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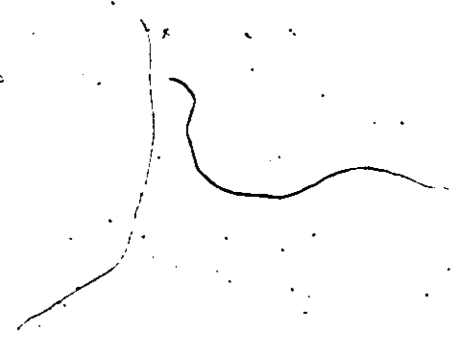

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

"The maturation of the Purkinje neuron in vivo and in culture"

A light and electron microscopic study.

By

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THESIS

Submitted to the School of Graduate Studies
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy.

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CHAPTER - 1

A: GENERAL INTRODUCTION

The cytoarchitectonics of the cerebellar cortex and the development of the Purkinje neuron in the intact animal are two of the most thoroughly investigated subjects in neuroanatomy. The mature cerebellar cortex is organized in layers: these are molecular, Purkinje cell and internal granular layers. However, such is not the case in the immature cerebellar cortex, which in the beginning consists of an external granular layer, a thin molecular layer, several layers of Purkinje cells, and a very thin layer of internal granule cells. The transition of the cerebellum from an unorganized immature state to a well organized mature state occurs in a very orderly sequence. The cytological phenomena which occur include the maturation of Purkinje neurons and interneurons, and the development of their synaptic contacts. In this sequence of cerebellar development, the maturation of Purkinje neurons is probably the most important single event.

The knowledge of the development of the Purkinje neuron is based upon evidence accumulated since 1897, in several species. At the earliest stage, examination of the Golgi impregnated sections reveals that the Purkinje neurons are small, approximately 8 - 10 μ in size: They form a 3 - 4 cell deep unorganized layer between a thick external granule cell layer and a thin internal granule cell layer (Dadoune 1966: Altman 1972). Soon they begin to align in a row and have many processes emerging from the soma region. (Athias 1897: Cajal 1911: Purpura et al., 1964: Meller and Glees 1969: Altman 1972: Kornguth and Scott 1972: Zecevic and Rakic 1976).

These processes have been measured and some of them have been found to be as long as 50 - 70 µ in length, in human material (Zecevic and Rakic 1976). During this period a small molecular layer also develops between the external granule cell and the internal granule cell layer (Altman 1972). The Purkinje neuron grows in size; an hypertrophied apical cone develops, and from it the main dendrite develops and grows towards the external granular layer (Altman 1972). Primary, secondary and tertiary dendritic branches develop from the main dendrite (Altman 1972). Processes emerging from the Purkinje soma disappear and the surface becomes smooth. (Athias 1897: Cajal 1911: Purpura et al., 1964: Meller and Glees 1969: Altman 1972: Korunguth and Scott 1972). The developing dendritic spines present on primary, secondary and tertiary dendrites begin to receive synaptic contacts from an increasing number of parallel fibers in the lower half of the molecular layer. Growth of the dendritic tree continues, until the maturation of the Purkinje neuron is complete. During this period, the external granular layer totally disappears and a tremendous increase in the molecular layer occurs.

On electronmicroscopic examination of the developing Purkinje neuron, it has been found, that spine - like processes - are present on the Purkinje soma (Larramendi and Victor 1967: Larramendi 1969: Meller and Glees 1969: Mugnaini 1969: Altman 1972: Korunguth and Scott 1972: Zecevic and Rakic 1976). It has been generally believed that long processes observed in light microscopy (LM) and spine - like processes observed in electronmicroscopy (EM) are similar, and that they receive only one type of afferent terminal - climbing fibers.

However, this concept of similarity of processes (LM) and spines (EM) appears very doubtful. The long processes (LM) measure 50 - 70 μ (Zecevic and Rakic 1976), where as the spine like processes (EM) measure 1 - 2 μ. Further, it is also generally believed that these long processes and somatic spines disappear by resorption during Purkinje neuron development. This concept of resorption was proposed by Athias 1897, and since then it has remained as the main explanation of the changes in appearance (Purpura et al., 1964: Dadoune 1966: Larramendi 1969: Mugnaini 1969: Meller and Glees 1969: Altman 1972). However, it seems rather difficult to accept that processes of such long length can be resorbed by Purkinje cells during their development.

A detailed review of the investigations of the development of the Purkinje neuron will analyze the developmental sequence, reveal the descrepancies reported, and explore the theories proposed to account for these events.

B: DEVELOPMENT OF THE PURKINJE NEURON IN ANIMAL.

In September 1837, J.E. Purkinje told the audience at a meeting in Prague that in free hand cut sections of fresh cerebellum, there were numerous fig-shaped corpuscles arranged in a row, with their rounded ends directed towards the white center of the folium; their tail-like apical processes were directed towards the surface, and disappeared in the grey matter. Since then, these fig-shaped corpuscles of the cerebellar cortex have been known as Purkinje neurons.

Johannes Müller reported Purkinje's discovery in almost identical words in his annual review of anatomy and physiology for the year 1836 (Müller, 1837). Purkinje's successors saw no more of this cell than he did until 1890, when staining with silver and Golgi methods came into use.

From 1890 onwards, many investigators took an interest in studying the morphology of Purkinje neurons. Prominent among those were Lugaro (1894) Schaper (1894) Popoff (1895, 1896) and Athias (1897).

In Nissl stained sections of the cerebellar cortex of the embryonic pig, Takasu (1905) found that it was organized in layers. In the beginning there was a small outer granular layer, which gradually got wider and later on seperated into 2 layers. Purkinje neurons were found to be lying in a row, between these two granular layers. In the early stages of development, Purkinje neurons were small and light in color. As they grew bigger they began to have many fine processes attached to the soma. At the same time, a small molecular layer also developed, which later on became very wide.

Addison (1911) found that Purkinje neurons in albino rats develop post-natally. In this study, the cerebellum was stained with (1) Carbol-Thionine and acid fuchsin (2) Cox-Golgi method, (3) rapid Golgi method,

and (4) Ramon Y Cajal's reduced silver nitrate method. He was able to identify, in the first post-natal day, Purkinje cells which measured 12 X 7 μ, and had a lightly staining nucleus. These cells were found in 2 - 3 irregular rows along the inner boundary of the molecular layer. At 3 days, Purkinje cells were found in 1 - 2 irregular rows and at 5 days in one continuous row. During this period, there was a great increase in size of both nucleus and cytoplasm also. The main bulk of the cytoplasm was at the ectal pole and from it several fine processes radiated into the molecular layer. At 8 days the cell increased in size and measured 18 X 12 μ. Between 8 - 10 days, there were definitive changes in form by elongation of the cytoplasm of the ectal pole to form the main dendrite. At the same time, all the dendrites became arranged in one plane, and this plane was parallel to sections directed across a folium. Nissl granules appeared in the cytoplasm at 8 - 10 days.

Although the investigations of Addison were very informative, he apparently failed to observe prolongations emerging from the surface of the Purkinje Soma. These prolongations were reported by Athias for the first time in 1897. He investigated the development of Purkinje neurons in cerebella obtained from cat, rabbit, dog and pig, stained with the rapid Golgi method. Thick and thin prolongations were found emerging from all over the immature Purkinje soma, especially from the lateral and superior surface area. Purkinje neurons at this stage were aligned in a row (Fig. 1a). Later on the Purkinje neuron matured and these prolongations were not found on the soma (Fig. 1b).

The formation of these prolongations followed by their disappearance led Athias to conclude that the prolongations disappear by resorption from the soma during development of the Purkinje neuron (Page 382, Athias 1897).

Figure 1a: 2 embryonic Purkinje cells from the cerebellum of newborn Rabbit. (Fig. 1 of Athias 1897).

Figure 1b: Purkinje cell from an nearly mature Rabbit. (Fig. V of Athias 1897).

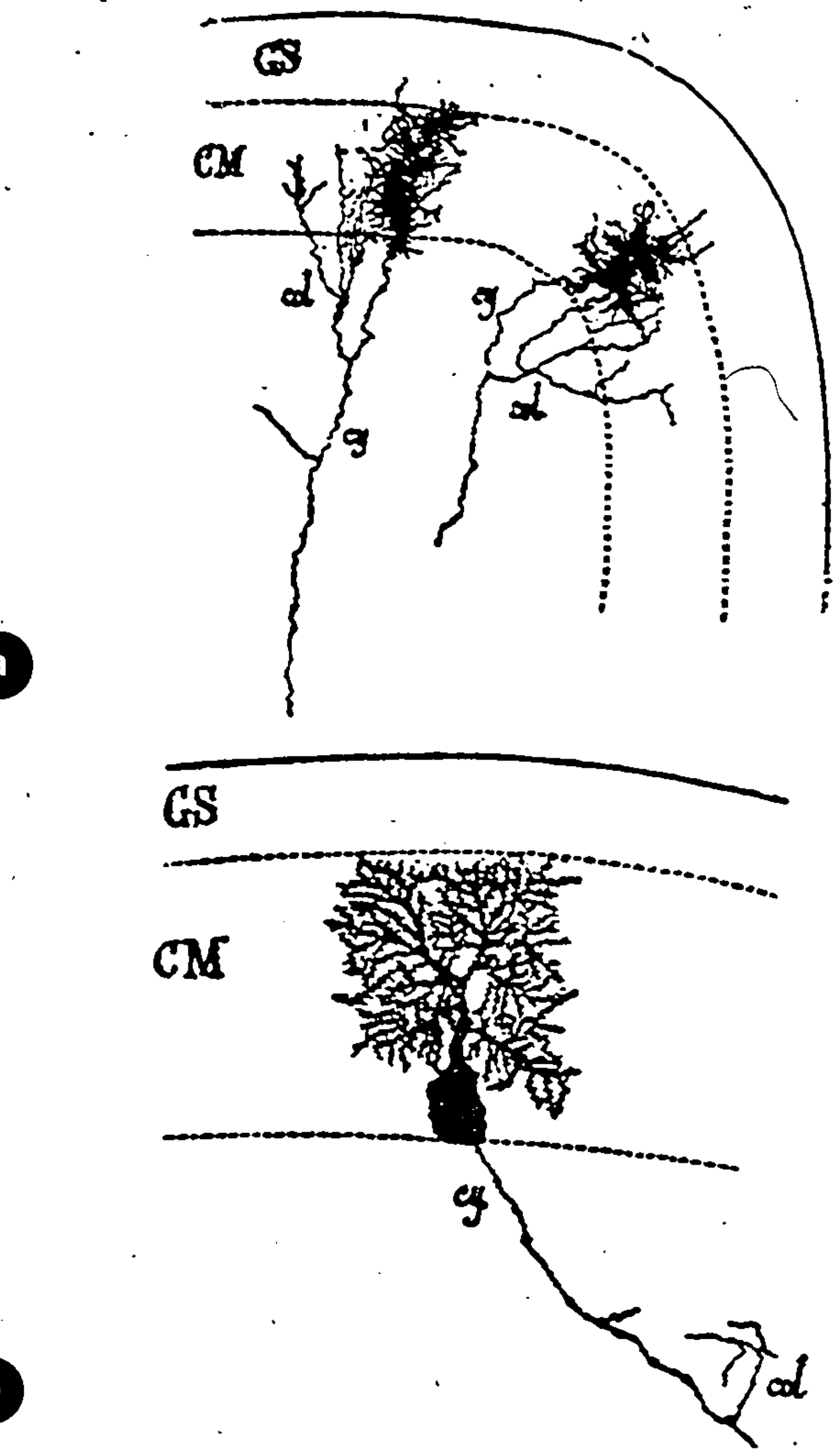


Figure 1

In 1911, Ramon y Cajal gave a detailed description of the development of the Purkinje neurons which was investigated in dog, cat and human infant cerebella. Cajal impregnated cerebella by the methods of Golgi and reduced silver nitrate, and divided the period of Purkinje neuron development into three phases. (Ramon y Cajal 1911; translated by Guth 1960).

1: Phase of the fusiform cells: In this phase, he described that the arrangement of Purkinje neurons was in several irregular strata. Nuclei were voluminous and were surrounded by a relatively abundant protoplasm, especially towards the superficial pole. The dendrons emerged from all sides of the soma.

2: Phase of stellate cells with disoriented dendrons: In this phase he observed an increase in the number of dendrons. The dendrons were located laterally on the soma. They were ascending, dichotomizing and terminating at diverse levels by means of conical points. The longest of them reached to the zone of bipolar cells.

3: Phase of orientation and of flattening of dendritic plume: In this phase, he observed that the dendrons have disappeared from the cell body. The cell body became an elongated pyriform configuration. The dendritic tuft became flat, and developed more of secondary and terminal branches.

It appears that the thick and thin prolongations of Athias are the same processes that Cajal termed disoriented dendrons. Cajal agreed that these processes disappear during development but expressed no opinion regarding the mechanism of this event.

Similar numerous short protoplasmic projections which looked like thin perisomatic dendritic ramifications were also observed by Purpura, Shofer, Housepian and Noback (1964). They found them emerging from the irregular contour of the Purkinje soma of newborn kitten. Within 4 - 5 days after birth, Purpura et al., also found that these perisomatic processes disappeared.

Dadoune's (1966) light microscopic investigations on the development of the Purkinje neuron in rat reconfirmed the results reported by earlier investigators. The presence of fine lateral processes on the Purkinje soma, was also reported.

Thus by the year of 1966, it became well established that during the development of Purkinje neurons, there was a period during which cytoplasmic expansions emerged from the soma. These have been given different names: thick and thin prolongations, Athias 1897; disoriented dendrons, Cajal 1911; perisomatic dendritic ramifications, Purpura et al., 1964; fine lateral processes, Dadoune 1966; Essentially all of them refer to the same event. It has also been noted that these cytoplasmic expansions persist for a short period and that they are not seen at later stages of development.

Further understanding of the morphological development of the Purkinje neuron began to occur with the application of electron-microscopic procedures by Larramendi and Victor 1967. They found cytoplasmic expansions, which fit the description of spines, emerging from the Purkinje soma of a 6 - 7 day old mouse. These spines were synaptically in contact with axon terminals identified as climbing fibers.

Larramendi (1969) continued to investigate the maturation of Purkinje neurons electronmicroscopically. He described 2 types of cytoplasmic processes: (1) long cytoplasmic processes having the characteristics of dendritic branchlets, and (2) spine-like cytoplasmic processes. Generally, it seems that he believed both of them to be the same type. The spine-like cytoplasmic processes contained only extensions of granular reticulum and sometimes bent a short distance from the cell body. They were embraced by fusiform axonal profiles, which contained many clear core round vesicles. Asymmetrical synaptic adhesions were observed between these spines and the axonal profiles. It was concluded that these axonal profiles were the terminals of climbing fibers. In Larramendi's text and photographs, the synaptic contacts of climbing fibers are always shown with the spine-like cytoplasmic extensions. This author assumed that both the longer dendritic branchlet and the spine like cytoplasmic process receive similar synaptic contacts. Therefore he did not investigate any other source of afferent terminal which might contact the dendritic branches.

Investigations continued on the spine like cytoplasmic extensions on the Purkinje soma after day 7 of development. The location of spines at day 10 was on the apical portion of the cell body and main dendrite; at day 14 they were found on the primary and secondary dendrites, and rarely on the soma region.

Larramendi proposed a combination of 2 mechanisms to account for this developmental sequence of the spines: (a) translocation, and (b) resorption.

He postulated that translocation of existing spines from the Purkinje cell body upwards to its apical portion and main dendrite occured due to the growth of cell surface, which was achieved by adding patches of new membrane to the existing one. Therefore the spines and their contacts are moved upwards from the soma to the main dendrite.

In the second proposal he postulated that some of the Purkinje cell somatic spines formed transient or temporary synapses and that these spines were resorbed by the Purkinje cells. However, he never observed any region of degenerating synapses or spines, but he noted that somatic spines did shrink in size during development. Although Larramendi could not resolve the actual mechanism of spine resorption, he finally concluded that the synapses observed in adult animals were remnants of synaptic contacts established earlier during development.

Larramendi tied the development of the Purkinje neuron to that of the climbing fiber, as described by Ramon y Cajal (Ramon y Cajal 1911; translated by Guth 1960). Cajal classified the development into three successive stages:

a) Pericellular nest stage: In this stage climbing fibers made their first contact with a Purkinje cell body by means of a rich plexus, formed on the lower part of the cell body.

b) Capuchon stage: During this period, there was displacement of the plexus towards the apical portion of the Purkinje cell body and the developing main dendrite.

c) Dendritic stage: Climbing fibers spread upwards onto primary, secondary and tertiary dendrites of Purkinje cells.

Larramendi equated the climbing fiber development with the morphogenesis of the Purkinje cell as seen in the Golgi preparations. He noted that the "pericellular nest" and "capuchon" stages corresponded with the "phase of stellate cells with disoriented dendrons", and the "dendritic stage" corresponded to the "phase of orientation and of flattening of dendritic plume".

Results similar to Larramendi (1969) were presented by Meller and Glees (1969), in the mouse. They found somatic spines synaptically in contact with climbing fibers. During the second week, somatic spines began to regress from the basal pole, then from the apical pole, and disappeared completely from the Purkinje soma by the end of the second week. From these observations, they also concluded that the somatic spines disappear by resorption.

In Golgi impregnated sections of 1 week old cerebellum, Meller and Glees found dendritic protrusions emerging from the Purkinje soma, which persisted for a period of only 2 - 3 days. (Fig. 2a). These dendritic projections appear to be similar to those described by Athias (1897) and Cajal (1911). Since the dendritic projections were not found on the mature Purkinje soma, therefore they also postulated that these have regressed.

A photograph of a Golgi-stained Purkinje neuron (Fig. 2b) clearly demonstrates small protrusions and long thick processes emerging from the soma at day 9. It appears that these authors failed to note the smaller protrusions and labelled the longer dendritic - like processes as "somatic spines". They made no attempt to relate their Golgi material to the electron microscopic results.

Figure 2a: Mouse cerebellum 8 days. (Fig. C of Meller and Glees 1969).

Figure 2b: Mouse cerebellum 9 days. (Fig. G of Meller and Glees 1969).

Figure 2

In chicken, the development of the Purkinje neurons was investigated electronmicroscopically by Mugnaini (1969). At embryonic day 15, he found processes emerging from the soma. These processes had the characteristics of dendrites and were synaptically in contact with parallel fibers (Fig. 3). On further investigation, Mugnaini found that the number of synaptic contacts between dendritic processes and parallel fibers increased with the maturation of Purkinje neurons. He did not investigate further the fate of these dendritic processes emerging from the Purkinje soma.

Mugnaini also found short cytoplasmic processes emerging from the Purkinje soma, which he labelled pseudopodia. According to Mugnaini, pseudopodia never attained the characteristics of dendrites, they never became populated by other organelles, and were synaptically in contact with climbing fibers.

It appears that Mugnaini successfully differentiated in electronmicroscopy two types of processes emerging from the developing Purkinje soma, each receiving a different fiber system. This analysis is based upon the cytological and synaptic similarity between Mugnaini's pseudopodia and the somatic spines described by others. It is possible to assume that both structures are equivalent. Similarly, Mugnaini's dendritic processes appear similar to the disoriented dendrons observed in light microscopic examination by earlier investigators. Nonetheless, Mugnaini proposed that the pseudopodia (EM) were equivalent to the disoriented dendrons (LM). Further he suggested that pseudopodia retract during development, but did not propose any mechanism.

In 1972, Altman investigated extensively the growth and synaptic maturation of Purkinje neurons of rat, using both Golgi and electronmicroscopic techniques. He divided the period of Purkinje cell maturation into 5 Phases:

Figure 3: Purkinje cell from an 15 day old embryonic chicken. (Fig. 14, of Mugnaini 1969).

Figure 3

Phase 1: Initially the Purkinje neurons were multilayered. By the 6th day, they were found to be aligned in a row. During this phase, Purkinje cells had few or no synapses.

Phase 2: Between day 5 - 7, in the Golgi preparations, two transient structures were found belonging to the Purkinje soma.

a) An hypertrophied apical cone composed of reticular cytoplasm.

b) Irregular long and thick cytoplasmic extensions which were termed "Lateral perisomatic processes".

In the electron-microscope, very early in development, climbing fibers were seen forming asymmetrical synapses with the smooth portion of the Purkinje soma, and later with spine-like processes. These appear to be similar to the spine-like processes of Larramendi (1969) and Meller and Glees (1969). Altman termed these spine like processes - "perisomatic processes" and failed to use a separate term to differentiate these electron-microscopic protrusions.

Phase 3: (Between day 8 - 12). By the 10th day, the hypertrophied apical cone attained its maximal enlargement, from which the outgrowth of the primary dendrite occured. Asymmetrical synapses of climbing fibers with "perisomatic processes" began to decline, and symmetrical inconspicuous basket cell synapses began to form on the smooth surface of the Purkinje soma. Parallel fiber - dendritic synapses were rarely observed.

Phase 4: (Between 12 - 15 days) The Purkinje soma became smooth, and perisomatic processes were no longer present. The growth of the dendritic system continued with an appreciable increase in the number of parallel fiber-dendritic spine contacts in the lower half of the molecular layer.

Phase 5: (Between 15 - 30 days) During this phase, the external granular layer disappeared, and the Purkinje dendrites continued to grow in the upper half of the molecular layer, where they were contacted by parallel fibers.

Altman accepted the arguements of Mugnaini (1969) that the disoriented dendrons of Cajal were equivalent to pseudopodia as seen electronmicroscopically. He proposed that both types of perisomatic processes observed in his studies are the equivalent of processes observed in the Golgi stained material by early investigators. Altman argued that since pseudopodia never became populated by organelles (Mugnaini 1969), therefore disoriented dendrons were not true dendrites and never develop into mature dendrites. He concluded that the dendritic tree grew from the apical cone; and that the perisomatic processes dissolve or translocate later in development.

In the Golgi impregnated sections of the cerebellum of 125 days old fetal macaque, Kornguth and Scott (1972) found apical dendrites and many long, thick somatic processes emerging from the Purkinje soma. These cells were in a single lamina at this stage and the apical dendrite was oriented towards the pia.

In the electronmicroscope, very early in development (100 day old fetal macaque), they found climbing fibers synaptically in contact with the smooth surface of the Purkinje soma, and small somatic processes. These small somatic processes are similar to somatic spines, as described by Larramendi (1969). Later on (125 days old fetal macaque), similar contacts were found on thick and long somatic processes. These processes have characteristics of large dendrites. Many of the climbing fiber

boutons were found capping the end of the somatic processes; the dendritic or somatic processes often penetrated into the boutons. No other terminals except presynaptic terminals of climbing fibers were identified around the Purkinje soma at this stage.

Using stereo-electronmicroscopy, they found fibrillar components in the presynaptic dense projections, in the synaptic cleft and in the post-synaptic thickening. These were continuous with each other, appeared to perforate both neuronal membranes, and were similar to the junctional plates described by Gray (1966).

From these results, the authors concluded that the Purkinje cell dendritic tree developed from the apical dendrite and somatic processes, which developed under the influence of climbing fibers. In order to emphasize their conclusion, Kornguth and Scott stated: "In no species can one find a Purkinje cell with a characteristically elaborate dendritic tree in the absence of climbing fibers".

They proposed the following mechanics to account for their observations and conclusions.

a) The interior pressure of the Purkinje soma is marginally higher than the exterior pressure.

b) The fibrillar twisted component in the junctional complex of climbing fibers and the Purkinje soma is contractile.

During the rapid growth of the Purkinje cell, the combined effect of these factors produces an evagination of the Purkinje cell membrane, resulting in the formation of a small somatic process: which later on becomes thicker and longer. Finally these processes develop into dendrites.

The investigations of Kornguth and Scott did add a new concept which was very different from the concept of others, concerning the development of the Purkinje neuron. However they failed to comment on the problem of the mechanism for the change of location of the somatic processes from the immature soma to the mature dendritic tree.

