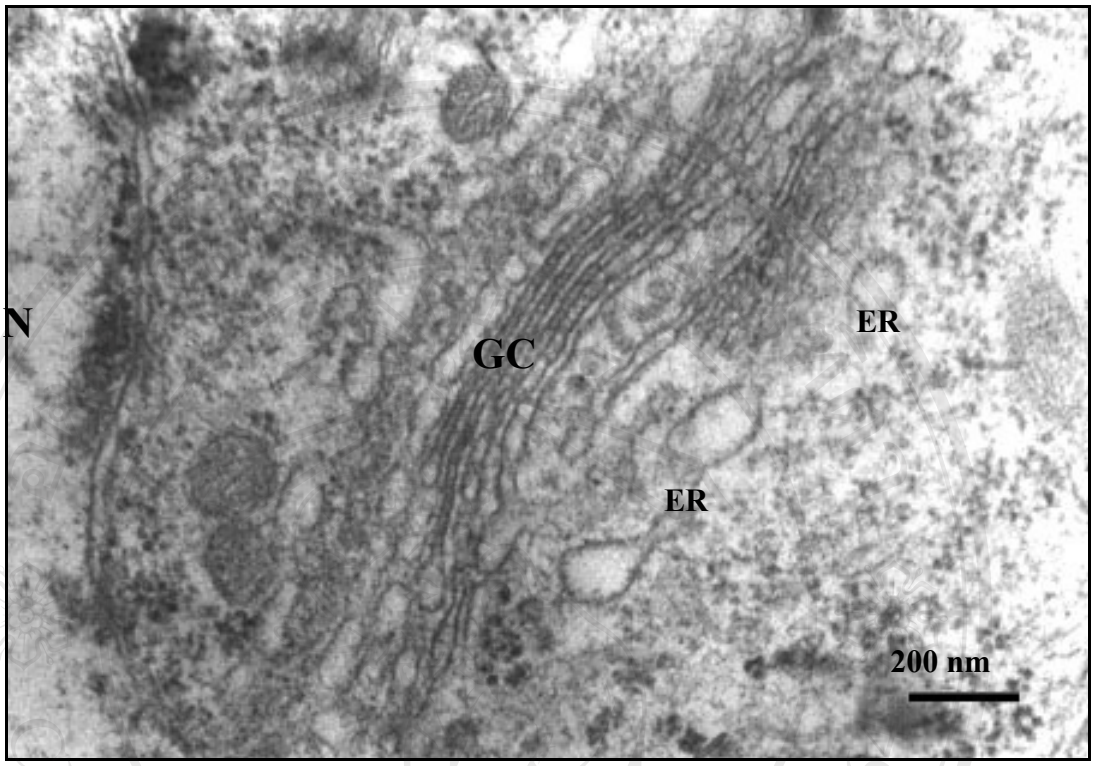



Figure 50 Intracellular organelles of the fly's neuron at a magnification of 30,000. N= nucleus; GC= Golgi complex; ER= endoplasmic reticulum.