Zecevic and Rakic (1976) investigated the differentiation of human Purkinje cells, by both Golgi and electronmicroscopic techniques. In Golgi preparations, in 12 - 18 weeks old embryos, they found many processes emerging from the Purkinje soma. Zecevic and Rakic classified them in 2 categories:

1: Long voluminous processes, oriented predominently towards the pia. These were classified as immature dendrites. They became longer and wider in distribution, between 18 - 22 weeks. They continued to grow and their fan-shaped distribution became quite apparent by the 28th week.

2: Fine varicose cytoplasmic expansion which emanated laterally from the Purkinje soma (Fig. 4a). Some of them were short, while others were 50 - 70 μ long. Many of the latter had enlargements at their tips, indicative of growth cones. The number of cytoplasmic expansions emerging from the Purkinje soma attained its peak by the 28th week. With further maturation of the Purkinje neuron, both short and long lateral expansions became less numerous, but more massive; and finally by the 35th week, they were absent completely from the Purkinje soma. At that time, the soma became smooth.

In electronmicroscopic examination, in the 12 - 14 week old fetus, they found a perisomatic plexus containing parallel fibers, basket cell axons and immature climbing fibers around the Purkinje cell soma. Parallel

fibers formed the largest component of this plexus. Between 16 - 18 weeks, synapses began to appear in the region of the perisomatic plexus, predominantly at the level of or slightly below the row of Purkinje cell bodies. Asymmetrical synaptic contacts were seen between terminals identified as climbing fibers and cytoplasmic expansions emerging from the Purkinje soma, cytoplasmic expansions emerging from immature dendrites and wide protrusions of the primary dendritic shaft. In 22 - 24 week old fetus, the pericellular plexus was still present, although the round profiles of parallel fibers were fewer in number, particularly around the basal pole of the Purkinje soma. At this stage, they observed electron-microscopically a process, also called a cytoplasmic expansion emerging from the Purkinje soma. These were approximately 1 - 2 μ in size, finger-like in shape, either straight or curved, usually containing agranular endoplasmic reticulum and no other organelle (Fig. 4b). Many of the climbing fibers were found to be synaptically in contact with this type of cytoplasmic expansion, and occasionally with dendritic spines.

Thus, these results concur generally with investigations in other species, which has shown that climbing fibers form contacts early in development with short cytoplasmic somatic expansions, i.e., somatic spines.

Zecevic and Rakic believed that all the lateral cytoplasmic expansions emerging from the Purkinje soma (observed in Golgi material) were similar to somatic spines (Larramendi 1969) or pseudopodia (Mugnaini 1969). Thus, they also failed to differentiate between the two types of cytoplasmic expansions. This could have been done on the basis of size alone i.e., long cytoplasmic expansions 50 - 70 μ long seen in Golgi (Fig. 4a) versus short cytoplasmic expansions 1 - 2 μ long seen in E.M. (Fig. 4b).

Figure 4a: Purkinje cell 32 weeks old human fetus.
(Fig. 12-A of Zecevic and Rakic 1976).

Figure 4b: Purkinje cell 24 weeks old human fetus
(Fig. 10 of Zecevic and Rakic 1976).

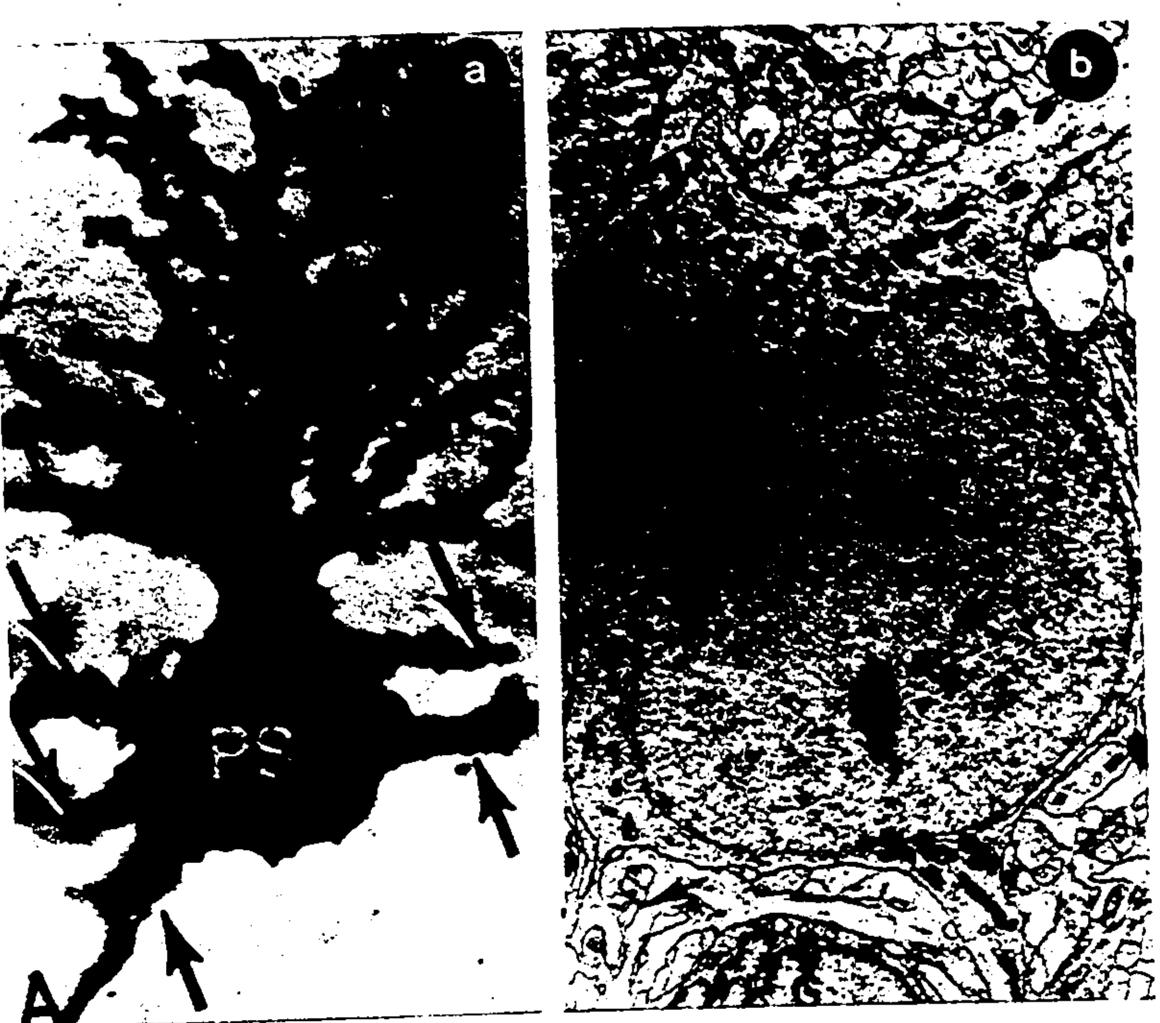


Figure 4

From the review of the literature of the development of the Purkinje neuron in vivo, it is possible to assume that there are 2 separate type of cytoplasmic expansions which emerge from the soma. These are:

1) Long, thick dendrite-like expansions. These have been observed both in Golgi material and electronmicroscopically,

2) Short spine-like expansions. These have been described only in the electron microscope.

However, all of the recent authors, have used the electronmicroscope for their investigations, have generally believed that there are not two but only one type of cytoplasmic expansions; and therefore they have used terms interchangebly to describe them. This variation in the terminology has created a confusion in the understanding of the development of the Purkinje neuron in vivo. Therefore in order to clarify the literature, it became necessary to introduce a classification scheme dividing these expansions into 2 types. This classification shall be used in this thesis and is based upon the size and the ultrastructural characteristics of these cytoplasmic expansions.

1) Perisomatic dendrites: In Golgi preparations, their size is between 5 - 70 μ. They are long, thick and branching. Many have enlargements at their tips. Ultrastructurally, these have all the characteristics of dendritic branchlets and contain several organelles.

2) Perisomatic spines: These have not been described in Golgi preparations. Ultrastructurally, these are 1 - 2 μ long, straight or curved and contain extensions of agranular reticulum only.

This classification has been illustrated further on the accompanying chart, in order to demonstrate the variety of terms used and that it is possible to place the previous descriptive terminology of the "cytoplasmic expansions" emerging from the Purkinje soma into one of the two types.

Type 1	Type 2	Author
& thin prolongations (Golgi)	------------	Athias 1897
iented dendrons (Golgi)	------------	Cajal 1911
omatic dendritic ications (Golgi)	------------	Purpura et al., 1964
lateral processes (Golgi)	------------	Dadoune 1966
--------------------	Cytoplasmic expansions (EM)	Larramendi & Victor 1967
cytoplasmic processes (EM)	Spine-like cytoplasmic expansions (EM)	Larramendi 1969
itic protrusions (Golgi)	Somatic spines (EM)	Meller and Glees (1969)
ites (EM)	Pseudopodia (EM)	Mugnaini (1969)
al perisomatic processes gi)	Spine-like perisomatic processes (EM)	Altman (1972)
thick somatic processes gi, EM)	Small somatic processes (EM)	Kornguth et al., 1972
cytoplasmic expansions gi)	Short cytoplasmic expansions (EM)	Zecevic & Rakic 1976
somatic dendrites (Golgi)	Perisomatic spines (Golgi)	Aggerwal 1976

VERY TIGHT BINDING

In view of the existing confusion in terminology and description of the cytoplasmic expansions emerging from the soma, it seemed necessary to repeat the Golgi study of the development of the Purkinje neuron in vivo. One of the main aims would be to differentiate between the perisomatic dendrites and the perisomatic spines: The results would also serve as a basis of comparison with the development of the Purkinje neuron in tissue culture.

It is generally believed that all of the cytoplasmic expansions receive only one type of afferent contact i.e., climbing fibers. However, climbing fibers have been demonstrated to be synaptically in contact with the perisomatic spines only; and have never been demonstrated to be synaptically in contact with the perisomatic dendrites. The electron-microscopical investigations of Mugnaini (1969) demonstrate that dendrites are synaptically in contact with parallel fibers. In the chart, this author has classified dendrites of Mugnaini in the category of the perisomatic dendrites. Therefore, it is possible to postulate that the perisomatic dendrites are contacted by parallel fibers and the perisomatic spines by climbing fibers.

All the authors reviewed agree that the immature Purkinje soma demonstrates numerous cytoplasmic expansions and the mature Purkinje soma shows a smooth contour. How does this morphological transition occur ? In order to explain this phenomenon, it has been proposed that the cytoplasmic expansions, whether dendritic and/or spinous, either resorb or translocate during development of the Purkinje neuron. Athias (1897) was the first investigator who postulated that the cytoplasmic expansions disappear by resorption. Since then, this concept has been generally accepted. However, there is no evidence in the literature to support the

postulate of Athias. Moreover, it seems rather difficult to conceive that 50 - 70 μ long perisomatic dendrites can be resorbed during development by the Purkinje neuron. Translocation of the spines from the soma onto the main dendrite was proposed by Larramendi (1969). In his postulate, Larramendi did not take into account the presence of the perisomatic dendrites. Kornguth and Scott (1972) reported that the cytoplasmic expansions continue to grow to become ultimately the dendrites. However, these authors failed to explain the movement of the dendrites from the soma. Therefore, to date, the mechanics of the movement of the perisomatic dendrites and the perisomatic spines from the immature Purkinje soma are not known.

This discussion leads into a general consideration of the factors which may influence and mold the shape of the mature Purkinje neuron and particularly, its dendritic tree. Assuming a precise knowledge of the development of a neuron such as the Purkinje cell, it is then possible to study the factors which influence and direct its development. For example, how much of the final pattern of a Purkinje cell is determined by extracerebellar afferents (in particular the climbing fibers), by local circuits (e.g., parallel fibers and basket cell axons), and by intrinsic (genetic) factors.

It is generally believed that the dendritic tree develops from the apical cone. In 1972 Kornguth and Scott postulated that in addition to the apical cone, the perisomatic dendrites also contribute in the formation of the dendritic tree under the influence of the climbing fibers. However, this postulate was neither documented with direct evidence, nor did it take into account the influence of parallel fibers. Herndon, Margolis and Kilham (1971) found that the degree of Purkinje cell alignement is dependent upon the granule cell development. It is also possible that there may be

enough intrinsic properties i.e., genetic, which enable the Purkinje neuron to develop on its own, the mature characteristic shape. Thus the factors which may influence the mature shape of the Purkinje neuron are also as yet unknown.

In order to investigate these questions, it is necessary to have a system where all or some of these factors can be manipulated experimentally. Tissue culture of the cerebellum offers a system in which one of the major proposed influence, the climbing fiber is lacking. It is well known that organotypic cultures of cerebellum have been reproduced in several laboratories. Much attention has been focussed on the so-called "mature" cultures of cerebellum as prepared in the Maximow double coverslip assembly. A review of the literature will indicate that much of the information concerning these cultures is either still in dispute and incomplete or that other data concerning these cultures is unavailable because of technical limitations.

C: TISSUE CULTURE STUDIES OF THE CEREBELLUM.

Light microscopically, in 3 weeks old mouse cerebellar cultures, Bornstein and Murray (1958) demonstrated, for the first time, myelin formation around newly grown axis cylinders of the Purkinje cell. Myelogenesis occurs at a similar age in the intact animal. Since then it is generally accepted that formation of myelin in cerebellar cultures indicates maturation of its neuronal elements. The ultrastructural similarity of myelin in cerebellar culture with myelin in vivo was established by Ross, Bornstein and Lehrer (1962).

In 1968, Allerand and Murray found that by pairing contiguous cerebellar explants, there was acceleration and increase in the formation of myelin, when compared with single explants. From this observation, they concluded that by pairing contiguous cerebellar explants, the potential of neuronal interaction could be increased; better neuronal specificity and selective affinity was retained in culture by this method.

Regarding the question of alignment of the Purkinje neurons in culture, there are very many conflicting opinions. In 1964, Wolf found that many Purkinje neurons remained in their multilayered organization in culture. However in 1970, (Wolf 1970; Wolf and Dubois-dalcq 1970) argued strongly for the alignment of Purkinje neurons in culture. This was indicated in their criticism of the observations of Seil and Herndon (1970), who did not find cell stratification. Similarly, Hendelman (1967) did not find cell stratification in his cerebellar cultures, but Kim (1970) did. Allerand (1971) claimed that many Purkinje cell tend to become oriented in a linear fashion, 1 - 2 cells thick, with increasing age of the culture; while other Purkinje cells retain their multilayered organization. She believed that the organization of the cerebellum in

vitro was dependent upon the position of the explant at explantation. According to her, explants placed with their cut surface against the coverslip were more likely to retain the organotypic cytoarchitectonics.

Seil (1972) reported from his experiments that the cortex was stratified in a mature cerebellar culture. He found a range of lamination varying from an essentially 2 layered structure consisting of outer granule and Purkinje cells, to a 4 layered structure, composed of outer and internal granular layer, a molecular layer and a layer of Purkinje cells. He proposed that the varied range of lamination in culture could occur due to little or partial migration of granule cells. Wolf (1970) proposed that the lamination in culture occured due to the process of angular toppling of the cortex and subsequent complete migration of granule cells past Purkinje cells in both directions. Thus to-date, it is still not certain, how much lamination of cortical neurons occur in cerebellar cultures, and which factors are responsible for this aspect of organotypicity. It is also not known whether lamination is one of the essential characteristics necessary to judge the maturity of a culture.

In 1971, Allerand reported the morphological characteristics of the living Purkinje cells. She studied them in brightfield microscopy. In the early days of the culture, she identified them by their prominent nucleus which contained one or two spherical nucleoli. The cell body was oval and the cytoplasmic portion became distinguishable only after several days. The delineation of the cytoplasm was considered to be an index of differentiation and maturation of the Purkinje neuron by Allerand. Within a month, she found, these neurons were flask-shaped in appearance. She measured and found them to be 20 - 30 μ in size in the living-state. Wolf (1964) measured and found that the mature Purkinje soma was between 12 - 20 μ. Seil (1972) reported that their size was 15 μ.

The shape of the mature Purkinje neuron in culture was described by Wolf (1964). With Holme's silver impregnation method, he found that the neuron was flask shaped. A similar shape with the same method was reported by Allerand (1971) also.

However, Wolf was not satisfied with the results obtained by the Holme's silver impregnation technique, because it was not providing him with the detailed morphological characteristics of the mature Purkinje neuron, in particular dendrites and spines. Therefore, he attempted to impregnate cerebellar cultures with a modification of the Golgi-Cox method. By this method, in 1969, Wolf and Holden found that more than one dendritic branch emerged from the Purkinje cell soma. This observation was considered an abnormal finding by them. Similar results were reported by Seil (1972) also, who impregnated his cultures with a modified Holme's method. Wolf and Holden (1969), and Wolf and Dubois-Dalcq (1970) did not publish any pictures of their findings. A detailed examination of Seil's (1972) photograph (Fig. 5) shows that the dendrites are originating from the apical portion of the Purkinje soma. The origin from the apical portion is within normal limits. Therefore, it remains to be confirmed whether many dendrites originate from the apical portion of the Purkinje soma or from the peripheral portion.

How much dendritic development of the Purkinje neuron actually occurs in culture is not known. Similarly, it is still debatable at what stage of the dendritic development the Purkinje neuron is considered to be mature, and whether an altered branching pattern should be considered normal or abnormal.

The presence of many spines on the dendrites is one of the several identifying characteristics of the Purkinje neuron in vivo. Using the Golgi-Cox method, the presence of many spines on the Purkinje cell dendrite in

culture was demonstrated by Wolf and Dubois-dalcq (1970). Although their observation is acceptable, generally their pictures were unsatisfactory because of the formation of a precipitate. The cell body was never clearly demonstrated in their material.

Figure 5: Purkinje cell with dendritic branching.

28 DIV. X 670 (Fig. 8 of Seil 1972).

Figure 5

The only study of the development of the Purkinje neuron in culture is by Allerand (1971). In silver impregnated cerebellar explants, the least differentiated neuronal stage was identified by the presence of prominent perinuclear accumulation of neurofibrils, which became continuous with an intensely argentophillic axon and a number of weakly impregnated ascending, basal and lateral dendritic projections which branched a few times before fading out. In the stage of dendritic differentiation, she found that there was increase in secondary and tertiary branching of the increasingly prominent ascending or apical processes and a disappearance of the more basal and lateral ones. The classical flask shaped Purkinje cell configuration with the relatively complex two dimensional dendritic branching was observed in cultures over one month of age. In addition, in mature cultures she found that many Purkinje neurons retained their primitive shape. This observation was described earlier by Wolf and Holden (1969); in their opinion, it was an abnormal developmental state. Allerand did not agree with their opinion. Instead, using the suggestions of Lenhossěk (1890) and Ramon y Cajal (1911), she proposed that the persistance of the primitive state of the Purkinje neuron in a mature culture was due to the failure of the climbing and parallel fibers to form synaptic contact with the neuron. Although this appears to be a reasonable argument, however it remains to be clarified as to why only a few Purkinje neurons receive adequate afferent contacts in culture ?

Efforts to investigate electronmicroscopically, the development of synapses in cerebellar culture were started by Perrier and De Harven in 1961. For fixation, osmium tetroxide alone was used. By this method, they found that few synaptic structures were present in mature cerebellar cultures. Callas and Hild (1963) did a comparison of the synapses observed in cerebellar cultures with synapses found in vivo. They concluded that these were identical, in both situations. Thus by 1963, it became

established that synapses develop in cultures, but the neuronal elements contributing to these synapses remained unknown.

Many ultrastructural characteristics of the Purkinje cell and development of synaptic contacts between the parallel fibers and Purkinje dendritic spines were demonstrated by Hendelman (1967). For his studies, he also used the same fixation method (osmium tetroxide). These results were confirmed later by Wolf and Dubois-Dalcq (1970) and Seil and Herndon (1970).

In addition to the parallel fiber synapse, Seil and Herndon (1970) found many dendritic spines, from which the presynaptic terminal was missing. Such spines were enveloped by a process of Bergman glia and they retained their normal post-synaptic thickening. Seil and Herndon (1970) explained that the presence of such spines was due to the reduced number of granule cells in culture. This explanation appears much more reasonable, because of the observations of Allerand (1971) and Hendelman et al., (1972). They found that toxicity of the feed selectively destroyed many granule cells in culture. However, this explanation was not accepted by Wolf and Dubois-Dalcq (1970). They argued that the deafferented spines (Seil and Herndon: 1970) were extremely close to the vesicle laden swellings of parallel fibers and might synapse with them in adjacent sections.

All of these electron microscopic studies do not describe the presence of other synaptic contacts in culture, like basket/stellate axons. It is possible to assume that the previous investigators failed to identify these contacts because of the fixation method (osmium tetroxide alone), other methods of fixation are needed to investigate these cultures electronmicroscopically. The advantage of using newer methods was demonstrated by Hendelman (1972). He adjusted the buffer for the aldehyde and osmium fixation to about 350 milliosmels at a ph of 7.3 - 7.4 . Using this method,

he differentiated successfully, the bouton of parallel fiber from the bouton of Purkinje axon collateral in culture.

The presence of spines on the Purkinje soma was also observed electronmicroscopically by Wolf and Dubois-Dalcq (1970). They assumed that the somatic spines in culture were similar to somatic spines developed by immature Purkinje cells in vivo; and since somatic spines "resorb" during development of the Purkinje neuron, therefore they did not pursue further the presence of Purkinje somatic spines in culture. However, it is of importance to find out if the Purkinje somatic spines in culture receive afferent terminals. During the development of the Purkinje neuron in vivo, climbing fibers form specific synaptic contacts with the Purkinje somatic spines. Climbing fibers originate from the inferior olivary neurons, which have not been explanted in culture with the cerebellum. Therefore, it is reasonable to assume that there are no extracerebellar climbing fibers in culture. If afferent contacts with the Purkinje somatic spines in culture are found, then it may be possible to consider one of the following:

1) Some climbing fibers originate within the cerebellum. Deep nuclear neurons have been suggested as a source of climbing fibers by Carrea et al., (1947).

2) During development, some other terminal has the ability to take over the post-synaptic site of the climbing fibers.

Studies of synaptogenesis may contribute to our understanding of the degree of synaptic specificity in culture, and the plasticity of the system during its development.

CHAPTER - 2

PROBLEM FORMULATION

A review of the development of the Purkinje neuron showed that there was a gap in the knowledge of its development. Using the Golgi method, the formation of the perisomatic dendrites has been demonstrated, but their synaptic connections have not been studied electronmicroscopically. It is only an assumption (of this author) to consider the dendrites of Mugnaini (1969) to be equivalent to the perisomatic dendrites; these were synaptically in contact with parallel fibers. Perisomatic spines have been demonstrated electronmicroscopically, but they never have been demonstrated in Golgi material. A clear understanding of both of them has been made very difficult, due to the variable terminology used by the previous authors. Therefore, it is not possible to be certain of the developmental pattern of the Purkinje neuron in vivo, described previously. A basis of comparison is not available, if one is to attempt to compare the development of material from in vivo versus culture. In order to establish this basic foundation, it is necessary to reinvestigate the normal developmental pattern in vivo, with the Golgi method.

A review of the morphological and synaptological characteristics of the Purkinje neuron in the so-called "mature" cerebellar culture does not reveal whether the Purkinje neuron attains its mature state, when compared with the intact animal, or whether there is a failure of the Purkinje neuron to organize at some stage. The question of lamination of the Purkinje neuron is as yet undecided. It remains to be established if the lamination is necessary for the formation of their mature flask shape; and if it should be considered as a sign of maturation. More information is needed regarding the dendrites: do these dendrites originate from the peripheral portion of the Purkinje soma or do they originate in an increased number from the apical portion of the cell ? Can the presence of spines indicate that the dendrites are mature ?

Electronmicroscopically, it has been established that the parallel fibers form synaptic contacts with dendritic spines. There is no information regarding the synaptic contacts of basket/stellate cells with the Purkinje neuron. Further it is not known whether Purkinje somatic spines in culture resorb or receive afferent terminals. All of these questions indicate that more accurate studies of the morphology and synaptology of the Purkinje neuron in culture are needed, in order to understand and compare them with their equivalents in vivo. These studies need to be done with improved and newer methods of impregnation and fixation.

It is known that in a cerebellar culture, Purkinje dendrites have many spines and Granule cells are reduced in number. In this situation, during development, is the system able to organize itself ? Would there be numerous spines without contacts, or is it possible that granule cell axons (parallel fibers) compensate by forming hypertrophic multiple contacts. This would represent a form of plasticity which could be seen in the electronmicroscope.

Similarly, it is possible to study the extent of this plasticity, by examining Purkinje somatic spines, which are known to receive specific afferent contacts of climbing fibers. It is assumed that there are no climbing fibers in a cerebellar culture; therefore is it possible for the parallel fibers to take over the post-synaptic site (Purkinje somatic spines) of the climbing fibers ? If so, what would be the influence of this interchange on the post-synaptic site, i.e., the shape of the perisomatic spines. This could be known by examining electronmicroscopically, in culture, the Purkinje perisomatic spines, and could demonstrate the extent of plasticity of a terminal during its development.

On the basis of the detailed investigations of the development and synaptic patterns, it is possible to formulate a theory concerning the development, and to describe the factors which are needed for the normal development of the Purkinje neuron.

CHAPTER - 3

MATERIALS AND METHODS:

The mice used in these experiments were from a colony of Paris R III strain.

1) Golgi Cox method: Intact animal

Mice were sacrificed after ether anaesthesia from the time of birth up to 30 days of age, at daily intervals. The cerebellum was removed without delay. It was fixed by immersing in a glutaraldehyde solution for 3 hours.

The solution of glutaraldehyde was a mixture of:

1: 25% glutaraldehyde (Sigma Grade IV)................................ 25 ml.

2: 0.8 gm Sodium hydroxide dissolved in 100 ml, Distilled water....... 19 ml.

3: 2.72 gm Potassium dihydro phosphate dissolved in 100 ml. D.W....... 25 ml.

4: Distilled water .... 31 ml.

The ingredients were mixed rapidly in a magnetic stirrer in the order specified above to avoid precipitation. The ph of the glutaraldehyde solution was adjusted to 7.2. During fixation in the glutaraldehyde solution, the cerebellum was cut in blocks of 3 - 4 mm size. The specimens were labelled as to their origin-vermis, paravermis, peduncle, tip region.

After fixation, blocks were rinsed twice in 0.9% Sodium chloride, and transferred to the Golgi-Cox solution.

The contents of the Golgi-Cox solution were:

a) 5% Potassium dichromate... 1 Part

b) 5% Mercuric chloride.. 1 Part

c) 4% Potassium chromate... 1 Part

d) Distilled water... 2 Parts

Mercuric chloride was dissolved in boiling water. In the boiling solution, potassium dichromate (completely dissolved) was added. The combination was further boiled for 8 - 10 minutes. Additional distilled water was added to account for evaporation. Boiling was stopped at this point and potassium chromate (completely dissolved) along with distilled water was added. The solution was allowed to cool for ½ hour. 1 Block of cerebellum was placed in a stender dish containing 25 ml. of Golgi-Cox solution. The stender dishes were kept in the dark for 18 - 22 days; blocks from newborn to 5 day old mice were kept for 30 - 35 days. After the required number of days, blocks were rinsed thoroughly in distilled water, until the rinse solution was totally clear (at least 20 rinses).

At this stage, blocks were embedded in celloidin, and parasagittal 75 - 100 μ thick sections were prepared.

The sections were blackened for 12 minutes in a freshly prepared solution of 5% potassium sulfite with 8 ml. of 5% oxalic acid per 100 ml. of potassium sulfite.

The sections were then rinsed twice in distilled water. Half of the sections were counterstained in cresyl echt violet for 2 - 3 minutes.

Following this, sections were rinsed in distilled water, rapidly dehydrated by using ascending series of alcohols, cleared in xylene and mounted with permount.

2) Tissue culture studies.

A. Cultures of cerebellum:

Newborn mice were sacrificed after ether anaesthesia. Parasagittal sections of the cerebellum containing cortex, deep nuclear neurons and at times few brain stem neurons (due to their location near the peduncles) were explanted first on the collagen-coated coverslips, and then into the Maximow double coverslip system (Fig. 6).

Figure 6: Plan of dissection of the newborn mouse cerebellum.

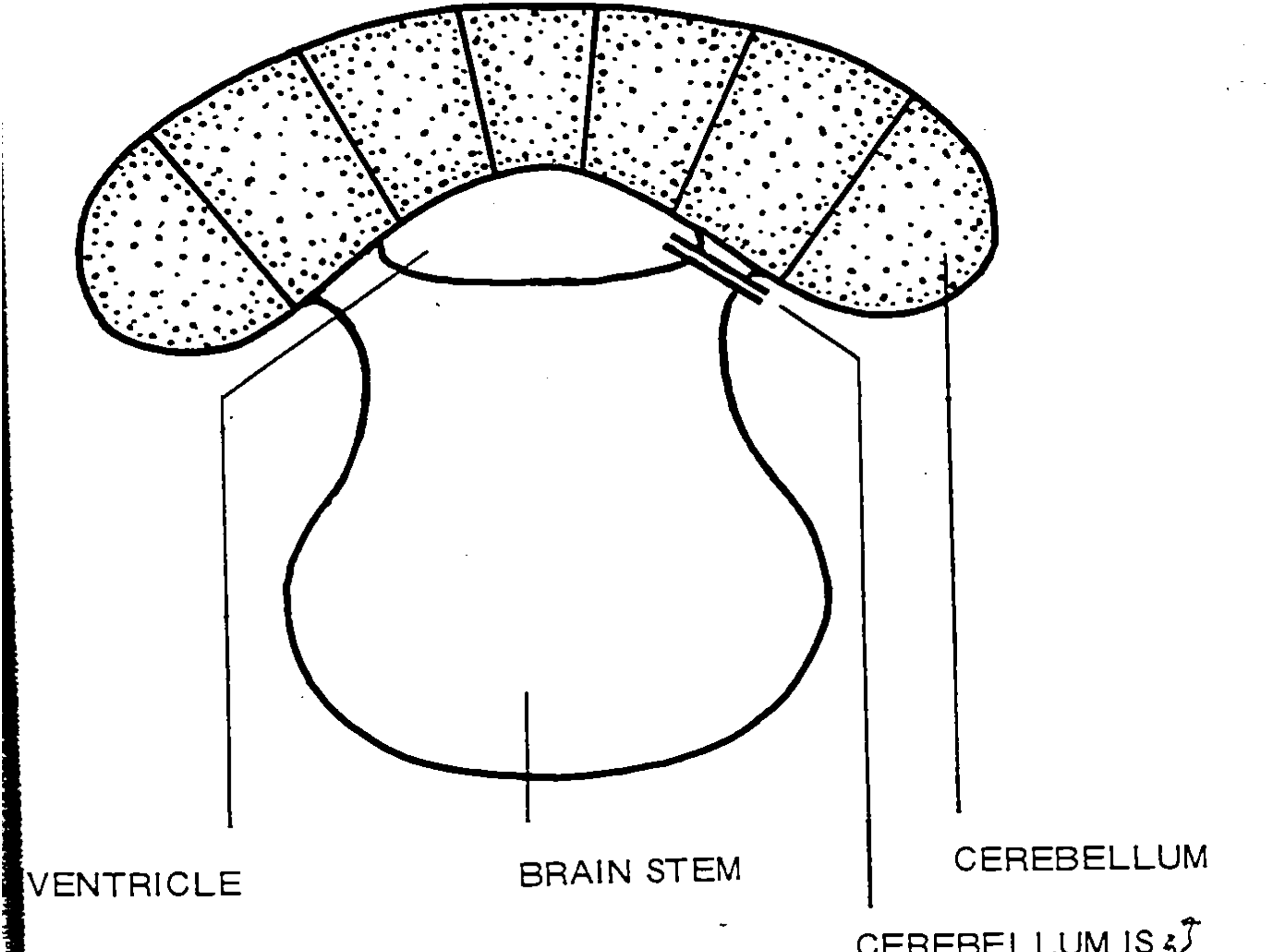


Figure 6

VERY TIGHT BINDING

Standard techniques for the maintainance of cerebellar cultures (Wolf 1964: Allerand 1971) were followed afterwards. The cultures were kept in the incubator set at 35.5 degree Centigrade. They were washed in B.S.S. (Balanced Salt Solution) and fed twice weekly. The nutrient medium consisted of 25% chick embryo extract, 25% human cord serum and 50% Eagles minimum essential medium. It was supplemented with glucose to produce a final concentration of 1000 mgm %. No antibiotics were employed.

Living cultures were examined regularly in the light microscope. Well myelinated cultures with cortical and deep nuclear neurons were selected and mapped. Some cultures also contained brain stem neurons.

B. Golgi-Cox method: Cerebellar culture

1 - 50 days old cultures were impregnated at daily intervals by the same Golgi-Cox method as was used for the in vivo study. Embedding in celloidin and sectioning of the explants was omitted. Cultures were mounted in permount as whole explants.

C. Electronmicroscopy of cerebellar cultures.

Mature cerebellar cultures from day 18 - 30 were washed in phosphate buffer 3 times and fixed in the glutaraldehyde fixative for 30 minutes. At an interval of 15 minutes the glutaraldehyde fixative was changed.

Cultures were then washed in the phosphate buffer 5 - 6 times, in order to remove the glutaraldehyde fixative completely.

The tissue was post-fixed in 1% osmium tetroxide for 1 hour at room temperature. The cultures were washed again 5 - 6 times in the phosphate buffer and were then dehydrated.

SOLUTIONS:

a) The phosphate buffer:

1: 2.26% Sodium phosphate monobasic.................................. 83 ml.

2: 2.52% Sodium hydroxide.. 17 ml.

Total 100 ml.

1.2 gms. of D-Glucose and 1 ml. of 1% Calcium chloride were added to each 100 ml. of the solution. The ph of the phosphate buffer was adjusted to 7.35. The osmolarity of the solution was measured to be 350 - 360 milliosmols.

b) The glutaraldehyde fixative:

In a beaker, to 17 ml. of 2.52% Sodium hydroxide, 1 gm. of Paraformaldehyde was added. The solution was heated to 40 - 60 degrees centigrade, because at this temperature the paraformaldehyde dissolves completely, without polymerizing. The beaker was removed from the hot water bath and following was added to the solution:

1: 83 ml. of 2.26% Sodium phosphate monobasic.

2: 1.2 gms of D-Glucose.

3: 1 ml. of 1% Calcium chloride.

4: 1.5 ml. of 70% glutaraldehyde (Ladd.)

The ph of the glutaraldehyde fixative was adjusted to 7.35.

c) The osmium tetroxide fixative:

To the 100 ml. of Phosphate buffer solution, 1 gm. of osmium tetroxide was added. Just before the start of the fixation, 1.2 gm. of D-Glucose and 1 ml. of 1% Calcium chloride was also added.

For dehydration ~~and~~ embedding, following procedure was done:

1: Wash in 60% Ethyly alcohol .... 3 times.

2: Keep in 60% Ethyl alcohol .... 5 minutes.

3: Change to 70% Ethyl alcohol .... 5 minutes.

4: Change to 95% Ethyl alcohol .... 5 minutes.

5: Change to 100% Ethyl alcohol .... 5 minutes.

6: Another change of 100% Ethyl alcohol for ... 5 minutes.

7: Another change of 100% Ethyl alcohol .... 5 minutes.

8: Change to Propylene Oxide .... 10 minutes.

9: Change to Propylene Oxide .... 10 minutes.

10: Propylene Oxide and Epon (equal parts) ..... 2 hours.

Propylene oxide and epon were changed midway at 1 hour interval. Cultures were kept in pure Epon overnight. The next day cultures were encapsulated in gelatin capsules. The epon was polymerized for 2 days at 35.5 degree centigrade, followed by 3 days at 60 degree centigrade.

For semithin sections, the glass coverslip was removed very carefully from the blocks. 1 μ thick sections were cut with a glass knife on a Reichert O.m-U3 microtome. The sections were stained with toludine blue and examined, until the appropriate area was identified.

For thin sectioning, blocks were further trimmed for the selected cortical area. These were sectioned with a Diamond knife. The sections were placed on Carbon coated grids and were stained with Uranyl acetate (saturated in alcohol) and Lead citrate. For the study of thin sections the Zeiss electron microscope 9A was used.

CHAPTER - 4

RESULTS

A: Golgi study of the development of the Purkinje neuron in vivo.

The postnatal development of the Purkinje neuron has been divided into 5 distinct phases. In fact, the maturation is a continuous process and the phases overlap each other. Thus, at any given time, a Purkinje neuron can be found that is either less mature or more advanced than the average cell of that particular stage. Variations in development do not exceed 1 - 2 days on either side of the average.

The 5 phases are divided as follows:

1: The immature cell (0 - 3 days).

2: The phase of the perisomatic dendrites (4 - 6 days).

3: The phase of the perisomatic spines (7 - 10 days).

4: The phase of the main dendrite (11 - 14 days).

5: The mature neuron (over 15 days).

1: The immature cell (0 - 3 days).

At birth and in the first few days after, the Purkinje neuron is present as an immature cell which rarely stains with the Golgi method. The cells have little or no dendritic plume. They form a zone, 3 - 5 cells wide. They measure 8 - 10 μ in size and are pear shaped. A lightly staining, relatively large nucleus fills the Purkinje soma (Fig. 7a). The axon often stains and even at this early stage, axon collaterals have been seen (Fig. 7b). The early perisomatic dendrites seen at day 1 grow longer and become more numerous by day 2 (Fig. 7c).

Figure 7a: Purkinje neuron from a new born mouse cerebellum. Note that the nucleus is unstained and the axon is emerging from the basal pole of the cell. Bar 20 μ.

Figure 7b: Purkinje neuron from a 1 day old mouse cerebellum. Hypertrophi apical cone is well developed. Bar 20 μ.

Figure 7c: Purkinje neuron from a 2 day old mouse cerebellum. Note the presence of many perisomatic dendrites, emerging from the soma. Bar 20 μ.

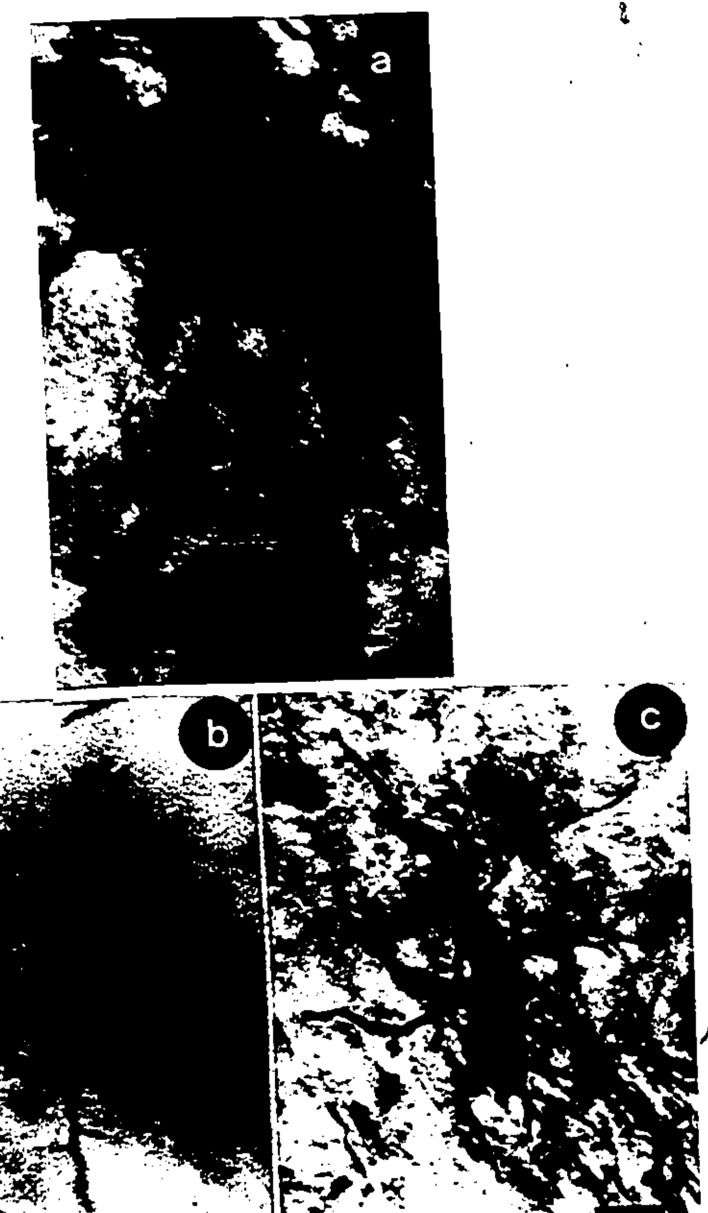


Figure 7

2: The phase of the perisomatic dendrites (4 - 6 days).

Starting in the previous phase, but increasingly apparent at about 4 days, the neuronal perikarya is characterized by laterally extending perisomatic dendrites (Fig. 8a). The perisomatic dendrites continue to grow longer and thicker; they have been measured to be 20 - 60 µ long. These dendrites dramatically alter the appearance of the cell, so much so that by the sixth day the Purkinje neuron resembles a hairy bush, totally lacking in orientation (Fig. 8b). Occasionally, growth cones at the tips of the perisomatic dendrites are also impregnated (Fig. 8b - arrow). Many of the perisomatic dendrites branch and occasionally spines have been seen (Fig. 8b). During this period the Purkinje neurons grow in size, and become aligned in a row. This is the beginning of lamination of the cerebellar cortex. The location of the Purkinje cells is between the forming internal granular and molecular layers. The perisomatic dendrites extend laterally into this molecular layer (Fig. 8a). Towards the later part of this phase, the dendrites are seen near the apical region of the neuron, radiating laterally and towards the pia (Fig. 8c). The basal portion of the soma has comparatively fewer processes (compare Figs. 8a, b and c).

3: The phase of the perisomatic spines (7 - 10 days).

The perisomatic dendrites are no longer present on the soma (Figs. 9a, b, c, d and e). The neuron has acquired an apparent orientation with a very short main dendrite at its apical portion (Fig. 9a). From the main dendrite, many dendritic branches radiate laterally and towards the pia,

Figure 8a: Purkinje neuron from a 4 day old mouse cerebellum. egl-external granular layer, mol-molecular layer, igl-internal granular layer. Bar 20 μ.

Figure 8b: Purkinje neuron from a 6 day old mouse cerebellum. Arrow is pointing to growth cone. Note the presence of few spines on the perisomatic dendrite. Bar 20 μ.

Figure 8c: Purkinje neuron from a 6 day old mouse cerebellum. Although it is from the same aged animal. It is slightly more mature than in Fig. 8b. Bar 20 μ.

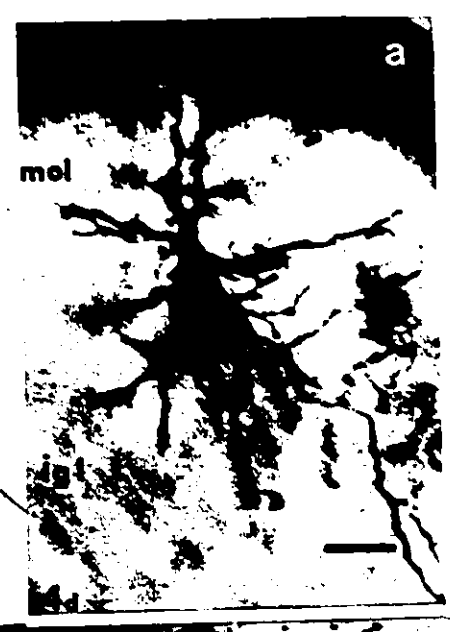


Figure 8

into the molecular layer (Figs. 9a and e). The region of the soma is now characterized by the presence of small, irregular surface projections - the perisomatic spines (Figs. 9b, c and d). These are present all over the soma and are seen clearly between 8 - 10 days of age. Towards the later part of this phase, the perisomatic spines are present mostly near the apical portion of the cell, and very few are present on the basal portion of the cell which appears relatively smooth (compare Figs. 9b, c, d and e).

4: The phase of the main dendrite (11 - 14 days).

The main characteristic of this phase is the development of a stout, long, single main dendrite which is oriented towards the pia (Figs. f, g and h). This main dendrite separates the soma from the spine-laden dendritic branches which are directed either laterally or towards the pia. The Purkinje somatic spines become fewer, in a basal-apical gradation and by day 13, the soma is almost devoid of spines (Fig. 9h). The spines are now seen along the main dendrite; and in all subsequent phases of the development, the main dendrite will continue to have them. At day 13, the main dendrite is much longer that at day 11 (compare Figs. 9f, g and h). The dendritic spines are present mostly on the spiny branchlets, and there are more dendritic spines and spiny branchlets in the lower part than in the upper part of the dendritic tree (Fig. 9g). The dendritic tuft continues to grow towards the pial surface and the molecular layer increases, while the external granular layer decreases.

Figure 9a: Purkinje neuron from a 10 day old mouse cerebellum. Bar 20 µ.

Figure 9b: Purkinje neuron from a 9 day old mouse cerebellum. Note the presence of many perisomatic spines on the soma. Bar 20 µ.

Figure 9c and d: Purkinje neuron from 8 day old mouse cerebellum. Many perisomatic spines are present on the soma.

Figure 9e: Purkinje neuron from a 10 day old mouse cerebellum. Bar 20 µ.

Figure 9f: Purkinje neuron from a 11 day old mouse cerebellum. Arrow is pointing to spines, present on the main dendrite. Bar 20 µ.

Figure 9g: Purkinje neuron from a 11 day old mouse cerebellum. M-main dendrite. 1-primary dendrite, 2-secondary dendrite, 3-tertiar dendrite, 4-spiny branchlets. Several spiny branchlets loaded with spines are present in the lower half of the molecular la Bar 20 µ.

Figure 9h: Purkinje neuron from a 13 day old mouse cerebellum. The long main dendrite is loaded with spines. Bar 20 µ.

a b c d e
10d
10d ANIMAL
f g h
1 2 3 4 M
11d

Figure 9

5: The mature neuron (over 15 days).

A mature neuron is characterized by a soma which is completely smooth in contour, and it measures 18 - 20 μ in size (Figs. 10a and b). The long main dendrite has some spines and many laterally oriented primary dendritic branches, which divide further into secondary and tertiary dendrites (Fig. 10a). Many small branches studded with spines and known as spiny branchlets (labelled 4 in Figs. 9g and 10a) emerge from the tertiary dendrites. In addition some of them originate from secondary and primary dendrites also (Fig. 10a). When this elaborate dendritic tree reaches the pial surface, the Purkinje neuron is considered to be fully mature, and at this time the external granular layer is no longer present.

Figure 10a: Purkinje neuron from a 15 day old mouse cerebellum. Arrows indicate spines on the main and primary dendrite. M-Main dendrite, 1-Primary dendrite, 2-Secondary dendrite, 3-Tertiary dendrite, 4 and 5-spiny branchlets. Bar 20 μ.

Figure 10b: Purkinje neuron from a 26 day old mouse cerebellum. M-Main dendrite. Bar 20 μ.