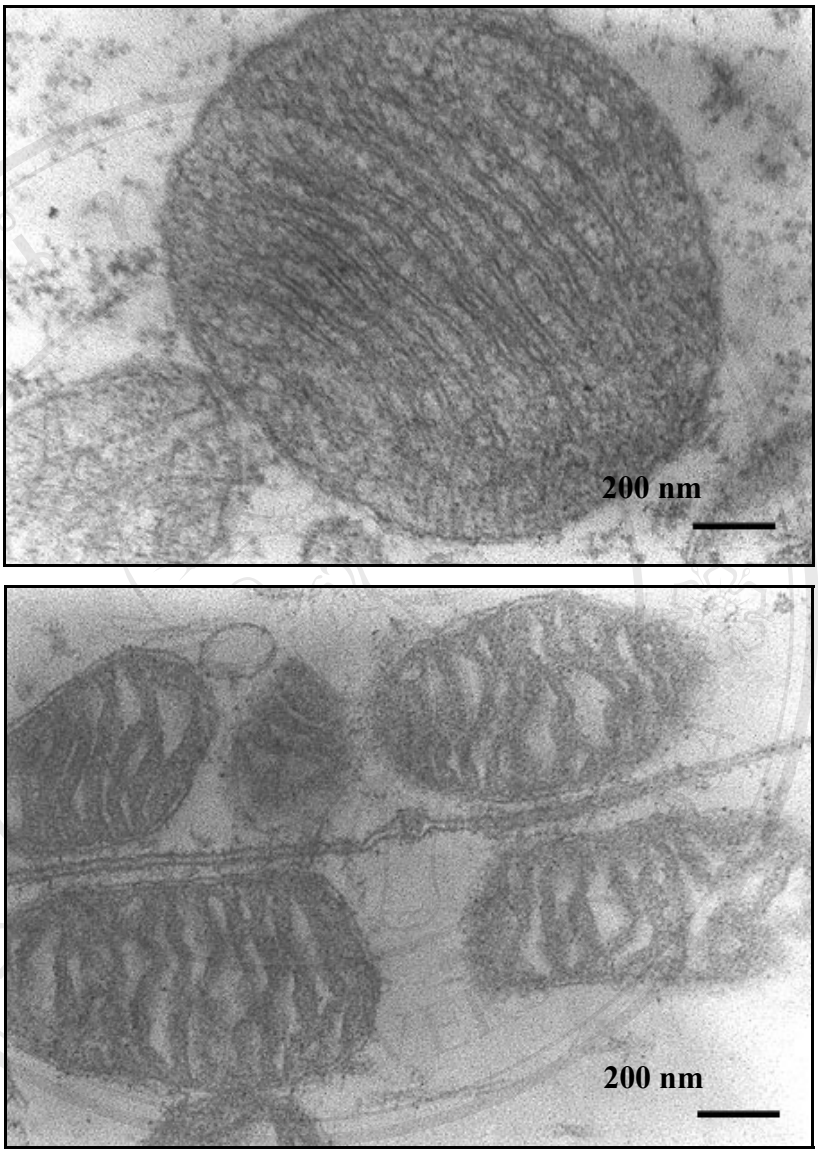



Figure 51 Structural alterations of mitochondria at a magnification of 40,000. **(a)** Spherical mitochondria in the Type 1 neuron. **(b)** A group of elongate mitochondria in the glial cell.

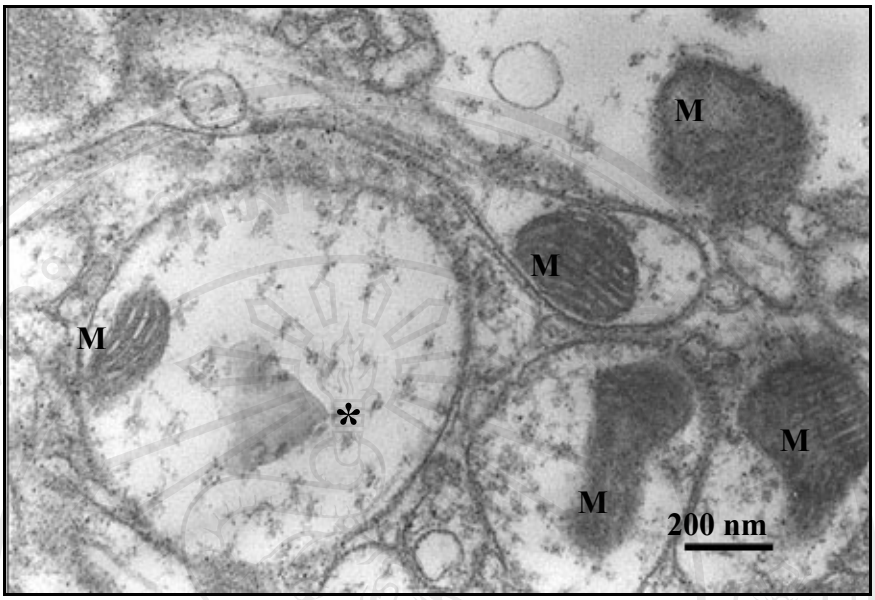



Figure 52 Axons showing size variations. The axonal neurotubules (indicated by the asterisk) were oriented parallel to the longitudinal axis of the axonal process and were thus seen in the section. Some mitochondria situated inside the axons were noted. A= axon; M=mitochondria.