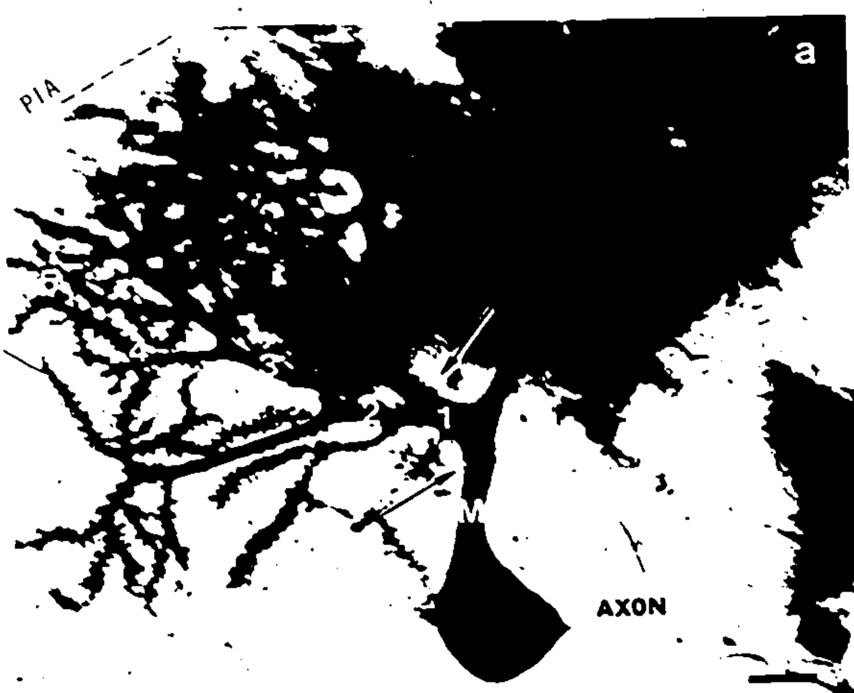


Figure 10

B: Purkinje neurons in culture

(1) Living culture:

In unstained living preparations, it is very difficult to identify cortical areas in a cerebellar explant until 12 - 14 days in vitro. Between day 13 - 15, myelin begins to appear (Fig. 11a). From day 18 onwards, the cortical area containing Purkinje neurons, interneurons and neuropil is visible (Figs. 11b, c and d). Purkinje neurons are usually not aligned in the cortical area, but are distributed in a random manner. They measure 18 - 20 µ in size and are round or oval in shape. A prominent nucleus, measuring 8 - 10 µ in size, is surrounded by cytoplasm which is faintly granular (Figs. 11b, c and d). One or two spherical nucleoli are also discernible in the nucleus. The neuropil which surrounds these neurons has a distinct mottled appearance (Figs. 11b, c and d).

(2) Golgi study of the development of the Purkinje neuron in vitro.

The analysis of the cultures in Golgi preparations was complicated by the fact that there was apparently a failure of the cortical elements to laminate (Fig. 12a). This was more of a problem in the earlier days of the culture, because the cells were less typical in appearance. In many cultures of 20 days or more, it was possible to identify a cortical area and a deep nuclear region (Figs. 12b and c). The cortical area was characterized by the presence of Purkinje neurons, Golgi cells, granule cells and stellate neurons. All of the neuronal elements in the cortical area were identified, using some of the morphological characteristics

Figure 11a: Myelinated axons in a unstained living preparation. 22 DIV.

Figure 11b: Cortical area in a culture. Arrow points towards a Purkinje neuron. 18 DIV.

Figure 11c: Cortical area in a culture. Arrow points towards a Purkinje neuron. 18 DIV.

Figure 11d: Cortical area in a culture. Arrow points towards a Purkinje neuron. Note the mottled appearance of the neuropil.

Bar. 20 μ and is applicable to Figs. 11a, b, c and d.

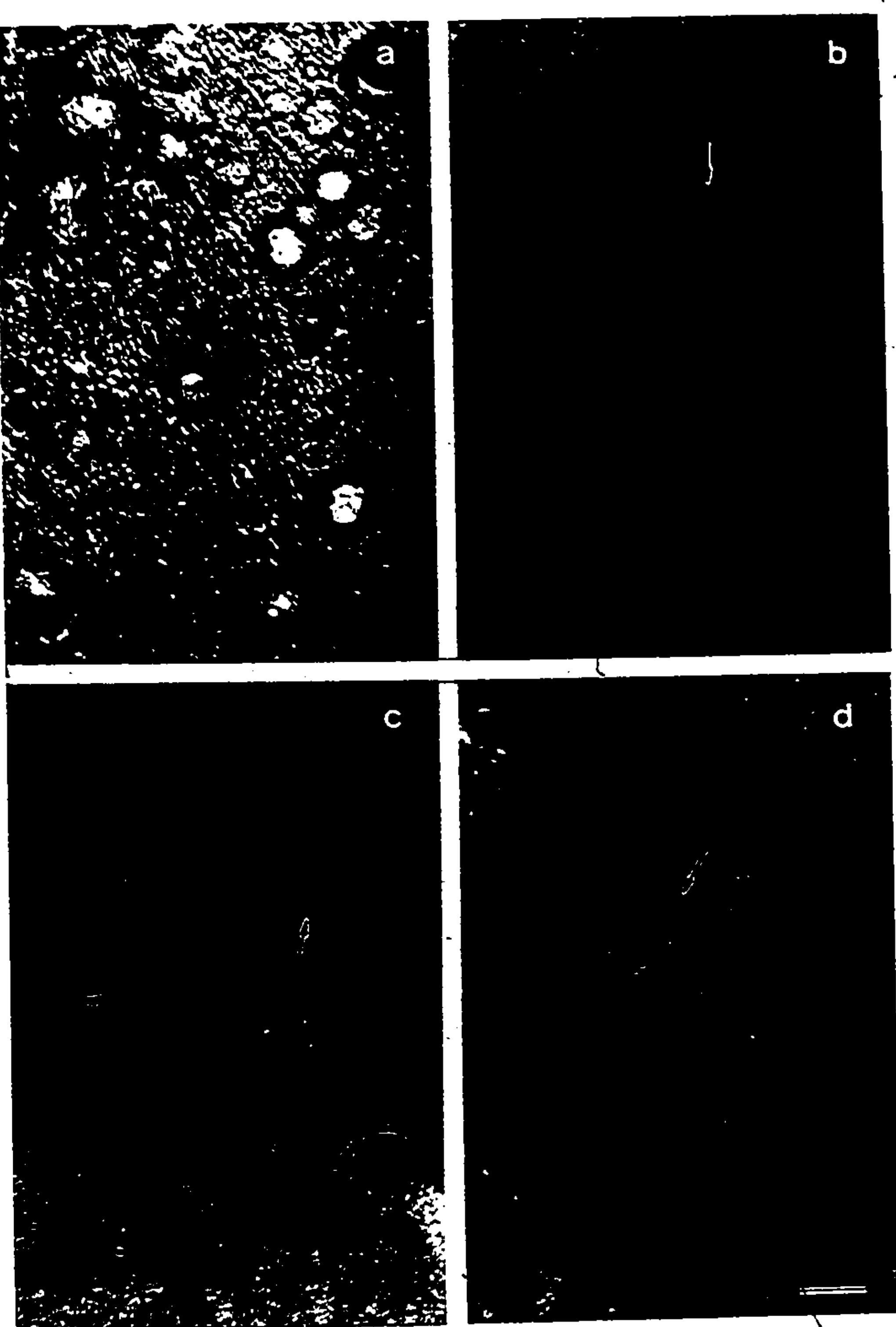


Figure 11

described in the adult animal (Palay and Chan-Palay 1974). Although a quantitative analysis has not been made, the Purkinje neurons were apparently impregnated more frequently in comparison to the other neuronal elements. The perikaryal measurements were found to correspond with the Purkinje neurons of the intact animal, and with the measurements cited in the literature (Addison 1911).

The developmental period of the Purkinje neurons in vitro could only be divided in 4 phases, because the 5th phase of the development of the Purkinje neuron could not be delineated from the 4th phase. Therefore, the 4th and the 5th phase have been described together.

1: The immature cell (0 - 3 days)

2: The phase of the perisomatic dendrites (4 - 6 days)

3: The phase of the perisomatic spines (7 - 10 days)

4: The phase of the main dendrite, and the mature neuron (over 11 days)

1: The immature cell (0 - 3 days)

In this phase, the appearance and size of the Purkinje cell match precisely with their counterparts in vivo (Fig. 13a). The axon in its usual manner emerges from the "basal" pole of the cell (Fig. 13b). A few perisomatic dendrites emerging from the Purkinje soma are also discernible (Fig. 13b).

2: The phase of the perisomatic dendrites (4 - 6 days)

In this phase, the Purkinje soma is characterized by the presence of long perisomatic dendrites (Fig. 13c), which emerge in all directions

Figure 12a: Low magnification of the cortex region of a cerebellar culture. 18 DIV. Numbered neurons are the Golgi impregnated Purkinje cells. Note the absence of the cortical lamination, and the absence of the alignement of the Purkinje neurons. Bar 20 μ.

Figure 12b: Low power view of a Golgi impregnated explant of cerebellum. cor-cortical region, DN-deep nuclear region. 18 DIV.

Figure 12c: Low power view of a Golgi impregnated explant of cerebellum. cor-cortical region, DN-deep nuclear region. 21 DIV. Bar 500 is applicable to Fig. 12 b also.

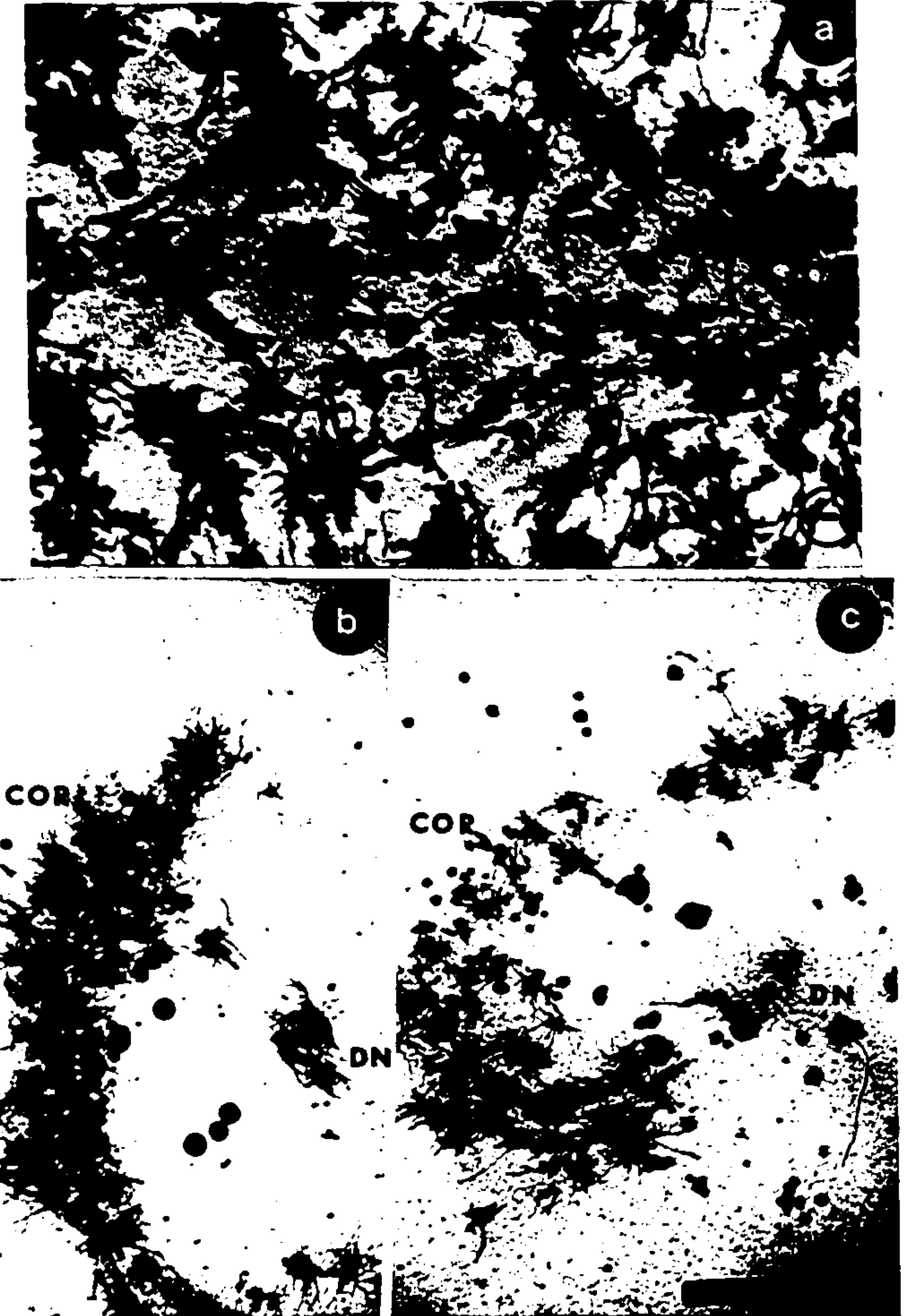


Figure 12

from the perikaryal region. They continue to grow thicker and longer (Fig. 13d). Although, in this phase, the individual neuronal morphology is similar to its counterpart in vivo, there is generally an absence of layer formation in culture (Fig. 12a). This feature is in a sharp contrast to the development in vivo.

3: The phase of the perisomatic spines (7 - 10 days)

In culture, the perisomatic dendrites remain attached to the soma in this phase (Fig. 14a). In addition, the perisomatic spines begin to emerge from the soma (Fig. 14b). There is an increase in size of the perikaryon also, which now measures 12 - 16 µ.

4: The phase of the main dendrite and the mature neuron (over 11 days)

In this phase, all Purkinje neurons from day 13 - 41 have been grouped together (Figs. 14c, d, e, 15a, b, c and d). The 4th phase cannot be seperated from the 5th phase, since the Purkinje neuron in culture usually fails to develop a stout, long main dendrite (Figs. 14c, d, e, 15a, b, c and d). However, sometimes a very short main dendrite can be found (Fig. 14c). The perikaryon increases further in size and is now between 18 - 20 µ. It is characterized by the presence of many perisomatic spines and perisomatic dendrites. The dendrites continue to increase in length and thickness, branch one or two times, and become loaded with spines (Figs. 14c, d, e, 15a, b, c and d). Generally, in culture the dendritic tree is much smaller, because of the underdevelopment of tertiary dendrites and spiny branchlets. Since there is no pial surface, there is no single time when maturation is considered complete.

Although in culture, all Purkinje neurons from day 13 - 41 look very different from their counterparts in vivo, essentially all of them have 5 characteristics in common.

These are:

1) The presence of the perisomatic spines on the soma.

2) The presence of the perisomatic dendrites on the soma.

3) The branching of and the presence of the spines on the perisomatic dendrites.

4) The absence of a stout, long, single main dendrite.

5) The perikaryal size which is between 18 - 20 μ.

Figure 13a: Purkinje cell. 1 DIV. The axon is emerging from the basal pole of the cell. Bar 20 μ.

Figure 13b: Purkinje cell. 2 DIV. The hypertrophic apical cone is well developed. Bar 20 μ.

Figure 13c: Purkinje cell. 4 DIV. Many perisomatic dendrites are emerging from the soma. Bar 20 μ.

Figure 13d: Purkinje cell. 7 DIV. Bar 20 μ.

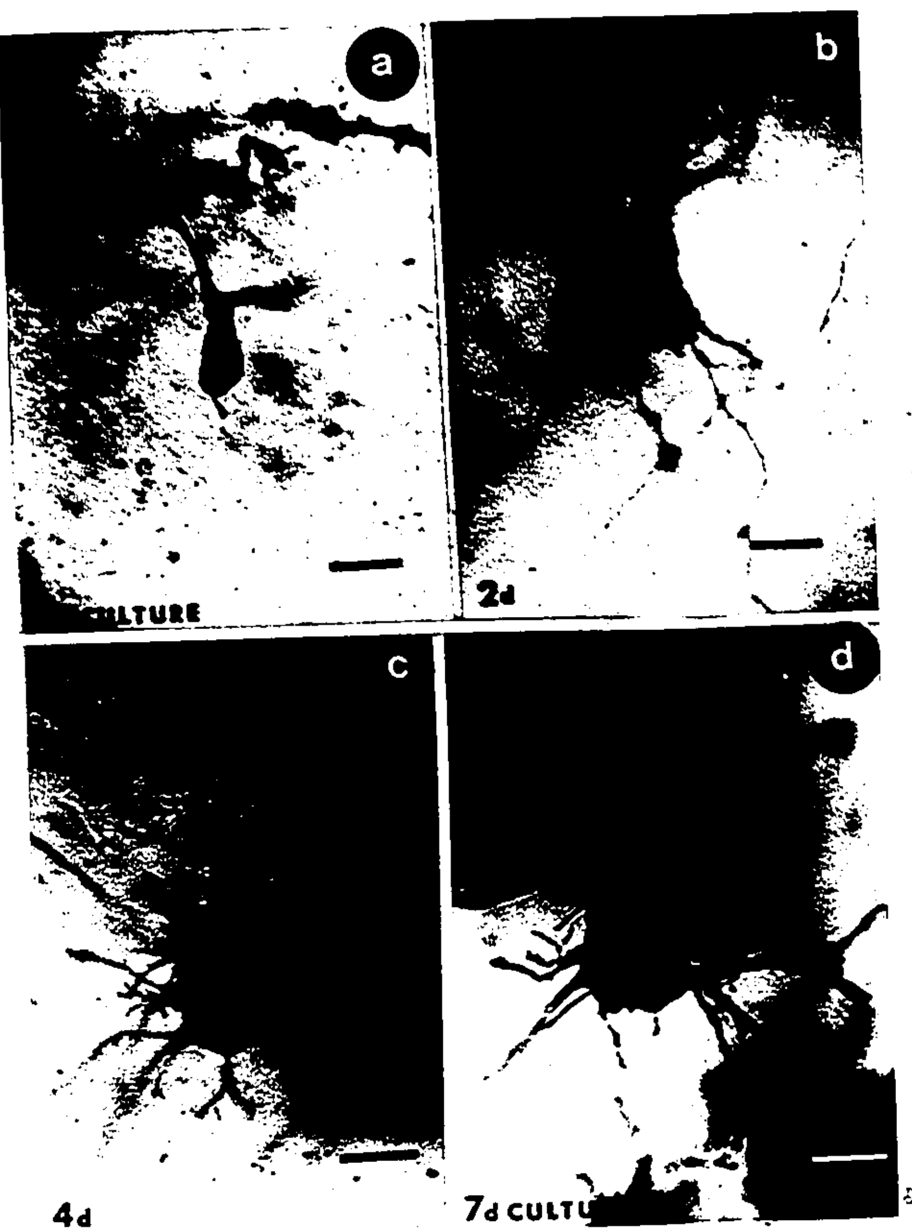


Figure 13

Figure 14a: Purkinje cell. 9 DIV. Many perisomatic dendrites are still present on the soma. Bar 20 μ.

Figure 14b: Purkinje cell. 9 DIV. Note the presence of many perisomatic spines on the soma. Bar 20 μ.

Figure 14c: Purkinje cell. 21 DIV. Many spines are present on the soma and dendrites, ax-axon. Bar 20 μ.

Figure 14d: Purkinje cell. 13 DIV. Note the presence of many spines on the perisomatic dendrites. Bar 20 μ.

Figure 14e: Purkinje cell. 18 DIV. Note branching of the perisomatic dendrites which are loaded with spines. Bar 20 μ.

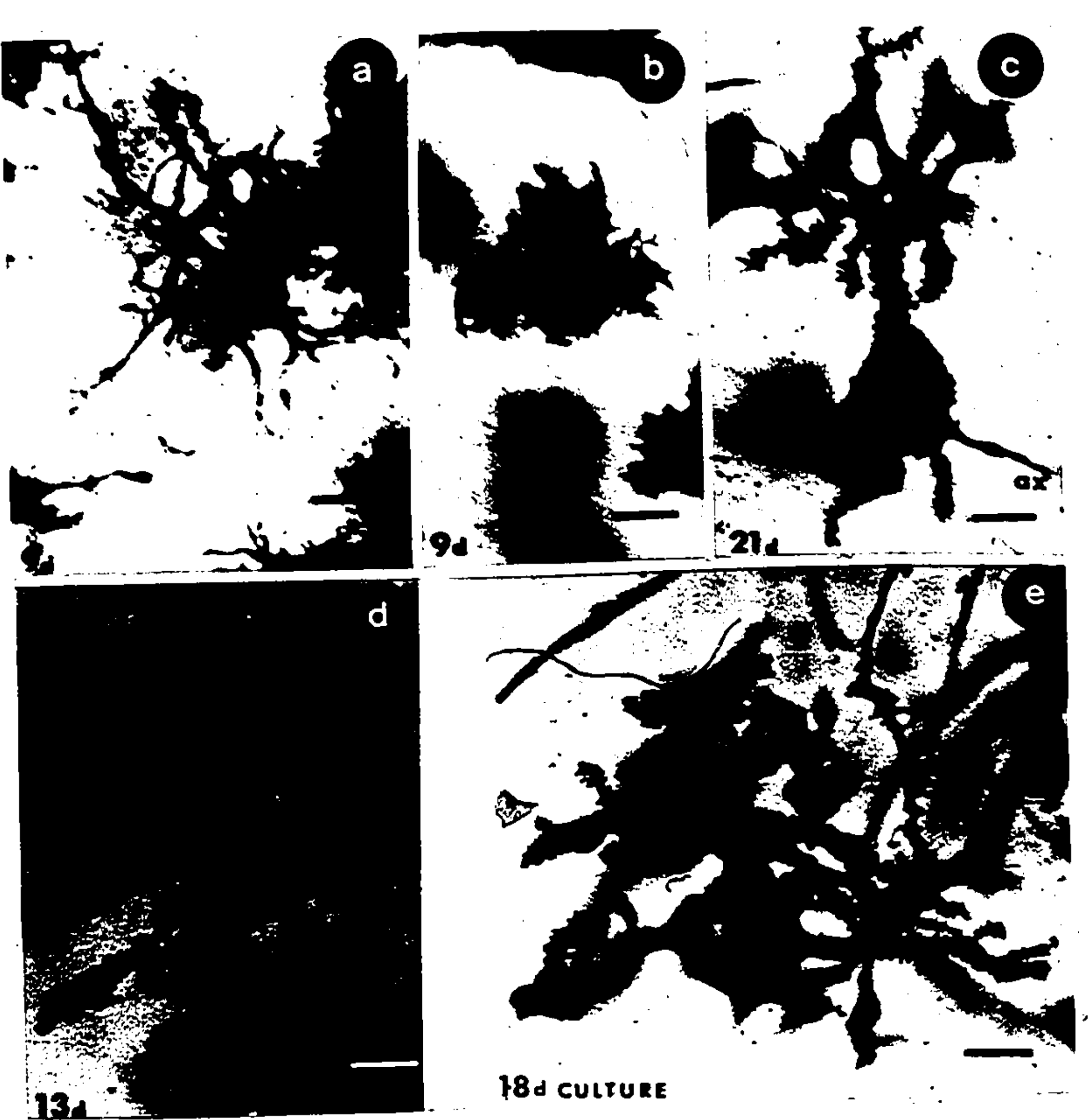


Figure 14

Figure 15a: Purkinje cell. 23 DIV. In the inset, note the presence of some perisomatic spines, seen in another plane of focus. Bar 20 μ.

Figure 15b: Purkinje cell. Arrows are pointing to perisomatic spines. 35 DIV. Bar 20 μ.

Figure 15c: Purkinje cell. Note the dendrites which are loaded with spines. 35 DIV. Bar 20 μ.

Figure 15d: Purkinje cell which has many dendrites. Other dendrites emerging from the soma are out of focus in this plane. 41 DIV. Bar 20 μ.

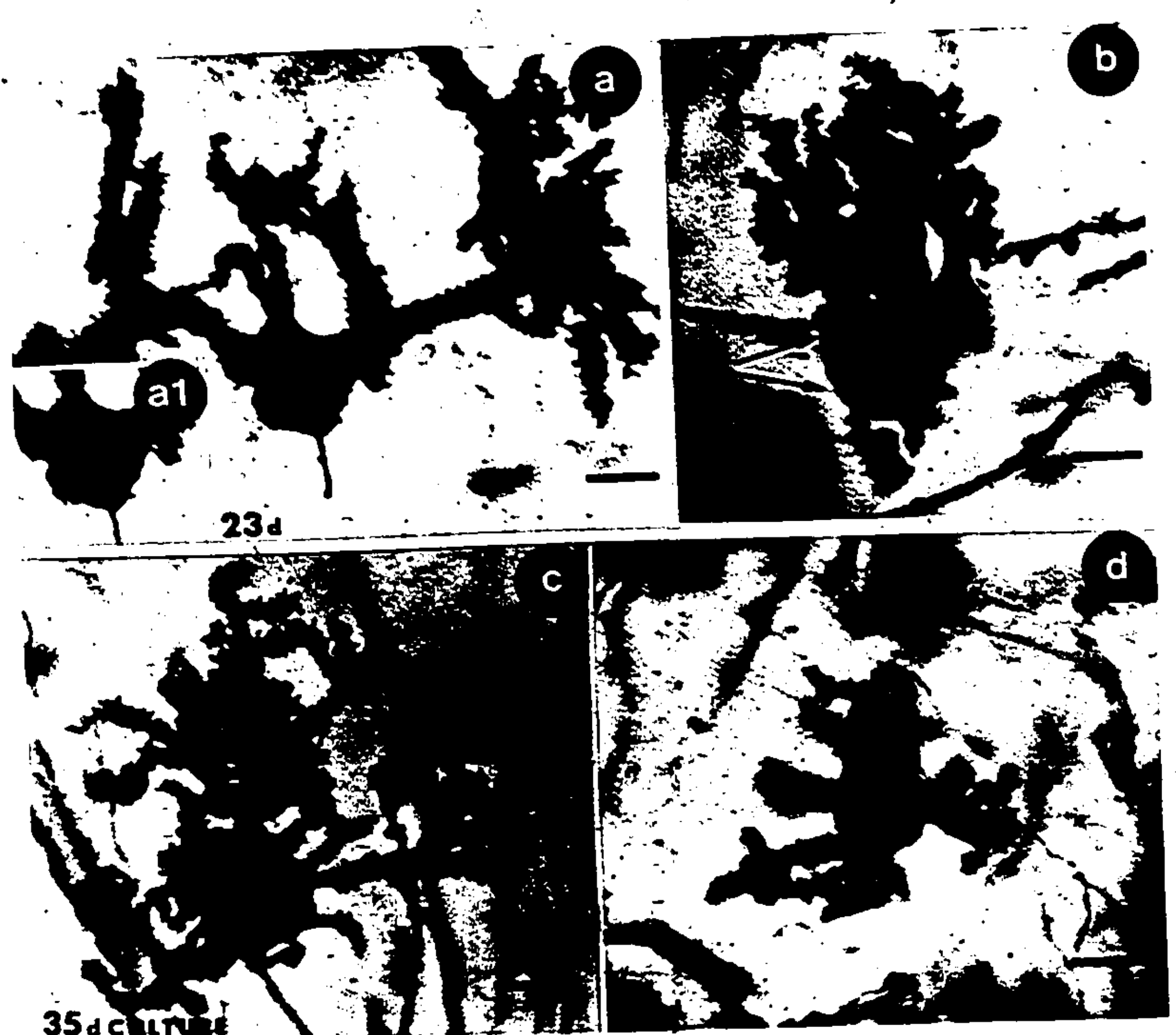


Figure 15

C: A comparison of the development of the Purkinje neuron in culture with in vivo:

In order to compare the developmental progress, the photographs of the Purkinje neuron of the same age, in culture and in vivo were arranged as follows. The developmental sequence of the Purkinje neuron in vivo has been placed in the upper row and that in culture in the lower row (Figs. 16, 17, 18 and 19). A comparison demonstrates that the individual neuronal morphology of the Purkinje neuron in culture develops at the same speed with which it develops in vivo up to 4 days (phase 2). The apical cone and many perisomatic dendrites emerging from the soma can be observed (compare Figs. 16D and d). Towards the later part of phase 2 in culture, the Purkinje neuron fails to reorganize its perisomatic dendrites towards the apical cone (compare Figs. 17F and e); and the long and thick perisomatic dendrites continue to be present on the "basal" portion of the soma. In phase 3, there is continued failure of the Purkinje neuron to reorganize and the perisomatic dendrites remain attached to the soma (compare Figs. 17G, H and f). In culture, amidst the perisomatic dendrites, between day 8 - 9, the perisomatic spines begin to emerge from the soma (Fig. 17g). Generally, in vivo the perisomatic spines begin to emerge from the soma, only after the location of the perisomatic dendrites has changed (Figs. 17G, G1, G2 and H). The formation of the perisomatic spines in culture indicates that the maturation process of the Purkinje neuron has continued to progress, in spite of the failure of the Purkinje neuron to reorganize. In phase 4 in vivo, the Purkinje neuron acquires a long main dendrite (Figs. 18J, k, and K1), whereas in culture, the Purkinje neuron fails to acquire a stout long

main dendrite (Figs. 17h, 18i, j, k, 19l, m and n). Occasionally, a very short main dendrite many form (Fig. 18j).

In culture, phase 4 could not be delineated from phase 5, because all Purkinje neurons from day 13 - 41 had similar characteristics (Figs. 17h, 18i, j, k, 19l, m and n). In phase 4 plus 5, there is further increase in the perikaryal size, elongation and branching of the perisomatic dendrites and formation of dendritic spines, indicating that the maturation of the Purkinje neuron has continued to progress in culture. Generally, in vivo a mature Purkinje neuron has a main dendrite, primary, secondary and tertiary branches, and spiny branchlets (Figs. 18k and 19L). The spines are present mostly on the spiny branchlets, and few are present on the tertiary, secondary and primary dendrites. In culture, all of the Purkinje dendrites and their branches are loaded with spines and there is a reduction in the number of tertiary dendrites and spiny branchlets (compare Figs. 18K, 19L and 17h, 18i, j, k, 19l, m and n). Thus, in culture the dendritic tree of the Purkinje neuron is not as elaborate as it is in vivo, it does not have a long, stout main dendrite, and all its branches are loaded with spines.

Since in culture, the Purkinje soma attains its mature size of 18 - 20 μ, develops the perisomatic spines, and the perisomatic dendrites which in turn branch and have spines therefore it is possible to conclude that it is mature. However, in culture the Purkinje neuron has not reorganized, it retains its perisomatic spines and perisomatic dendrites on the soma, and it fails to develop an apical main dendrite. Therefore the mature shape appears very different and modified from its counterpart in vivo.

Figure 16A: Purkinje cell. New born mouse. Bar 20 μ.

Figure 16B: Purkinje cell. 1 day old mouse. Bar 20 μ.

Figure 16b: Purkinje cell. 1 day culture. Bar 20 μ.

Figure 16C: Purkinje cell. 2 day old mouse. Bar 20 μ.

Figure 16c: Purkinje cell. 2 day culture. Bar 20 μ.

Figure 16D: Purkinje cell, egl-external granular layer, mol-molecular layer, igl-internal granular layer. 4 day old mouse. Bar 20 μ.

Figure 16d: Purkinje cell. 4 day culture. Bar 20 μ.

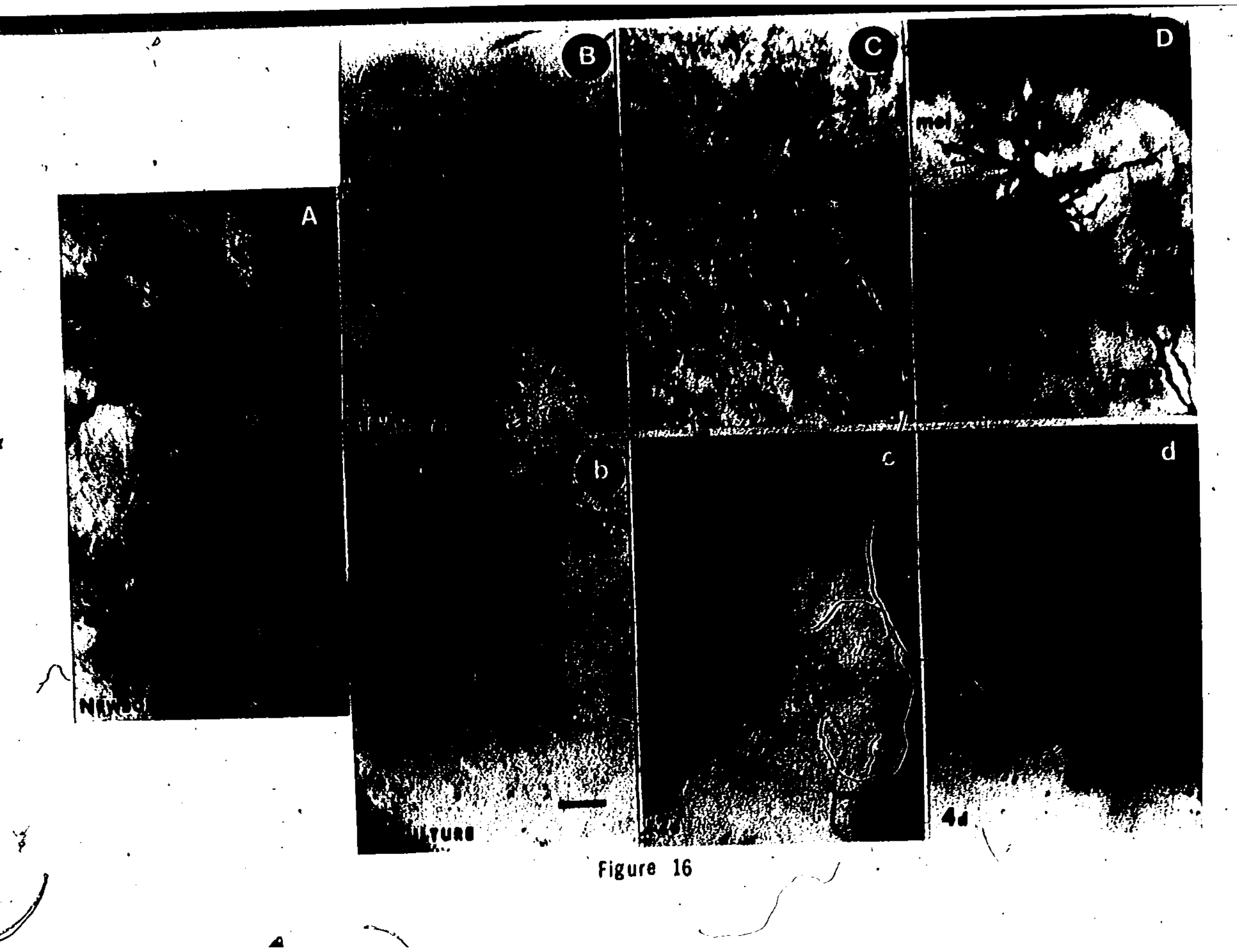


Figure 16

Figure 17E: Purkinje cell. Arrow is pointing towards a growth cone. 6 day old mouse. Bar 20 μ.

Figure 17e: Purkinje cell. 7 day culture. Bar 20 μ.

Figure 17F: Purkinje cell. 6 day old mouse. Bar 20 μ.

Figure 17f: Purkinje cell. 9 day culture. Bar 20 μ.

Figure 17G: Purkinje cell. 9 day old mouse. Bar 20 μ.

Figure 17 Gland G2: Purkinje cell. 8 day old mouse.

Figure 17g: Purkinje cell. Arrow is pointing towards perisomatic spines. 9 day culture. Bar 20 μ.

Figure 17H: Purkinje cell. 10 day old mouse. Bar 20 μ.

Figure 17h: Purkinje cell. 13 day culture. Bar 20 μ.

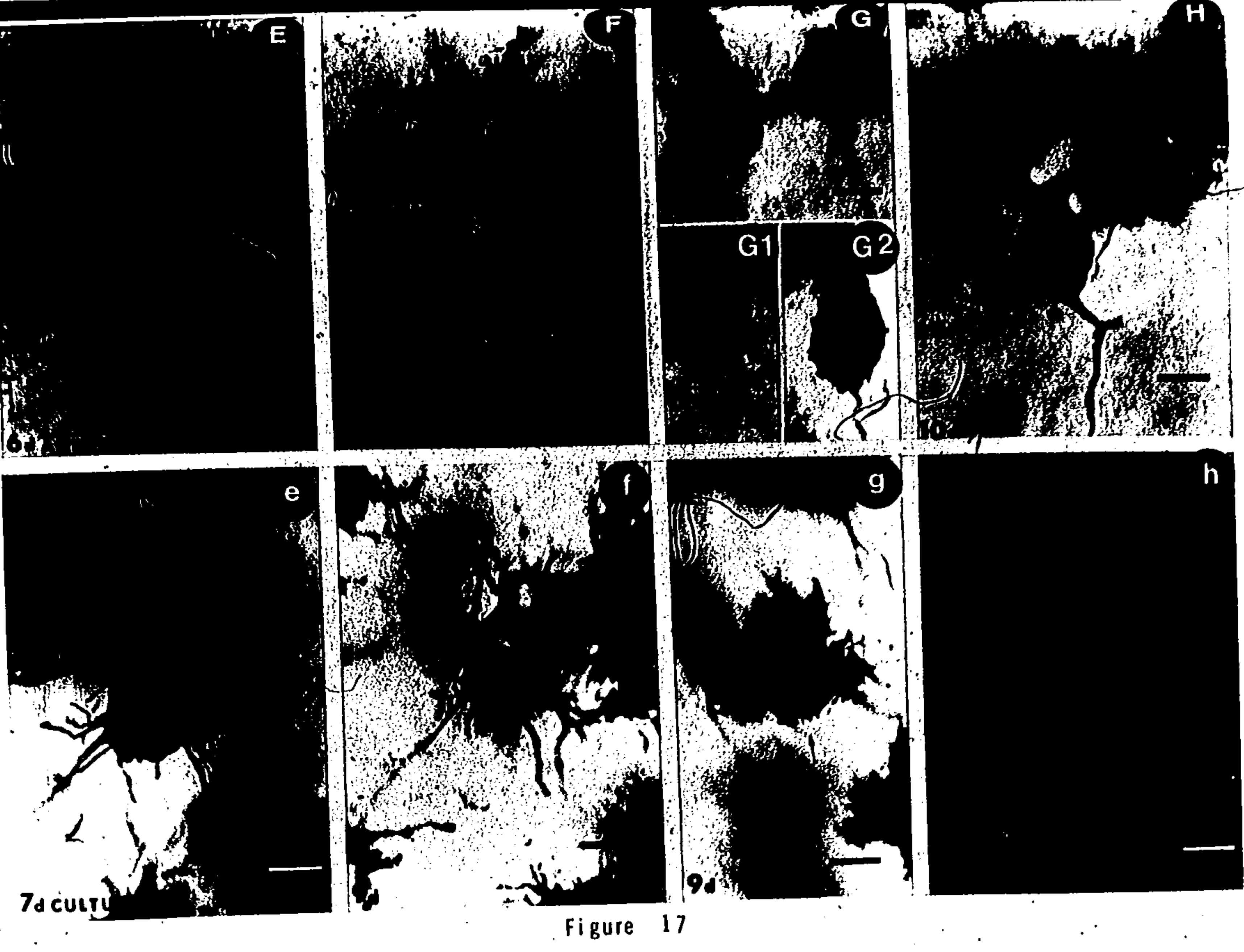


Figure 17

Figure 18I: Purkinje cell. 10 day old mouse. Bar 20 μ.

Figure 18i: Purkinje cell. 18 day culture. Bar 20 μ.

Figure 18J: Purkinje cell. 11 day old mouse. Arrow is pointing to spines on the main dendrite. Bar 20 μ.

Figure 18j: Purkinje cell. 21 day culture. ax-axon, Bar 20 μ.

Figure 18K: Purkinje cell. 11 day old mouse. M-main dendrite, 1-primary dendrite, 2-secondary dendrite, 3-tertiary dendrite, 4-spiny branchlet. Bar 20 μ.

Figure 18K1: Purkinje cell. 13 day old mouse. Bar 20 μ.

Figure 18 K: Purkinje cell. 23 day culture. Inset K1-Soma of the same Purkinje neuron as in Fig. 18K, in another plane of focus. Bar 20 μ.

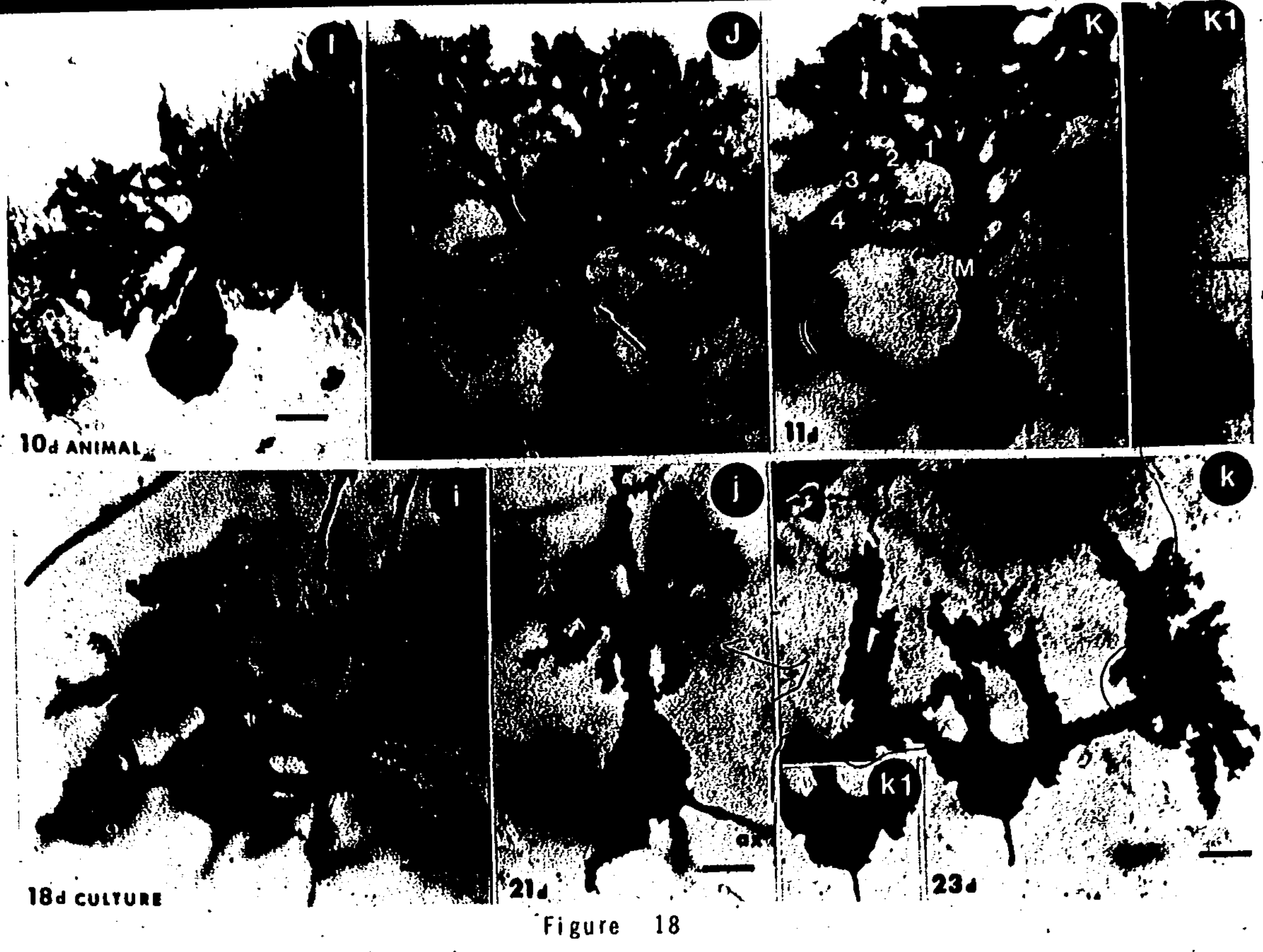


Figure 18

Figure 19L: Purkinje cell. 15 day old mouse. Arrows are pointing to spines, present on the main and primary dendrite. M-main dendrite, 1-primary dendrite, 2-secondary dendrite, 3-tertiary dendrite, 4 and 5-spiny branchlets. Bar 20 μ.

Figure 19l: Purkinje cell, 35 day culture. Bar 20 μ.

Figure 19M: Purkinje cell, 26 day old mouse. Bar 20 μ.

Figure 19m: Purkinje cell. 35 day culture. Arrows are pointing towards perisomatic spines. Bar 20 μ.

Figure 19n: Purkinje cell. 41 day culture. Some dendrites are out of focus in this plane. Bar 20 μ.

Figure 19

D: Study of the semithin sections of the cortical area of the mature cerebellar culture.

Light microscopically in semithin sections, the cortical area is recognized by the presence of small granule cells which are grouped in clusters of 3 - 5 (Figs. 20a and b). Scattered among these are the Purkinje neurons. They are identified on the basis of their large size (Figs. 20a and b). In majority of the cultures, the trilaminar pattern of the cerebellar cortex has not been seen. However, rarely in a culture, a small area which gives the appearance of a trilaminar pattern may be found. Similar to the results of Golgi material in culture many dendrites emerge from the Purkinje soma (Figs. 14c, d, e, 15a, b, c and d) and they radiate in all directions; in a given plane of a semithin section, 2 dendrites can be seen emerging from the Purkinje soma (labelled Pu. in Fig. 20b). The Purkinje neurons measure 18 - 20 μ in size and contain a lightly staining pale nucleus which is surrounded by a moderately basophillic rim of cytoplasm. The nucleoli present within the nucleus are intensely basophillic. Amongst the smaller group of cells, there are few stellate neurons, and in the larger group there are a few Golgi neurons and glia. All of the neurons and glia are surrounded by neuropil which gives a mottled appearance to the cortical area in a living preparation.

Figure 20 a: 1 μ section of the cortical area of a culture. 21 DIV. Bar 20 μ.

Figure 20 b: High magnification of the same area, seen in Fig. 20 a. Pu-Purkinje cell, Gr-clumps of granule cells. Bar 20 μ.