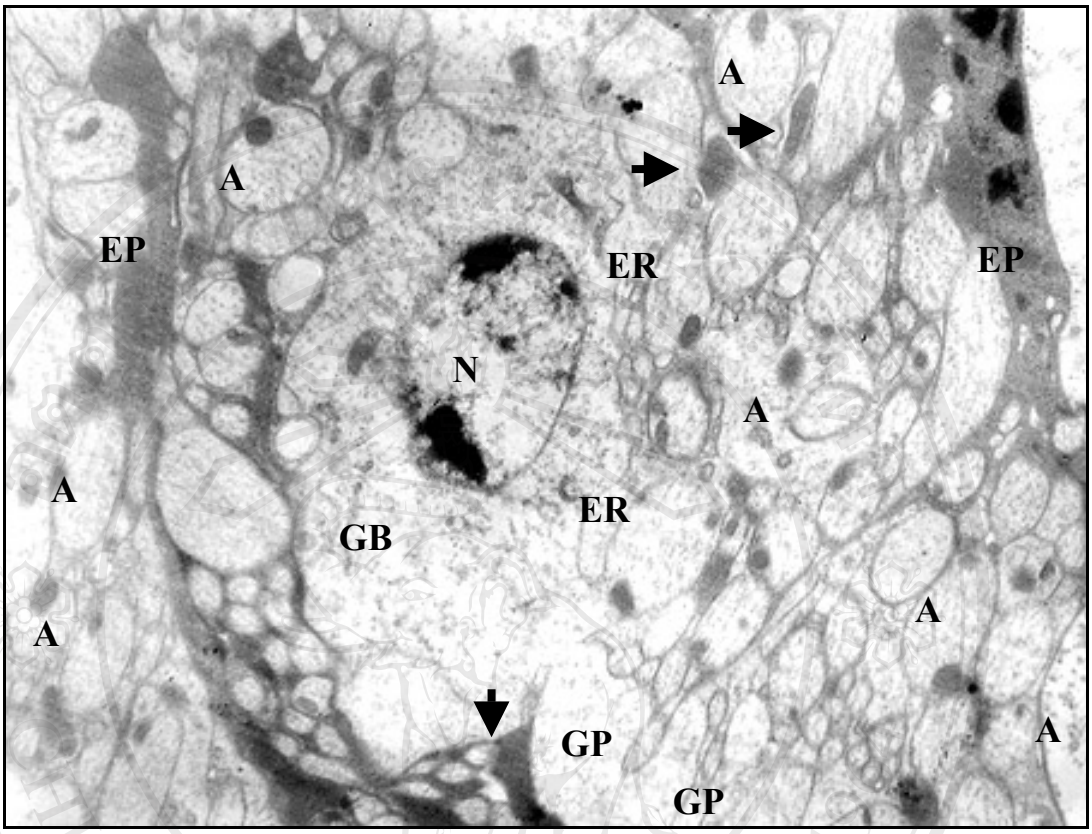



Figure 53 Arrangement of the glial cell processes surrounding numerous axons at a magnification of 5,000. The electron dense material was noted (arrows) lying in the distended extra cellular spaces (EP) between the glial cells. A= axon; N= nucleus of the glial cell; GB= the glial cell body; GP= the glial cell process; ER= distended endoplasmic reticulum.

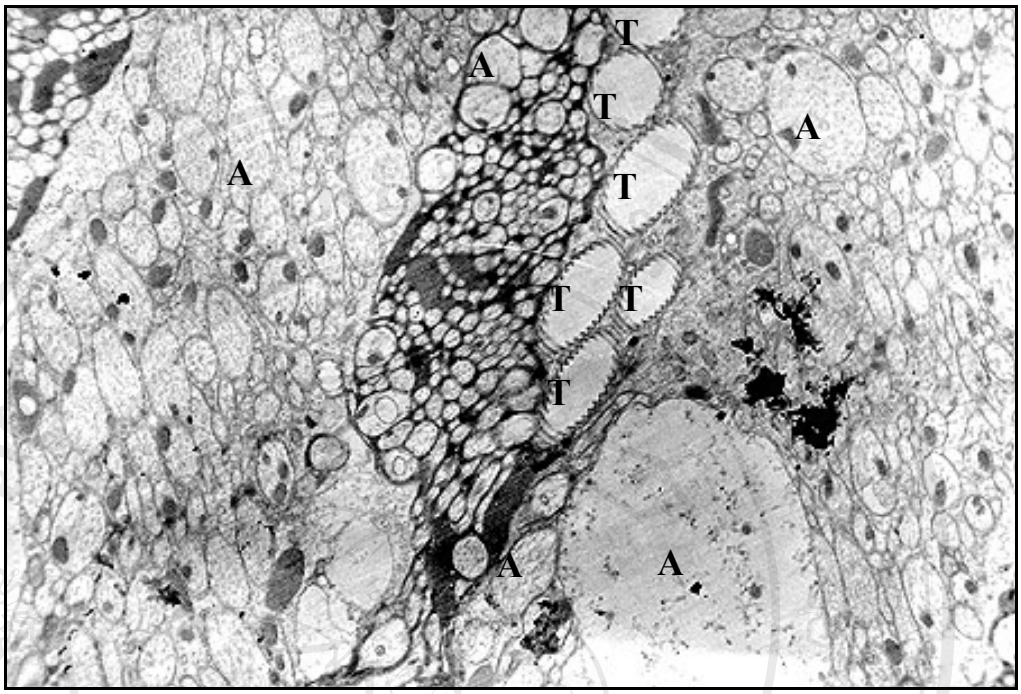



Figure 54 A number of tracheoles found near the bundles of axon in the fly brain at a magnification of 3,150.