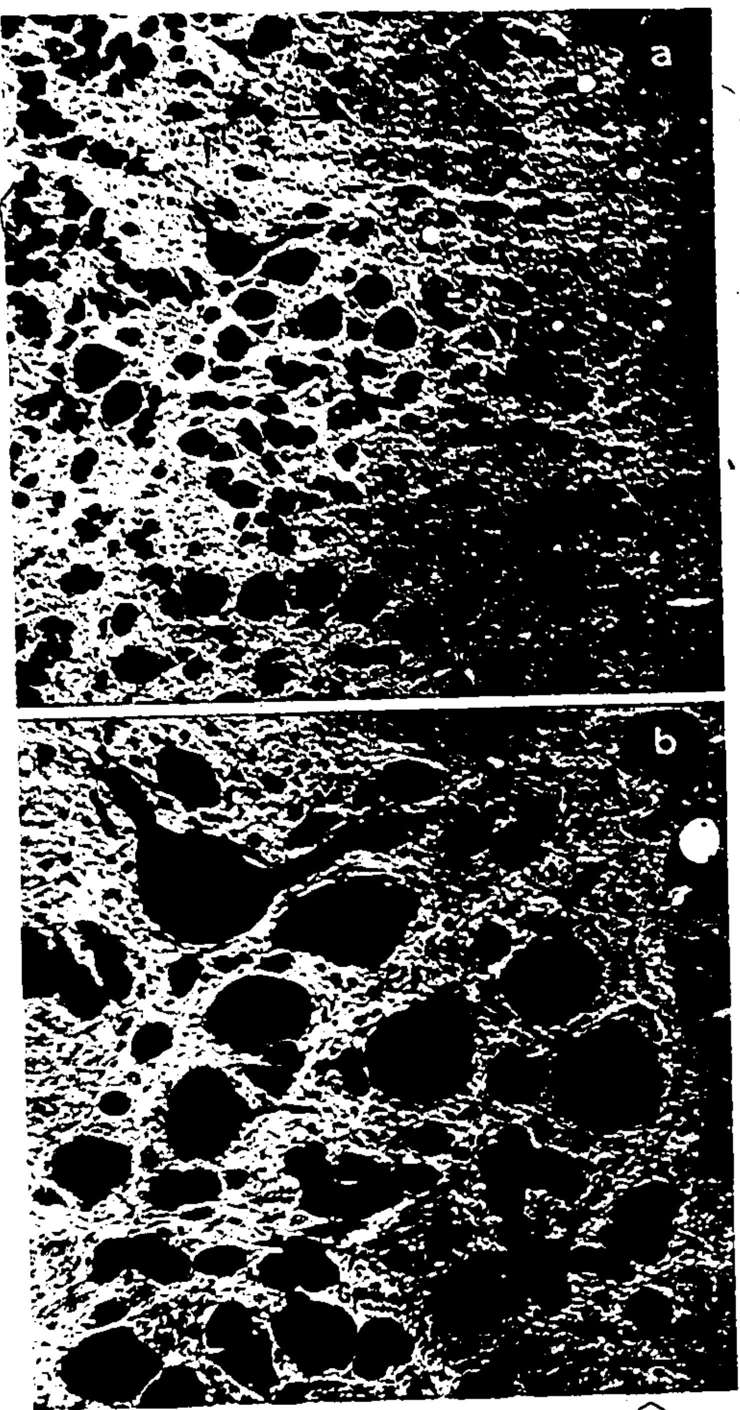


Figure 20

E: Electron microscopical study of the Purkinje neuron in culture.

1) Ultrastructure of the Purkinje soma:

In electronmicroscopic examination, Purkinje neurons were found to be large (Figs. 21 and 22). The nuclear chromatin was homogenously and thinly dispersed throughout the karyoplasm. The nucleus contained 1 - 2 spherical nucleoli which were occasionally located near the nuclear envelope. The nuclear cap consisting of a wrinkled nuclear membrane and Nissl substance was also present. In the cytoplasm of the Purkinje cell, the Nissl substance consisting of granular endoplasmic reticulum and ribosomes was highly diffuse. The agranular endoplasmic reticulum formed a loose-mesh network throughout. At the periphery of the cell, the agranular reticulum was spread into broad, discontinuous cisternae which lay parallel and beneath the plasmalemma. These have been called hypolemmal cisternae, and have been considered a specific characteristic of the Purkinje neuron. They were first reported in the Purkinje cell of the mormyrid fish (Kaiserman-Abramof and Palay 1969). Other organelles, such as mitochondria, Golgi apparatus and lysosomes were also present in the cytoplasm.

Figure 21: Electron micrograph of a Purkinje neuron.
38 DIV. Bar 1 µ.

Figure 21

Figure 22: Electron micrograph of a Purkinje neuron.

38 DIV. Bar 1 μ.

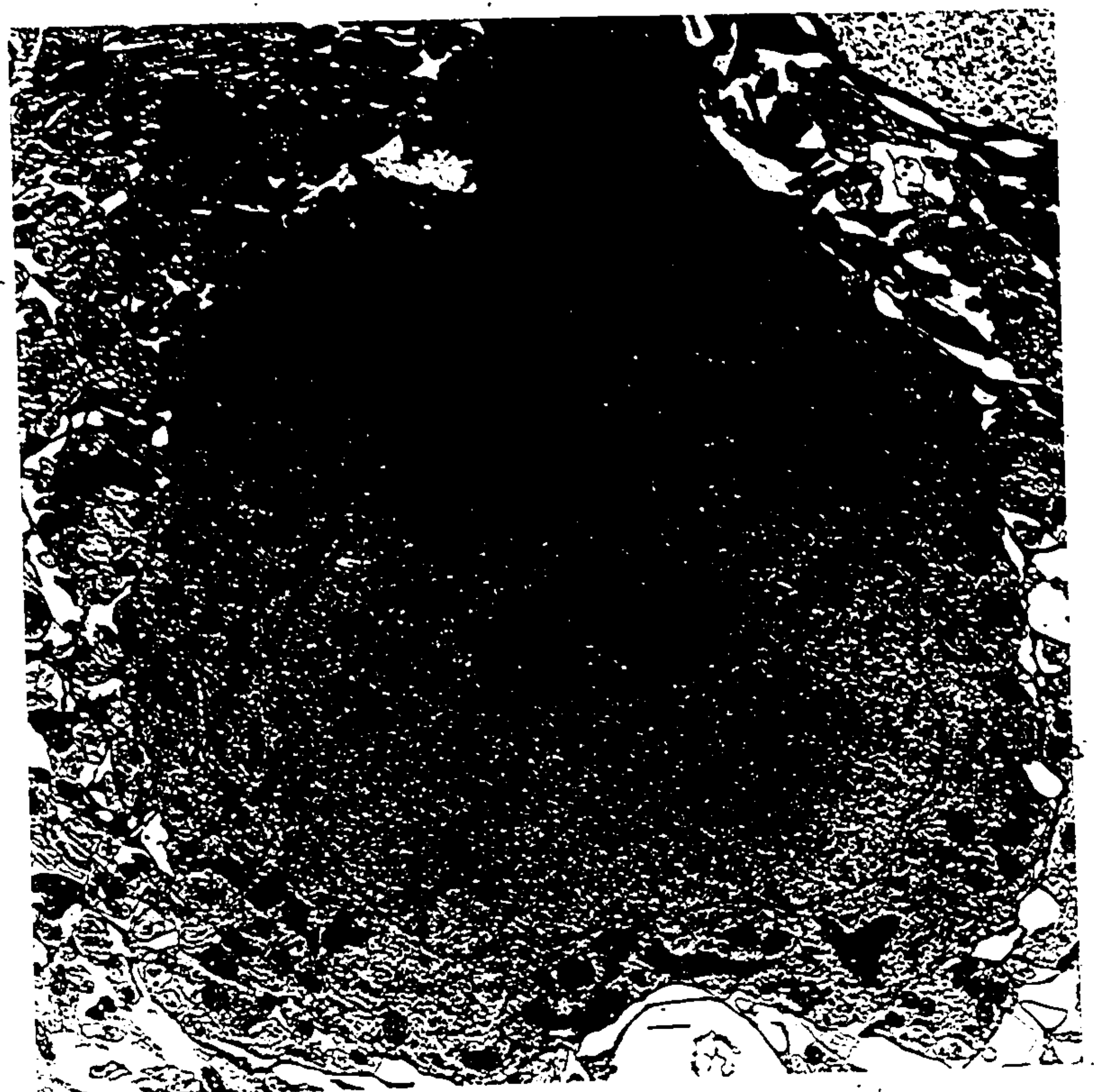


Figure 22

2) Electron microscopic study of the surface of the Purkinje soma.

Many perisomatic spines were present on the surface of the Purkinje soma (Figs. 21, 22, 23a, b, c, 24a, b, c, d and e). These were long and had a rounded head, and were either straight or slightly curved like a hook. Sometimes, they occured alone, but generally they were found in clusters of 2 - 5, and typically contained extensions of agranular reticulum. All of the perisomatic spines have been classified into 4 types. The classification is based upon the presence of either a pre-synaptic terminal or glia, and the presence of the post-synaptic web.

a) Perisomatic spine without a sheath of glia, post-synaptic web or a pre-synaptic terminal (Fig. 23a).

b) Perisomatic spine surrounded by a sheath of glia, but without post-synaptic web and a pre-synaptic terminal (Fig. 23b).

c) Perisomatic spine with a post-synaptic web and surrounded by a sheath of glia (Fig. 23c). The intersynaptic cleft is wide and the post-synaptic density is clearly visible.

d) Perisomatic spines surrounded by a pre-synaptic terminal and with a post-synaptic web (Figs. 21, 22, 24a, b, c, d and e). This type of perisomatic spine was seen most frequently. The pre-synaptic terminals contained mitochondria and were filled with round vesicles, which aggregated near the pre-synaptic membrane. The synaptic cleft was wide with a prominent post-synaptic web (Fig. 24b- arrow). This type of synaptic contact has been classified as the asymmetric type (Colonnier 1968). Generally, the pre-synaptic terminal surrounded a cluster of 2 to 5 perisomatic spines. At times 2 pre-synaptic terminals were seen synaptically in contact with 2 clusters of perisomatic

Figure 23a: Electron micrograph of a Purkinje perisomatic spine which is without a sheath of glia, post-synaptic web or a pre-synaptic terminal. 21 DIV. Bar 1 μ.

Figure 23b: Electron micrograph of a Purkinje perisomatic spine which is surrounded by a sheath of glia, but is without a post-synaptic web and pre-synaptic terminal. 38 DIV. Bar 1 μ.

Figure 23c: Electron micrograph of a Purkinje perisomatic spine which is enveloped in a sheath of glia. Note the presence of the inter-synaptic cleft and the post-synaptic density, indicated by arrow. 38 DIV. Bar 1 μ.

Figure 23

Figure 24a: Electron micrograph of 2 clusters of Purkinje perisomatic spines. They are receiving synaptic contact from pre-synaptic terminals. 38 DIV. Bar 1 μ.

Figure 24b: Electron micrograph of two pre-synaptic terminals which are contacting two clusters of perisomatic spines. One of the spine is attached to the soma in this plane of section. Arrow is pointing towards asymmetrical-type synapse. 38 DIV. Bar 1 μ.

Figure 24c: Electron micrograph of a cluster of Purkinje perisomatic spines seen in Fig. 22. A pre-synaptic terminal is forming synaptic contacts with them. 38 DIV. Bar 1 μ.

Figure 24d: Electron micrograph of a cluster of Purkinje perisomatic spines seen in Fig. 24a (lower cluster). 38 DIV. Bar 1 μ.

Figure 24e: Electron micrograph of a long Purkinje perisomatic spine which is receiving an afferent fiber. 21 DIV. Bar 1 μ.

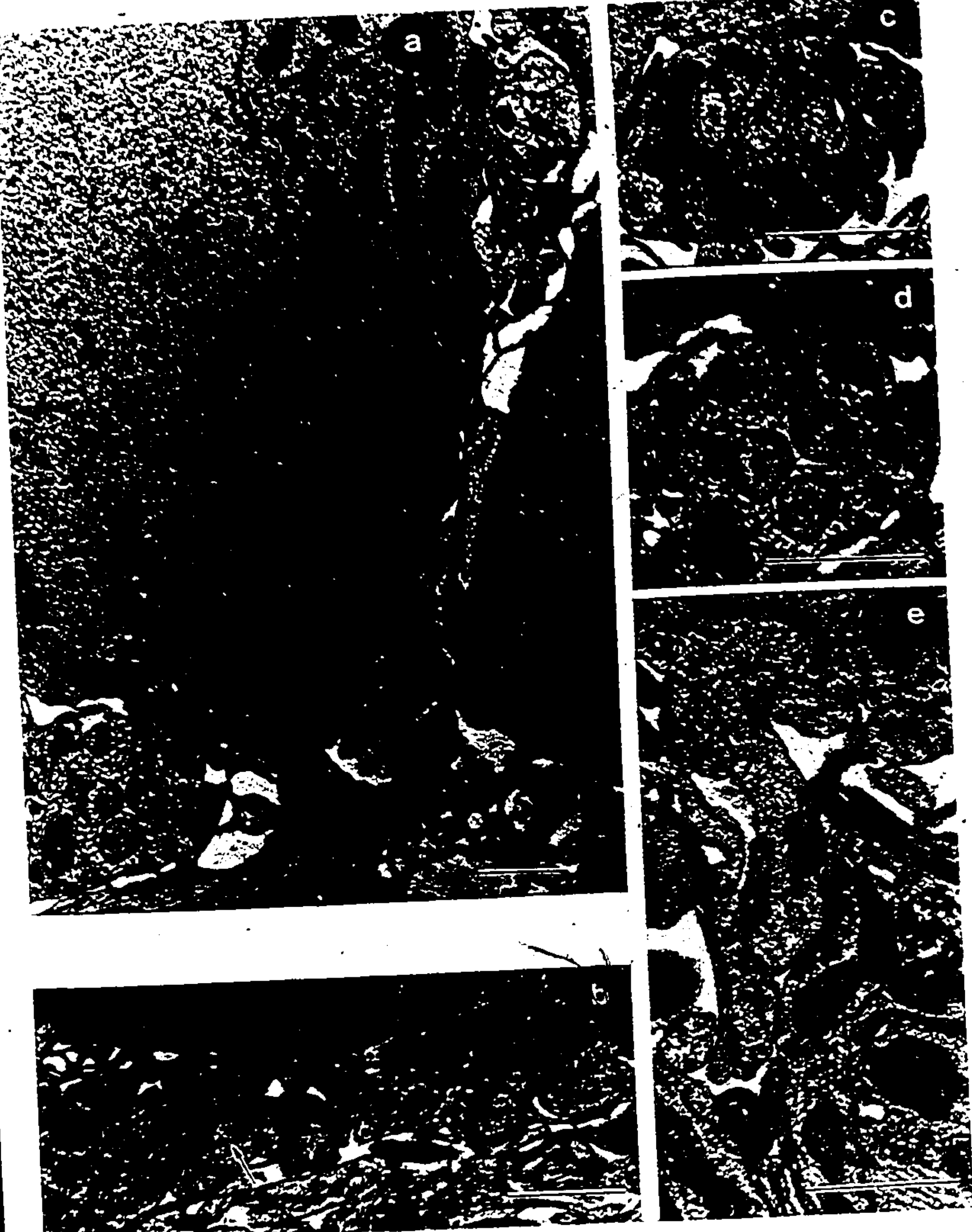


Figure 24

spines, often adjacent to each other (Fig. 24 b). A long perisomatic spine was also seen, which was synaptically in contact with a long afferent fiber (Fig. 24 e).

Other types of terminals were also present on the smooth surface of the Purkinje soma. These are:

A) Basket/stellate type

B) Purkinje recurrent collateral type

A) Basket/stellate type: These terminals contained mitochondria and their axoplasm was light in color. At times, neurofilaments in the axolemma could be seen very clearly (Fig. 25a). Many elliptical or "flat" vesicles were present in the bouton and the vesicles aggregated near the pre-synaptic membrane. The synaptic cleft was not widened and the post-synaptic density was not very dense (Fig. 25b). This type of synaptic junction has been classified previously as the symmetric type (Colonnier 1968); and the characteristics are consistent with those of basket/stellate type terminals as described in vivo (Palay and Chan Palay 1974). Rarely, from 1 - 4 basket/stellate type boutons were seen together and synaptically in contact with the smooth surface of the Purkinje soma (Fig. 25b). They are reminiscent of the electron microscopical appearance of a basket formation in vivo. However it is of interest to note that basket cells with their characteristic axons have not been seen in Golgi material.

Figure 25a: Electron micrograph of a basket/stellate type terminal, present along the smooth surface of a Purkinje soma. Note the aggregation of vesicles near the pre-synaptic membrane. Vesicles are small round and few "flat". 38 DIV Bar 1 μ.

Figure 25b: Electron micrograph of 4 basket/stellate type boutons, present together along the smooth surface of a Purkinje soma. The arrows point towards the bouton. 21 DIV Bar 1 μ.

Figure 25c: Electron micrograph of a Purkinje recurrent collateral type terminal, synaptically in contact with the smooth surface of a Purkinje soma. Note the symmetrical type synaptic junction. 21 DIV-Bar 1 μ.

Figure 25d: Electron micrograph of a Purkinje recurrent collateral type terminal which is forming a symmetrical type synaptic contact with the smooth surface of a Purkinje soma. The vesicles are pleomorphic. 22 DIV Bar 1 μ.

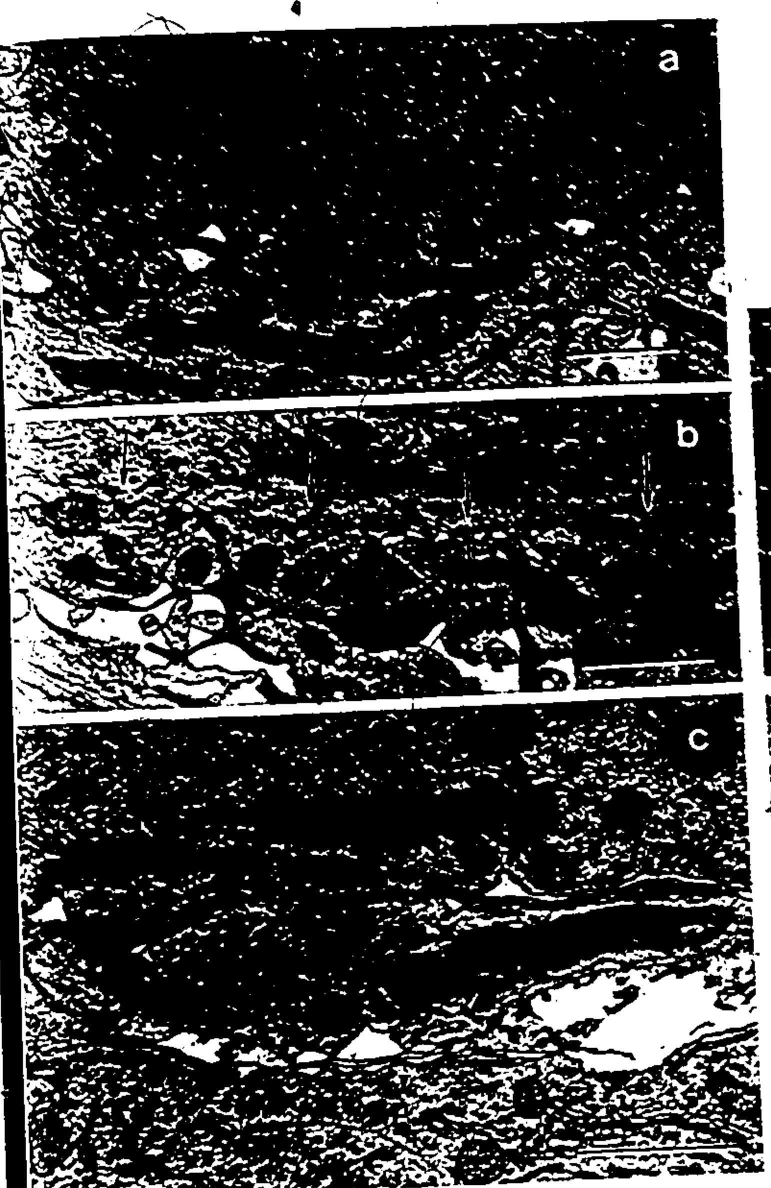


Figure 25

B) Purkinje recurrent collateral type: These boutons also contained mitochondria and their axoplasm was generally dark. They were filled with a mixed population of vesicles and formed a long symmetrical type synaptic junctions with the smooth surface of the Purkinje soma. (Figs. 25c and d).

3: Ultrastructure of the Purkinje dendrite

It has already been demonstrated in Golgi material that in culture, many dendrites emerge from the Purkinje soma (Figs. 14c, d, e, 15a, b, c, and d), and they radiate in all directions. Similarly, in a given plane of section, in the electron microscope, 1 - 2 thick dendrites were seen emerging from the Purkinje soma (Figs. 26a and b). Similar thick long dendrites were seen in the neuropil, not connected to the soma. They contained the usual organelles, i.e., mitochondria, granular and agranular endoplasmic reticulum and lysosomes (Figs. 26c, 27a, b, c and 28a). Multivesicular bodies were also seen (Figs. 27a and 29a - arrow). Hypolemmal cisternae, a characteristic of the Purkinje neuron were found to be present, parallel and beneath the plasmalemma (Fig. 27a). Many dendritic spines containing sacs of endoplasmic reticulum were seen emerging from the dendrite (Figs. 26c, 27a and b). Generally, the spines were long, with a narrow neck and a rounded head. Mostly, they were seen in clusters of 2 - 12 (Figs. 26a, 27a and b).

On the smooth surface of large dendrites, two types of pre-synaptic terminals were seen. These were basket/stellate cell axons (Fig. 28a)

Figure 26a: Electron micrograph of a Purkinje cell. Two dendrites (arrows) are emerging from the soma, at this plane of sectioning. 30 DIV. Bar 1 μ.

Figure 26b: Electron micrograph of a Purkinje cell. Again two dendrites (arrow) are emerging from the soma. 22 DIV Bar 1 μ.

Figure 26c: Electron micrograph of a large size (5 - 6 μ) Purkinje dendrite. 38 DIV. Bar 1 μ.

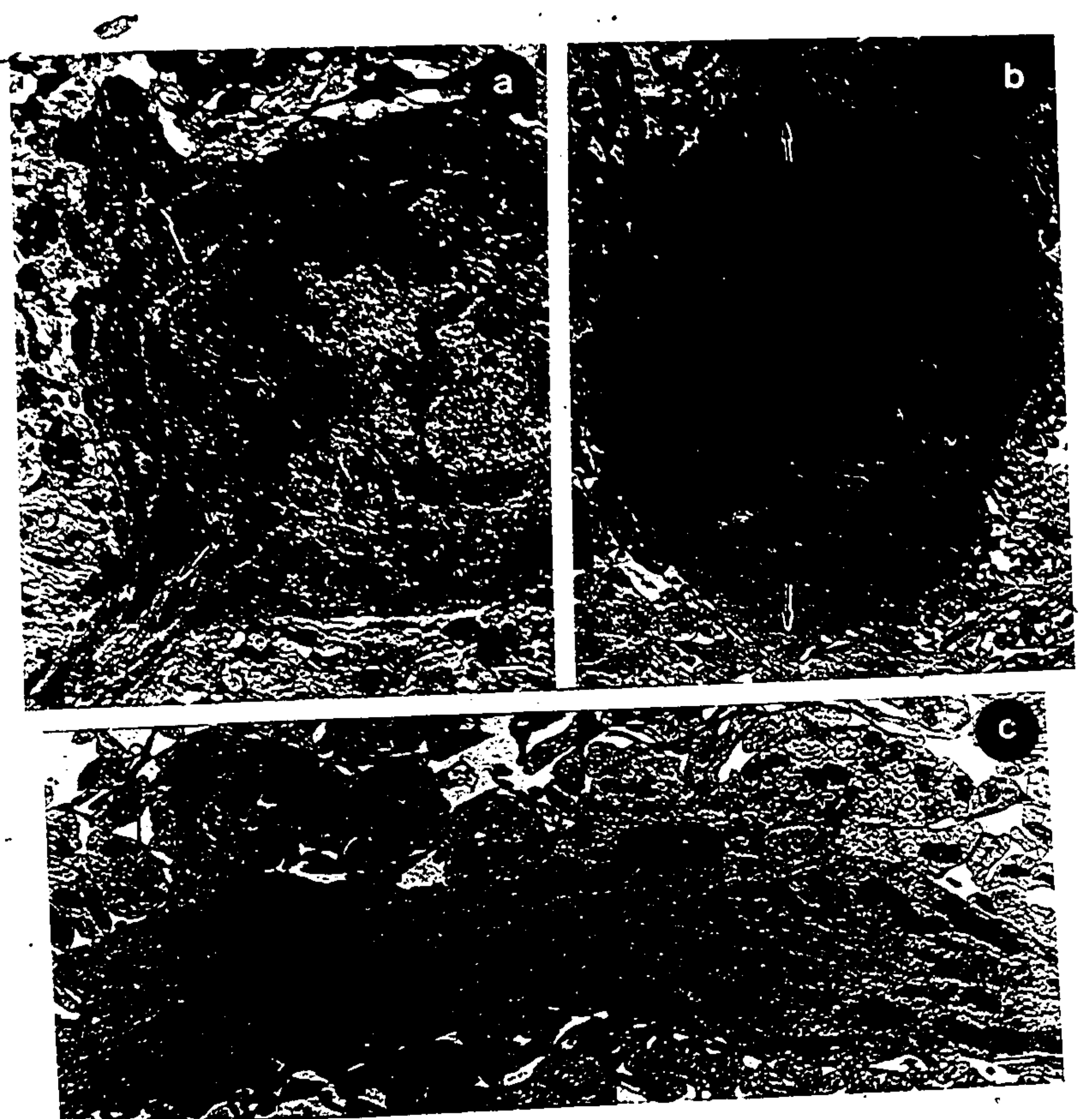


Figure 26

Figure 27a: Electron micrograph of a Purkinje dendrite. Note the presence of multivesicular body (arrow). Approximately 12 dendritic spines are present, one of which is emerging from the dendrite. The spines are surrounded by single large pre-synaptic terminal. 22 DIV. Bar 1 µ.