**3. Nerve cell count**

The total number of neurons that filled in the given grid area, which calculated to 948.64 $\mu m^2$, was conducted in a classified 4 depth sectioning level and 8 brain locations. Only the vertical plane of the fly brain was employed to determine the number of neurons. The measurement of 4 (location 2,3,6 and 8) of the specific 8 brain locations was carried out owing to a lack of neurons in some areas at different depth cut levels.

The median value comparisons of the nerve cell count at accomplished locations among the 2 age groups of the blowfly *C. megacephala* are shown in Table 1-4. In both young and old flies, Type 1 and Type 2 neurons were found at all 4 locations, while Type 3 neuron was barely found at location 6 and 8. For location 2, the Type 1 neuron and total number of neurons of 3-day-old flies tended to decrease at all depth levels, except the total neurons at depth level 4, when the flies reached 30 days old. However, only the number of neurons at depth level 2 and 3, calculated in the young and old group, was statistically significant using the Mann-Whitney *U* test ($P$=0.000). The Type 2 neuron between both age groups was seemingly stable at all depths. In the senescent group, neurons at various depths were significantly different at only depth 2 and 4. Although the differences between the total number of neurons in young flies compared at all depths were significant, those in the old group were not the same. The comparison of total neurons in young and old flies was notably different at all depths except for depth 1. For location 3, the differences in neurons between depth levels in both the young and old group were probably not important. The Type 1 neuron had a statistically significant difference between the 2 age groups only at depth 1, while the Type 2 neuron had significant differences at both depth 1

and 2. Total neurons at location 3 for 3-day-old flies had no statistically significant variation at all depths, but those for 30-day-old flies were extremely different. A striking difference in the number of total neurons between 2 age groups was observed only at depth 2 ($P$=0.000). For location 6 and 8, most of the 3 types of neuron were found only at depth level 2 and 3. Significant differences in the number of location 6 neurons compared between all depths were noted in all neuron types, except for Type 3 of the young age group and Type 1 of old age group. However, the Type 3 neuron at depth 3 had no variations when compared between the 2 age groups. Statistically significant differences in the number of all neuron types were noticed at depth 2 and 3 when comparing between the 2 groups. The number of neurons at location 8 had significant differences when comparing between all depths and both age groups. All neuron types at all depth levels were found to be almost significantly different ($P$ <0.05) when compared between the young and old age group using One-Way ANOVA. In conclusion, the number of Type 1 and Type 3 neurons in young flies was smaller when compared to that in old flies at all locations and all depth levels except for Type 2 neurons.

Table 1 Comparison of the number of neurons per unit area (948.64 μm$^2$) at location 2 between 3- and 30-day old *C. megacephala*

| | 3-day-old | 30-day-old | |
|---|---|---|---|
| | Median (range) | Median (range) | $P^*$ |
| **Type 1 neuron** | | | |
| Depth 1 | 27 [a] (20-54) | 25 [k] (8-40) | 0.181 |
| Depth 2 | 50 [b] (45-60) | 13 [l] (8-33) | 0.000 |
| Depth 3 | 51 [b] (43-55) | 11.5 [l] (5-37) | 0.000 |
| Depth 4 | 36 [c] (30-40) | 35 [m] (5-41) | 0.685 |
| **Type 2 neuron** | | | |
| Depth 1 | 9 [d] (5-16) | 9 [n] (4-21) | 0.164 |
| Depth 2 | 12 [e] (7-20) | 17 [o] (9-24) | 0.001 |
| Depth 3 | 17 [f] (12-21) | 17.5 [o] (9-26) | 0.777 |
| Depth 4 | 9 [d] (6-14) | 19 [o] (9-28) | 0.000 |
| **Total Neuron** | | | |
| Depth 1 | 35 [g] (25-67) | 35 [p] (23-55) | 0.669 |
| Depth 2 | 63.5 [h] (55-80) | 33.5 [p] (21-49) | 0.000 |
| Depth 3 | 68 [i] (58-73) | 30.5 [p] (18-56) | 0.000 |
| Depth 4 | 46 [j] (38-52) | 51 [q] (24-66) | 0.000 |

[a,b,...,q] The different letters indicate a significant difference between values at various depths of the fly's brain (Mann-Whitney *U* test; $P<0.05$)

$P^*$ from the Mann-Whitney *U* test of neuron density comparison between the 2 age groups

Table 2 Comparison of the number of neurons per unit area (948.64 μm$^2$) at location 3 between 3- and 30-day-old *C. megacephala*

| | 3-day-old | 30-day-old | |
|---|---|---|---|
| | Median (range) | Median (range) | $P^*$ |
| **Type 1 neuron** | | | |
| Depth 1 | 33 [a] (28-40) | 18 [h] (12-35) | 0.000 |
| Depth 2 | 28 [b] (19-39) | 24 [h] (16-35) | 0.171 |
| Depth 3 | 21 [c] (9-29) | 20 [h] (13-42) | 0.670 |
| Depth 4 | 31 [b] (10-47) | 34 [i] (18-45) | 0.122 |
| **Type 2 neuron** | | | |
| Depth 1 | 14 [d] (5-23) | 22 [j] (15-39) | 0.000 |
| Depth 2 | 20.5 [e] (11-31) | 31.5 [k] (26-42) | 0.000 |
| Depth 3 | 28 [f] (12-36) | 27.5 [l] (21-35) | 0.175 |
| Depth 4 | 22.5 [e] (6-30) | 19 [m] (12-26) | 0.558 |
| **Total Neuron** | | | |
| Depth 1 | 46 [g] (37-63) | 41 [n] (30-61) | 0.053 |
| Depth 2 | 49 [g] (39-61) | 56 [o] (42-76) | 0.000 |
| Depth 3 | 47.5 [g] (38-61) | 47.5 [p] (37-77) | 0.184 |
| Depth 4 | 51.5 [g] (30-72) | 51 [p] (38-66) | 0.261 |

[a,b,..,p] The different letters indicate a significant difference between values at various depths of the fly's brain (Independent Samples T test; $P<0.05$)

$P^*$ from the Independent Samples T test of neuron density comparison between the 2 age groups

Table 3 Comparison of the number of neurons per unit area (948.64 μm$^2$) at location 6 between 3- and 30-day-old *C. megacephala*

| | 3-day-old | 30-day-old | |
|---|---|---|---|
| | Median (range) | Median (range) | $P^*$ |
| **Type 1 neuron** | | | |
| Depth 2 | 13 [a] (11-18) | 5 [h] (2-8) | 0.000 |
| Depth 3 | 16 [b] (12-19) | 4 [h] (2-9) | 0.000 |
| **Type 2 neuron** | | | |
| Depth 2 | 5 [c] (2-6) | 22 [i] (17-26) | 0.000 |
| Depth 3 | 3 [d] (2-5) | 15 [j] (11-26) | 0.000 |
| **Type 3 neuron** | | | |
| Depth 2 | 1 [e] (0-3) | 2 [k] (0-3) | 0.000 |
| Depth 3 | 1 [e] (0-3) | 1 [l] (0-3) | 0.116 |
| **Total Neuron** | | | |
| Depth 2 | 19 [f] (15-24) | 29 [m] (22-35) | 0.000 |
| Depth 3 | 20.5 [g] (16-25) | 22 [n] (17-34) | 0.007 |

[a,b,..,n] The different letters indicate a significant difference between values at various depths of the fly's brain (Mann-Whitney *U* test; $P<0.05$)

$P^*$ from the Mann-Whitney *U* test of neuron density comparison between the 2 age groups

Table 4 Comparison of the number of neurons per unit area (948.64 μm$^2$) at location 8 between 3- and 30-day-old *C. megacephala*

| | 3-day-old | 30-day-old | |
|---|---|---|---|
| | Median (range) | Median (range) | $P^*$ |
| **Type 1 neuron** | | | |
| Depth 2 | 36 [a] (24-41) | 9 [i] (6-12) | 0.000 |
| Depth 3 | 26 [b] (20-39) | 9 [j] (7-12) | 0.000 |
| **Type 2 neuron** | | | |
| Depth 2 | 11 [c] (6-15) | 14 [k] (11-23) | 0.000 |
| Depth 3 | 10 [d] (4-17) | 18 [l] (12-24) | 0.000 |
| **Type 3 neuron** | | | |
| Depth 2 | 3 [e] (0-4) | 2 [m] (0-3) | 0.001 |
| Depth 3 | 4 [f] (0-5) | 3 [n] (0-4) | 0.035 |
| **Total Neuron** | | | |
| Depth 2 | 49 [g] (39-56) | 25 [o] (20-36) | 0.000 |
| Depth 3 | 40 [h] (30-50) | 31 [p] (24-35) | 0.000 |

[a,b,..p] The different letters indicate a significant difference between values at various depths (Mann-Whitney *U* test; $P<0.05$)

$P^*$ from Mann-Whitney *U* test using in neuron density comparison between the 2 age groups