Figure 27b: Electron micrograph of a large Purkinje dendrite (7 µ). Many spines (arrows) are emerging from the dendrite. Note the presence of hypolemmal cisternae. 22 DIV. Bar 1 µ.

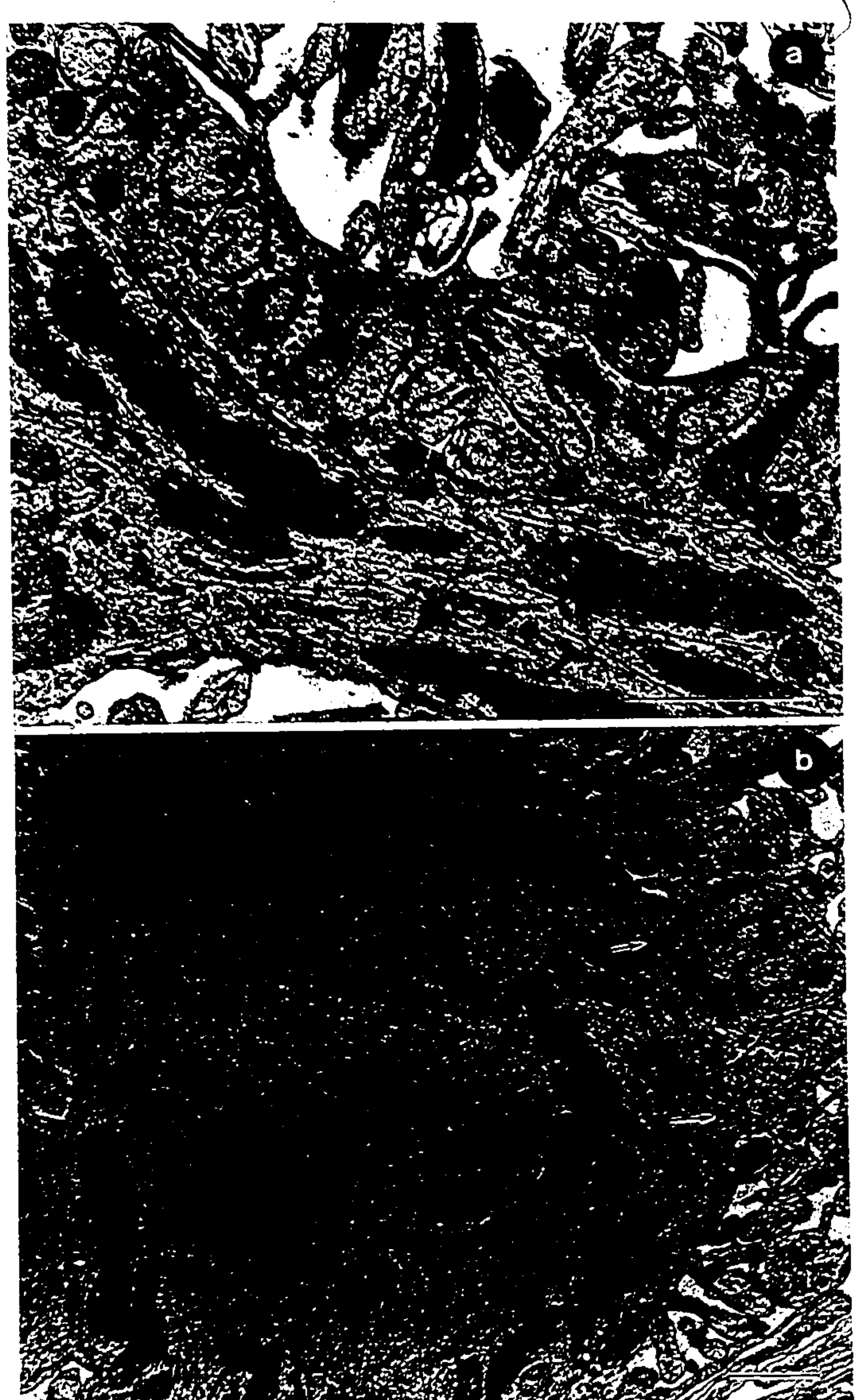


Figure 27

and Purkinje recurrent collateral type (Fig. 28b). Both of them formed symmetrical-type synaptic contacts with the smooth surface of the Purkinje dendrite, and were similar to the ones described earlier.

Very small dendrites were seen also in the neuropil (Figs. 28c and d). The ultrastructural characteristics of the small dendrites were similar to those seen in the large dendrites. Hypolemmal cisternae were present and were seen extending into the spines (Fig. 28d). Many spines which radiated in all directions were present on these small dendrites (Figs. 28c and d).

Based upon the criteria used to classify the perisomatic spines, all of the dendritic spines were also classified into 4 types.

a: Purkinje dendritic spines without a sheath of glia, post-synaptic web or a pre-synaptic terminal (Fig. 29a).

b: Purkinje dendritic spines surrounded by a sheath of glia. These were without a post-synaptic web or a pre-synaptic terminal (Figs. 29b and c).

c: Purkinje dendritic spines surrounded by a sheath of glia and with a post-synaptic web (Figs. 29c and d). Between the glia and the spine, the synaptic cleft had dense material and the post-synaptic web was easy to identify.

d: Purkinje dendritic spines surrounded by a pre-synaptic terminal and with a post-synaptic density. These were seen in clusters of

Figure 28a: Electron micrograph of basket/stellate type terminal which is forming a symmetrical type synaptic contact with the smooth surface of a large Purkinje dendrite. 38 DIV. Bar 1 μ.

Figure 28b: Electron micrograph of a Purkinje recurrent collateral type terminal which is forming a symmetrical type synaptic contact with the smooth surface of a large dendrite (Asterick). 21 DIV Bar 1 μ.

Figure 28c: Electron micrograph of a small dendrite, loaded with spines. A large bouton is synaptically in contact with the spines, and is forming asymmetrical type contact. 38 DIV. Bar 1 μ.

Figure 28d: Electron micrograph of a small dendrite. Note the presence of hypolemmal cisternae. These are extending into a dendritic spine (arrow). 38 DIV. Bar 1 μ.

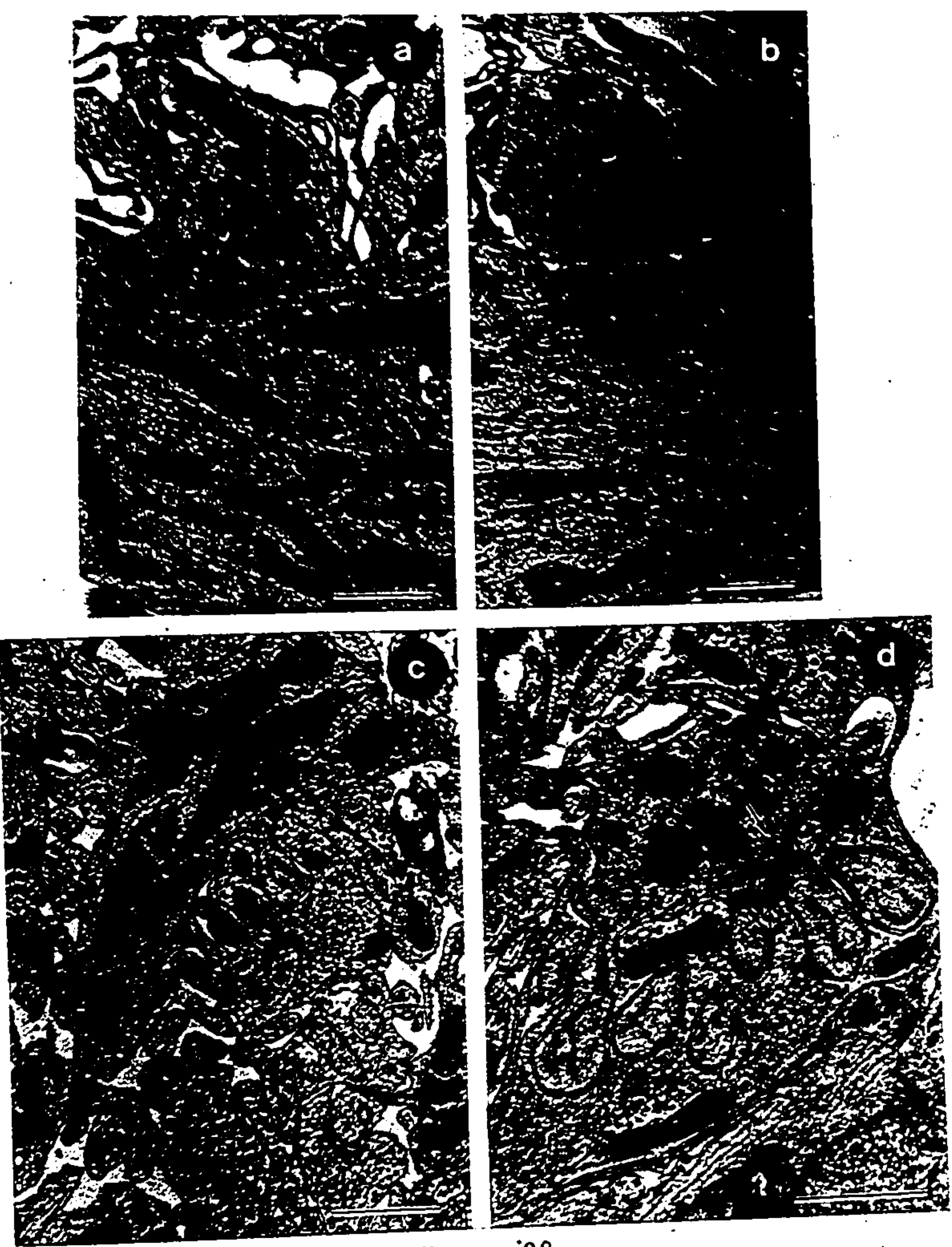


Figure 28

2 - 12 (Figs. 26c, 27a, b, 28c, d, 30a, b and c). A large pre-synaptic terminal was found to be synaptically in contact with these spines. The terminal was filled with round vesicles which aggregated near the pre-synaptic membrane. The synaptic cleft was wide. Both the synaptic density and the post-synaptic web were present (Fig. 28d). This type of synaptic contact has been classified as the asymmetric type (Colonnier 1968). Fig. 30a demonstrates a Purkinje dendrite in culture which is surrounded by two clusters of spines. Each cluster of dendritic spines had only one terminal which was synaptically in contact with many spines (Fig. 30a). Similarly, in the neuropil, clusters of 2 - 12 dendritic spines were also found (Figs. 30b and c). These were synaptically in contact with a large pre-synaptic terminal. Typical parallel fiber-dendritic spine contacts were seen also (Fig. 30b). Generally, in vivo parallel fibers form synaptic contacts with 1 - 2 dendritic spines only. Very rarely, 3 - 4 spines are contacted by one parallel fiber. The number of spines contacted by one parallel fiber never exceeds more than 4. Therefore, a cluster of more than 5 dendritic spines, synaptically in contact with one parallel fiber represents a new observation in culture.

In addition, synapse en-passant of parallel fiber was seen in the neuropil (Fig. 31a). At times, parallel fibers were found pushing into a sheath of glia which surrounded a cluster of Purkinje dendritic spines (Fig. 31b). In the neuropil, other types of Purkinje dendritic spine clusters were found also. In these clusters, many dendritic spines were contacted by a terminal, while others were surrounded by a sheath of glia (Fig. 31c).

Figure 29a: Electron micrograph of Purkinje dendritic spines. They are without any sheath of glia, pre-synaptic terminal or post-synaptic web. 38 DIV. Bar 1 μ.

Figure 29b: Electron micrograph of a cluster of Purkinje dendritic spines, covered by a sheath of glia; but without post-synaptic web. Other spines are receiving synaptic contact from parallel fibers. 21 DIV. Bar 1 μ.

Figure 29c: Electron micrograph of Purkinje dendritic spines covered by a sheath of glia. Arrow indicates the spine with post-synaptic density. 38 DIV. Bar 1 μ.

Figure 29d: Electron micrograph of Purkinje dendritic spines, surrounded by a sheath of glia. Arrow indicates the spine with a post-synaptic density. 38 DIV. Bar 1 μ.

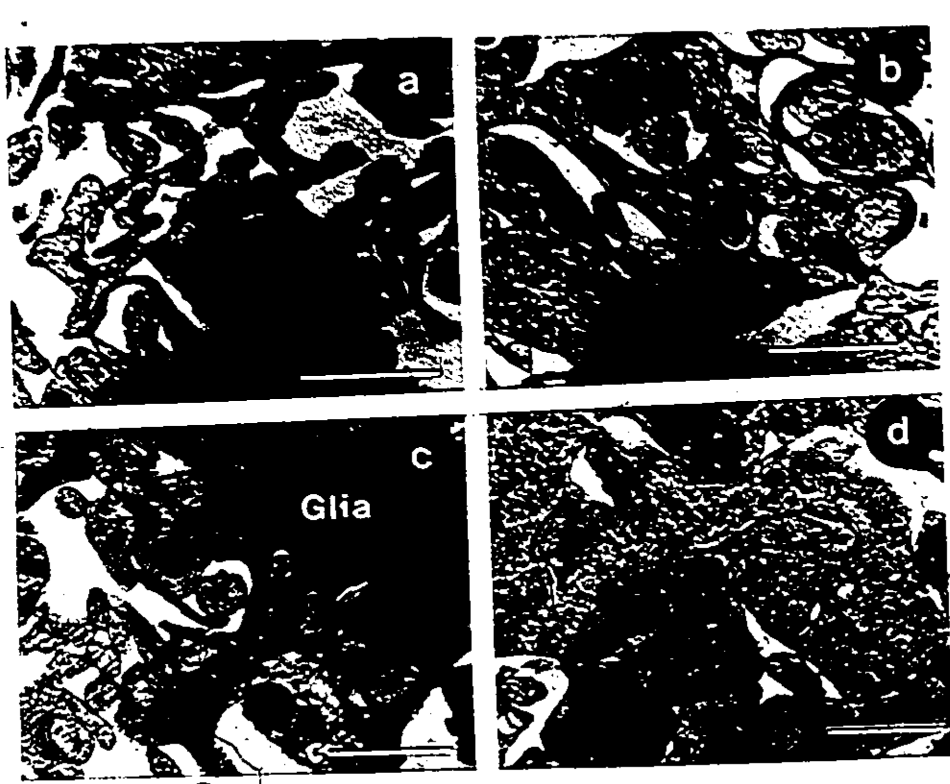


Figure 29

Figure 30a: Electron micrograph of two clusters of Purkinje dendritic spines, present near a Purkinje dendrite. Each cluster of spines is contacted by only one terminal. 38 DIV. Bar 1 μ.

Figure 30b: Electron micrograph of a cluster of dendritic spines in the neuropil. Again these are synaptically in contact with one terminal. Arrow indicates a normal 1: 1 parallel fiber-dendritic spine contact. 38 DIV. Bar 1 μ.

Figure 30c: Electron micrograph of another cluster of dendritic spines in neuropil. These are also surrounded by one terminal. 38 DIV. Bar 1 μ.

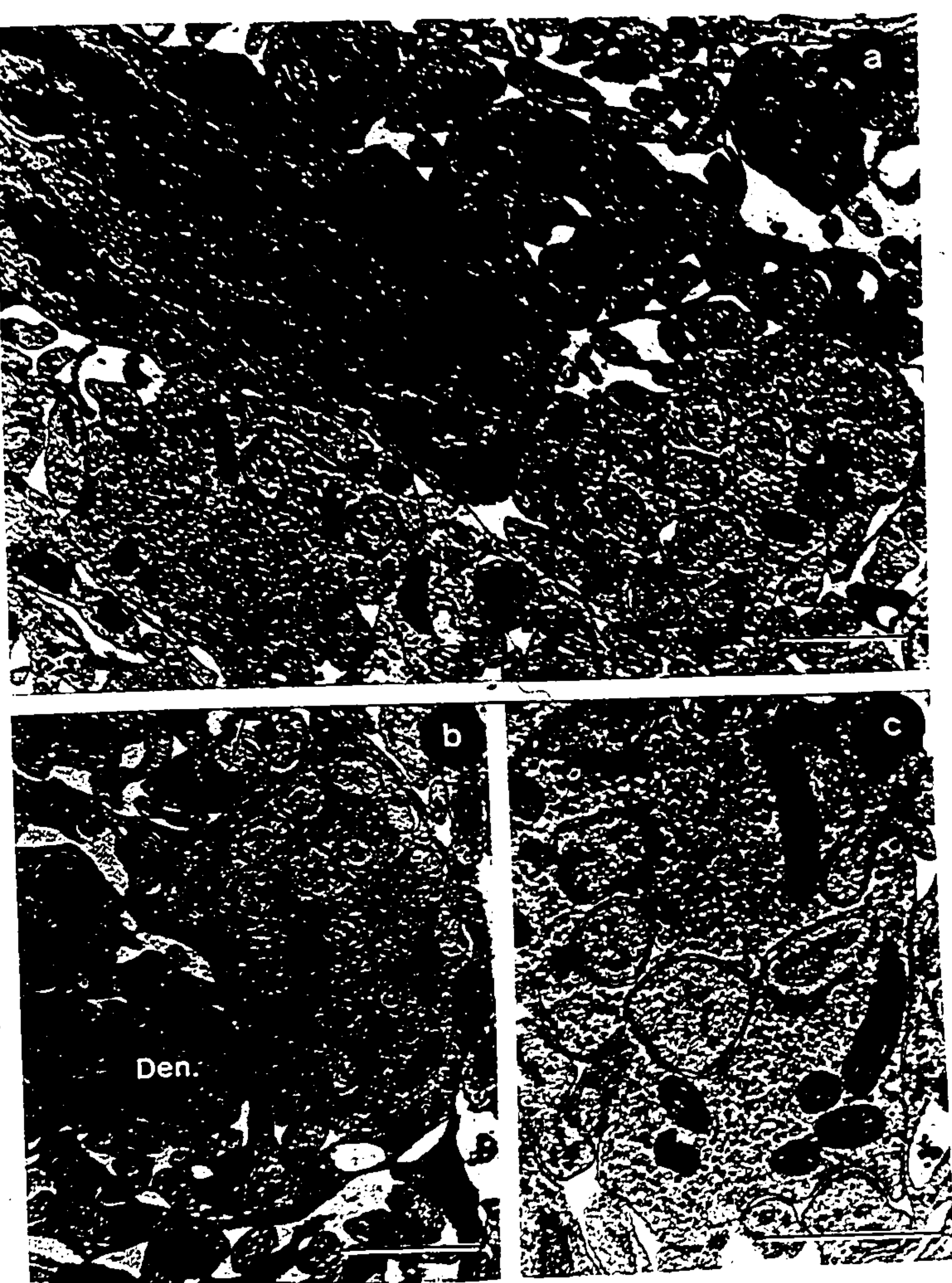


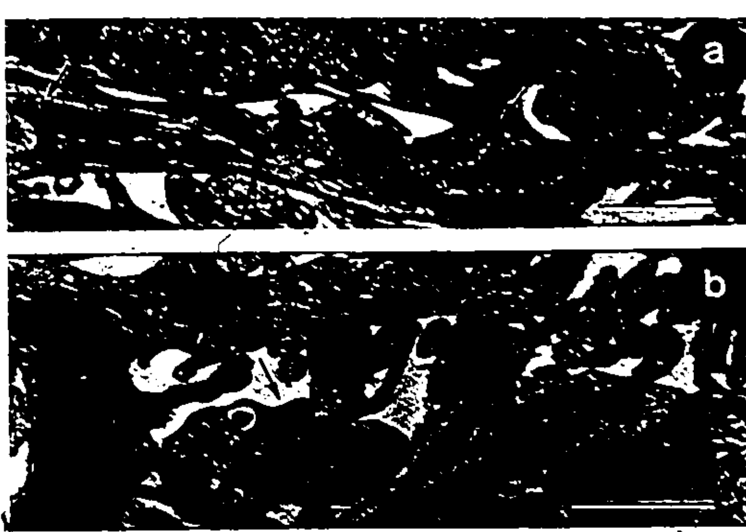
Figure 30

Figure 31a: Electron micrograph of a parallel fiber which is forming synapse en-passant in neuropil. 22 DIV. Arrows refer to parallel fiber-dendritic spine contacts. Bar 1 μ.

Figure 31b: Electron micrograph of a parallel fiber (arrow) which is pushing into a sheath of glia; while the glia surrounds a cluster of Purkinje dendritic spines. 38 DIV. Bar 1 μ.

Figure 31c: Electron micrograph of a cluster of Purkinje dendritic spines. Some of them are synaptically in contact with a parallel fiber while others are surrounded by a sheath of glia. 38 DIV. Bar 1

Figure 31d: Electron micrograph of mossy fiber in culture. Arrow indicates a Purkinje dendritic spine which is receiving asymmetrical type synaptic contact from the mossy fiber. Arrow heads indicate mossy fiber-granule cell dendrite synapses. 38 DIV. Bar 1 μ.



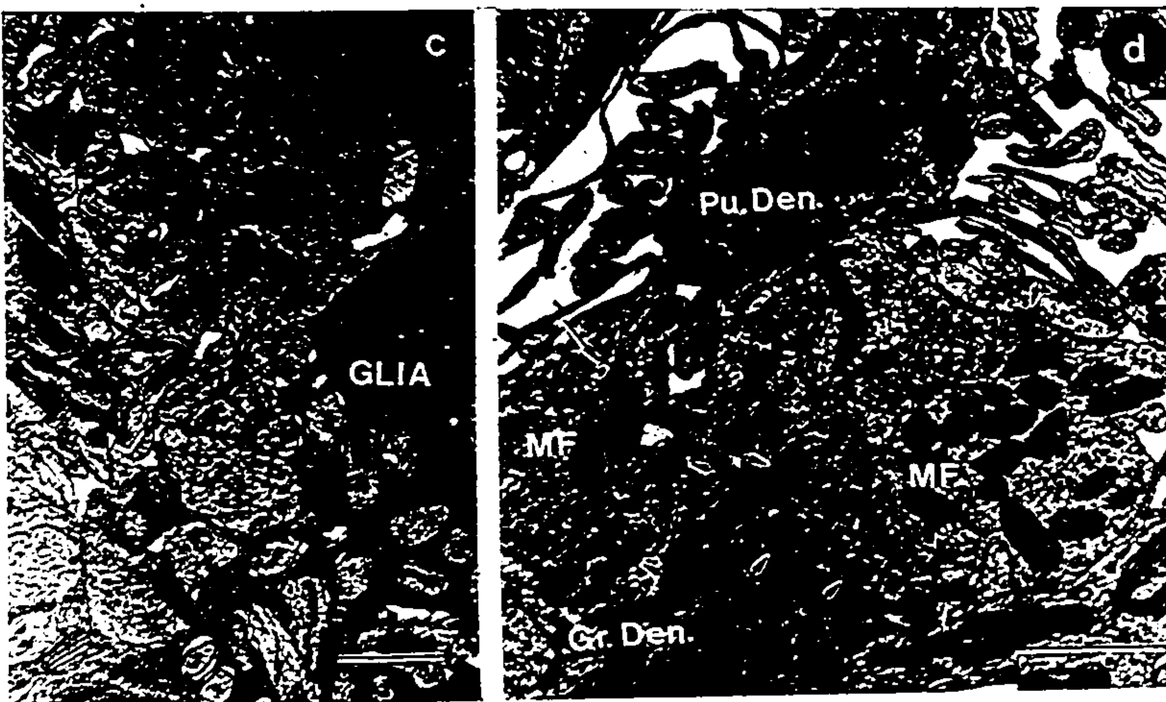


Figure 31

Many large terminals containing round vesicles and forming asymmetrical type synaptic contacts with the granule cell dendrites were seen also in the neuropil (Fig. 31d). This type of configuration has been described in vivo, and is very similar to mossy fiber glomerulus (Palay and Chan-Palay 1974). Besides forming their known asymmetrical type synaptic contacts with the granule cell dendrites, these terminals were found to be synaptically in contact with the Purkinje dendritic spines also (Fig. 31d), and the contacts were the asymmetric type.

4: Unknown type of terminals:

In culture, another type of terminal was also observed. This terminal contained mitochondria and a mixed population of vesicles. It was seen forming symmetrical-type synaptic contact with the smooth surface of the Purkinje soma and the perisomatic spine (Fig. 32a). For a comparison in Fig. 32a, there are two perisomatic spines. One of them is receiving an asymmetrical - type synaptic contact from a terminal containing round vesicles, while the other is receiving a symmetrical - type contact from a terminal containing mixed type of vesicles (Asterik) Fig. 32b shows another similar terminal which is forming a symmetrical type synaptic contact with the smooth surface of the Purkinje soma and asymmetrical type with the perisomatic spine.

Figure 32a: Electron micrograph of two different type of pre-synaptic terminals which are synaptically in contact with the perisomat spines. Large asterik indicates a terminal which contains a mixed population of vesicles and forms a symmetrical-type synaptic contact with the perisomatic spines. 38 DIV. Bar 1 μ.

Figure 32b: Electron micrograph of a terminal with a mixed type of vesicle .It is forming a symmetrical-type synaptic contact with the smo surface of the Purkinje soma; and the contact with the spine appears to be asymmetric. 38 DIV. Bar 1 μ.

Figure 32

CHAPTER - 5

DISCUSSION

(a) Development of the Purkinje neuron in vivo.

Using the Golgi method, the postnatal maturation of the Purkinje neuron of the mouse has been investigated. The study shows that it is a process of continuous development. During the maturation period, the Purkinje neuron acquires a very complicated mature morphological shape which is very different from its shape at day 0.

In phase 1, beginning from day 1, the Purkinje neuron begins to have small processes which emerge from its soma (Figs. 7B and c). In addition, an [illegible]phic cone begins to develop. Very few Purkinje neurons are impregnated in this phase. However, Cajal (1911) described similar cells, which were called fusiform cells. Altman (1972) also found cells with similar morphological characteristics; but he failed to identify the presence of small processes emerging from the soma, during phase 1.

During phase 2 and the later part of phase 1, the processes emerging from the soma become longer, branch and extend laterally and towards the pia, into the molecular layer (Figs. 8a and b). Light microscopically, many previous investigators have seen and described them (Athias 1897; Takasu 1905; Addison 1911; Cajal 1911; Purpura et al., 1964; Dadoune 1966; Meller and Glees 1969; Korunguth and Scott 1972; Altman 1972; Zecevic and Rakic 1976). Cajal described similar processes, and named this the phase of the disoriented dendrons. In Altman's work, this is equivalent to phase 2 (see table on page 22). A comparison of the present Golgi results (Figs. 8a, b and c) with those of Meller and Glees (Fig. 2a - page 12) and of Zecevic and Rakic (Fig. 4a - page 20) demonstrates the similarity, and morphological resemblance to dendrites. The processes become longer, they branch and have growth cones at their tips. In addition, they develop spines. Therefore, it can be concluded that long and thick processes present on the Purkinje soma up to day 6 are the perisomatic dendrites.

Electron microscopically, the perisomatic dendrites have been investigated in at least 3 different species. Mugnaini (1969) found them in 15 day old chicken embryo (Fig. 3 - page 14). They had the ultrastructural characteristics of dendrites and were receiving synaptic contacts from parallel fibers. While investigating the somatic spines in the mouse, Larramendi (1969) found long cytoplasmic processes which had the characteristics of dendrites. Similarly, Kornguth and Scott (1972) observed them in fetal macaque. Therefore, it can be concluded that the perisomatic dendrites have ultrastructural characteristics of dendrites and receive synaptic contacts from parallel fibers.

Between day 4 and 6, all of the Purkinje neurons become aligned in a row. This is a well documented observation. It is of interest to note that at day 4, when the Purkinje neurons become aligned in a row, the perisomatic dendrites are quite well developed (Fig. 8a). They are approximately 30 - 50 μ long and extend laterally and towards the pia, into the molecular layer. In addition, the cerebellar cortex becomes laminated during phase 2, because the external granular, molecular, Purkinje cell and internal granular layers can be demarcated from each other.

A second type of process, the spines,emerge from the developing Purkinje soma (Figs. 9b, c and d). In Golgi material, the spines are present all over the soma, between day 8 - 10; and they characterize phase 3 of development. A comparison of Figs. 8a, b with Figs. 9b, c, demonstrates that morphologically the spines are different from the perisomatic dendrites, and as such they should be considered a separate entity. The perisomatic spines have not been demonstrated in Golgi material before (Preliminary results were reported by Hendelman and Rouf 1974; Hendelman and Aggerwal 1975). In the Golgi material of

Meller and Glees (1969) and Kornguth and Scott (1972), the perisomatic spines are present, but they were not recognized.

Electron microscopically, the perisomatic spines have been investigated, thoroughly; their ultrastructure and synaptology is very well documented (Larramendi and Victor 1967; Larramendi 1969; Mugnaini 1969; Meller and Glees 1969; Kornguth and Scott 1972; Altman 1972; Zecevic and Rakic 1976). The perisomatic spines receive synaptic contacts from terminals which have been identified as "climbing fibers". The climbing fiber-perisomatic spine contacts are very specific, during development. Thus, the Golgi study demonstrates that during the development of the Purkinje neuron, it is possible to differentiate the perisomatic dendrites from the perisomatic spines. Further the study supports the classification made by this author earlier (table on page 22).

During the 4th phase, a very significant development is the formation of a stout long main dendrite (Figs. 9f, g and h). The Purkinje soma becomes completely smooth in contour and the perisomatic spines are now present on the main dendrite. At this point, the nomenclature of the perisomatic spine changes to main dendritic spines. By a careful examination of Figs. 9f, g and h, it can be concluded that the perisomatic spines on the soma are reduced gradually, in a basal towards apical gradient. Similar results have been reported by Meller and Glees (1969), electron microscopically.

In addition, during the 4th phase, the spiny branchlets begin to appear in the lower half of the molecular layer (Fig. 9g). This observation indicates that the parallel fiber - dendritic spine contacts are formed first in the lower half of the molecular layer, around day 11. A similar

observation has been reported by Altman (1972), during his 4th phase. However, in his opinion the earliest parallel fiber-dendritic spine contacts are formed, around day 15. Altman reported that during the 3rd phase, the perisomatic spines disappear from the soma (in electron microscopy), and that the smooth surface of the soma is invaded by basket cell axons. Therefore, the analysis of development reported here would include both phase 3 and phase 4 of Altman.

From day 11 onwards, it is possible to identify and sub-divide the dendritic tree (Fig. 9g and 10a). The branching patten of the Purkinje cell dendritic tree is dichotomous (Palay and Chan-Palay 1974). Therefore, following the same principle of dichotomization, the dendrites originating from the main dendrite (M) are the primary dendrites (1), which divide (secondary dendrites - 2), and sub-divide (tertiary dendrites - 3). The tertiary dendrites give rise to smaller branches (spiny branchlets - 4 and 5). The spiny branchlets originate mostly from the tertiary dendrites, although few arise from the secondary and primary dendrites. Generally, the spiny branchlets are loaded with spines which are long; and the spines present on the tertiary, secondary, primary dendrites and main dendrite are short and stubby.

The classification of the dendritic tree is similar to the classification of Ramon y Cajal (1911). He has referred to the dendrite emerging from the mature soma as dendritic trunk. The primary, secondary and tertiary dendrites have been noted to be smooth branches; and the smaller branches originating from the tertiary dendrites are known to be spiny branchlets. In addition, it is of interest to note that it was Cajal (1911), who postulated that specific parts of the Purkinje neuron were contacted

by specific afferents; the cell body by the basket cell axons; the "smooth" branches by climbing fibers; and the spiny branchlets by parallel fibers.

During the 5th phase, additional spiny branchlets along with parallel fiber - dendritic spine contacts are formed in the upper half of the molecular layer. When the dendritic tree reaches the pia, and the external granular layer totally disappears, the Purkinje neuron is said to be mature. The formation of spiny branchlets, along with parallel fiber-dendritic spine contacts first in the lower half and than in the upper half of the molecular layer again demonstrates a lower towards upper gradient of development. Similar results, were reported by Altman (1972), during the 5th phase.

During the maturation of the Purkinje neuron, it has been demonstrated that the perisomatic dendrites and the perisomatic spines emerge from the immature Purkinje soma, and these are not present on the mature Purkinje soma. How does this morphological transition occur ?

In an effort to explain this occurence, the earlier investigators proposed that the processes emerging from the immature Purkinje soma were either resorbed (Athias 1897) or translocated (Larramendi 1969). However, it has already been demonstrated in the review of the literature that both of these concepts are not convincing. Therefore, it is important to formulate a theory of the development of the Purkinje neuron, which could explain the normal developmental sequence.

(b) The proposed theory of the development of the Purkinje neuron.

In an effort to unravel the complicated mechanics of the development of the Purkinje neuron, the development in vivo was reviewed again.

It seems that at day 1, the small processes emerging from the Purkinje soma are the early perisomatic dendrites; they grow longer at day 2; and at day 4, there is a considerable increase in their length and thickness. The direction of growth of the perisomatic dendrites is lateral and towards the external granular layer. A small molecular layer can be identified at day 4; and the perisomatic dendrites can be seen extending into it. The presence of the molecular layer at day 4 in the mouse indicates that many granule cells have completed their migration; and many parallel fibers have been formed.

It appears that at this stage, in the development of the Purkinje neuron, parallel fibers play a very important role. In order for the normal shape of the Purkinje neuron to develop, it is essential that parallel fibers form a molecular layer, and form adequate synaptic contacts with dendritic spines. In addition, in the molecular layer, parallel fibers must grow parallel to the pia. It is proposed that in vivo, during this stage all the 3 steps are carried out simultaneously by the parallel fibers. As a result of the 3 steps, the Purkinje neurons become aligned in a row, and become fixed in place. In order to illustrate the postulate, imagine that at day 3, there is a loose fisherman's net (developing parallel fibers which are wavy), in space. In the net, there are many fish (Purkinje neurons); they are held strongly by the net and cannot escape (due to the formation of parallel fiber dendritic spines contacts). Imagine further that the net is loose in space at day 3, and all fish hanging from the net are in an irregular strata (The Purkinje neurons are in irregular rows at day 3). Between day 4 and 6, the net become stretched (due to the growth of the parallel fibers which is parallel to the pia): Therefore all fish also become levelled (the

Purkinje neurons become aligned in one plane). While the fisherman's net is becoming stretched, it is also developing more contacts with the fish (parallel fiber- dendritic spine contacts). Thus at day 6, the fish are being held more firmly than they were at day 4. Due to this development, the fish (the Purkinje neuron) are fixed in place, and cannot move.

Therefore, it is proposed that between day 3 and 6, many synaptic contacts are formed between the parallel fibers and Purkinje dendritic spines. Due to the development of the contacts; the formation of the molecular layer; and growth of the parallel fibers parallel to the pia, the Purkinje neurons will be held firmly in their place. From then on, the mature normal shape of the Purkinje neuron develops due to:

1) The descent of the nucleus towards axon i.e. towards white matter. The movement results in the formation of the main dendrite and the smooth soma of the mature Purkinje neuron.

2) The growth of the perisomatic dendrites and the apical dendrites. With the help of their growth cones, they grow longer and branch, and also develop spines. Therefore the perisomatic dendrites and the apical dendrites form the dendritic tree in a mature Purkinje neuron, resulting in the primary, secondary and tertiary dendrites and the spiny branchlets.

According to the postulate, if the nuclear descent towards the axon occurs, there would be an apparent shift of the perisomatic dendrites; and they would appear to emerge from the apical portion of the soma. This is in fact what is seen, in the late 2nd phase (compare Figs. 8a, b and c). In Fig. 8c, most of the dendrites are present near the apical portion of the soma. With further descent, the soma region is cleared of all dendrites.

In the next phase, there is a development of the perisomatic spines. With continued descent, there should be a relative change of the position of the perisomatic spines, in a basal towards apical gradient. This is in fact consistent with observations (compare Purkinje soma in Figs. 9a, b, c, d and e). The soma is again cleared of all its spines, and is now presumably ready to receive basket synapses on its smooth surface (Purkinje soma in Figs. 9f, g and h).

The descent of the nucleus leaves above it a stout process which now forms the main dendrite. Since the spines have not moved, they will be located on the main dendrite; and this is in fact seen in phase 4 (Figs. 9g and h).

Further according to the postulate, the mature dendritic tree develops from the perisomatic dendrites and the apical dendrites. It has already been demonstrated that the developing perisomatic dendrites have growth cones at their tips (Fig. 8b - arrow), and that all dendrites are present near the apical portion of the soma (Fig. 8c), in the later part of the phase 2. The dendrites grow at their tips both laterally and towards the external granular layer (but not into the layer), into the molecular layer. Their branching pattern follows the principle of dichotomization. In Fig. 9e, the main dendrite is small, and from it many primary dendrites are originating. The primary dendrites have undergone at least one division, as a result many secondary dendrites are also present. In Fig. 9g, the main dendrite is longer. Many secondary dendrites and their branches i.e. the tertiary dendrites along with their spiny branchlets can be seen.

Fig. 9g demonstrates further that the formation of the tertiary dendrites and the spiny branchlets is earlier in the lower half of the molecular layer than in the upper half of the molecular layer, because the tertiary dendrites and the spiny branchlets are not formed as yet in the upper half of the

molecular layer. The full bloom of the mature dendritic tree i.e. the primary (1), secondary (2) and the tertiary dendrites (3) and the spiny branchlets (4 and 5), is present in Figs. 10a, at 15 days post-natally.

From the review of the results, it seems that the full bloom of the mature dendritic tree requires for its development the continued presence and increase in number of the parallel fibers. In addition, it seems that until day 10, the descent of the nucleus and development of the dendritic tree is slow, and from day 11 onwards, the development of both becomes accelerated.

A visual impression of the descent of the nucleus can be made, by placing an imaginary nucleus in the middle of the main dendrite in Fig. 9e, and by erasing the soma. The neuron begins to look like the Purkinje neuron at day 6 in Fig. 8b. Similarly, a visual impression of the postulate i.e. fixation of the neuron, descent of the nucleus, and growth of the dendrites can be made by placing all Purkinje neurons of the various developmental ages, side by side (Fig. 33). The pictures were taken at the same magnification and the pia was aligned. With maturation, the nuclear region (the soma) is situated increasingly further from the pial surface.

Figure 33: Purkinje neurons of the various developmental ages, at the same magnification. The pia is marked by the black dotted line at the top of each figure. Bar 20 μ is applicable to all figures.

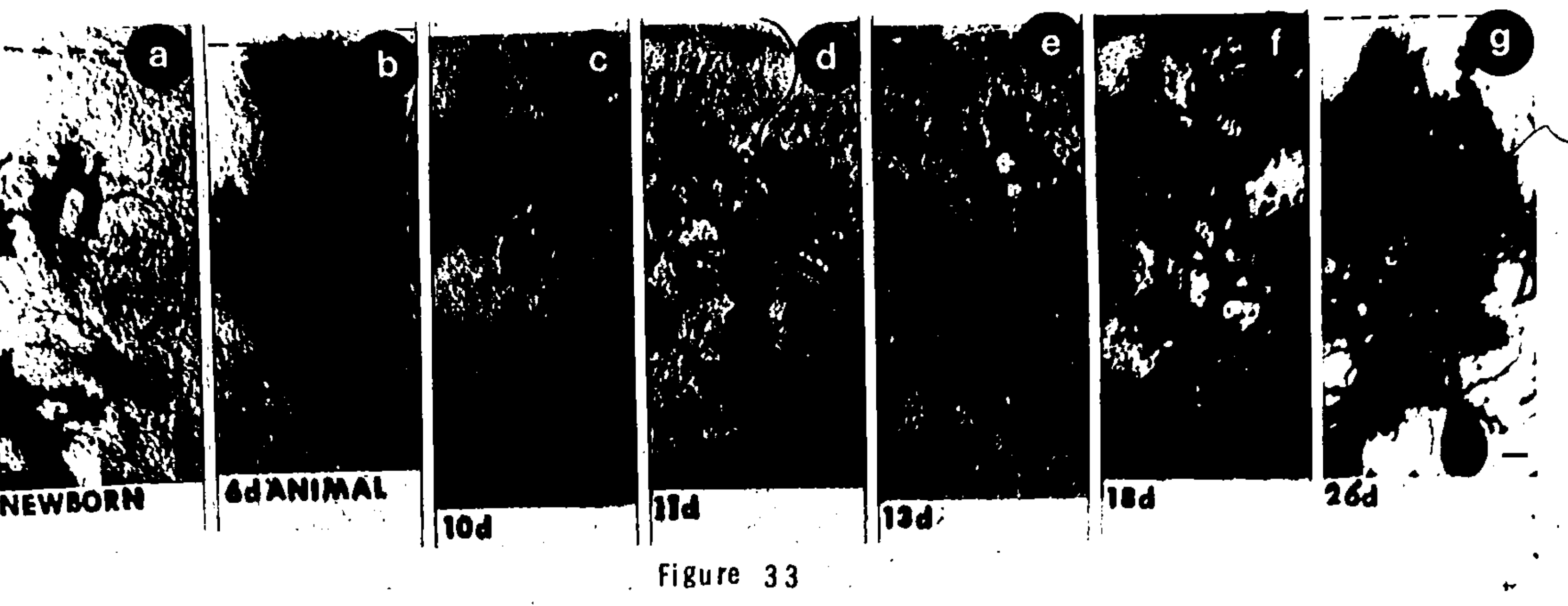


Figure 33

Thus, on the basis of the theory, it is possible to conclude that the present results of the development of the Purkinje neuron in vivo, can be explained. The descent of the nucleus and relative change in position of the perisomatic dendrites and the perisomatic spines, makes it unnecessary to propose translocation of spines, as postulated by Larramendi (1969). Similarly, it can be proposed that they do not resorb, as postulated by Athias (1897).

(c) The development of the Purkinje neuron in culture.

According to the theory, for the normal shape of the Purkinje neuron to develop, it is essential that parallel fiber - dendritic spine contacts form, that the molecular layer develops, and that the parallel fibers grow parallel to the pia. As a test of the theory, it was decided to study the development of the Purkinje neurons in culture. Two events occur in these cultures. First, it is known that a toxicity of the feed selectively decimates granule cells (Allerand 1971: Hendelman et al., 1972), although a quantitative analysis of the decimated versus surviving granule cells has not been done. The surviving granule cells mature normally (Wolf and Holden 1969: Wolf and Dubois-Dalcq 1970: Kim 1970: Allerand 1971: Hendelman et al., 1972: Aggerwal and Hendelman 1976). Secondly, a molecular layer is not formed, and the parallel fibers do not become stretched. Generally, in culture the parallel fibers follow a wavy course. Even in such altered conditions, the Golgi study of the development of the Purkinje neuron has demonstrated that the maturation occurs continuously. The Purkinje soma is 8 - 10 μ in size at day 1, it becomes mature and attains a size of 18 - 20 μ (compare Figs. 16b with 18i, j, k, 19l, m, n in culture with Figs. 19L, M in vivo).

In culture, the perisomatic dendrites begin to emerge from the Purkinje soma according to their in vivo schedule i.e. at day 1-2 (compare Figs. 16B with b). They continue to grow and increase in number on the Purkinje soma, up to day 4 (compare Figs. 16D with d). According to the proposed theory, by this time in vivo enough contacts between the parallel fibers and the perisomatic dendrites develop, and an identifiable molecular layer is formed. These contacts in turn hold and fixate the Purkinje neurons in place. However, such is not the case in culture, because the toxicity of the feed is continuously destroying the granule cells, and the molecular layer formation is not occuring. Therefore, according to the theory, there should not be enough synaptic contacts between the parallel fibers and the perisomatic dendrites, at this stage in culture. Consequently, the Purkinje neuron should not be fixed in place, and they should be lying at random in the cerebellar cortex. Fig. 12a demonstrates exactly, what has been anticipated i.e. the Purkinje neurons are not aligned in a row and the cerebellar cortex is not laminated. Similar results have been reported by other investigators also (Hendelman 1967; Seil and Herndon 1970; Hendelman 1972; Aggerwal 1974; Aggerwal and Hendelman 1975, 1976; Privat and Drian 1976).

It has been proposed that after the Purkinje neuron is fixed by the parallel fibers, the nucleus begins to move slowly towards the axon. The descent of the nucleus is responsible for the relative shift of the perisomatic dendrites from the soma, towards the apical portion of the soma (Fig. 17F). The basal portion of the soma become free and from it many perisomatic spines begin to emerge (Figs. 17G, G1 and G2).

Since the Purkinje neuron is not fixed in culture, therefore according to the theory, the descent of the nucleus should not occur, and the perisomatic dendrites should continue to be present on the soma. Concurring with the postulate, the results in culture demonstrate that in this stage the perisomatic dendrites continue to be present on the peripheral portion of the soma, along with few on the apical portion of the soma (Figs. 17e and f). While being present on the soma, the perisomatic dendrites become thick and branch further. A preliminary report of the results has been made earlier (Aggerwal 1974; Aggerwal and Hendelman 1975, 1976). In addition, amidst the perisomatic dendrites, the perisomatic spines also have begun to emerge from the soma (Fig. 17g). This event indicates further that the relative shift of the perisomatic dendrites did not occur in culture i.e. the nucleus failed to descend in culture. However, the neuron has continued to mature, because the perisomatic spines have been formed on schedule. In this phase, the overcrowding of the Purkinje soma in culture occurs and is shown by the presence of both the perisomatic dendrites and the perisomatic spines. In culture, the presence of many perisomatic dendrites on the Purkinje soma has been mentioned earlier by others (Wolf and Holden 1969; Wolf and Dubois-Dalcq 1970; Seil 1972; Privat and Drian 1976). Similarly, the presence of many perisomatic spines on the mature Purkinje soma has been reported (Wolf and Dubois-Dalcq 1970; Privat and Drian 1976). It is interesting to note that Wolf and Dubois-Dalcq (1970) assumed that the perisomatic spines in culture, resorb.

In the theory, it has been proposed that from day 11 onwards, due to the rapid descent of the nucleus towards the axon, a long main dendrite is formed, and a relative shift of the perisomatic spines onto the main

dendrite occurs. Since in culture, the nucleus fails to descend, therefore it cannot get into the stage of the rapid descent. As a consequence, it is predicted that there will not be the formation of a stout, long main dendrite in culture. Again the anticipation is confirmed, i.e. the mature Purkinje neuron in culture fails to have a stout, long main dendrite (Figs. 18i, j, k, 19l, m and n).

Therefore, it can be concluded that the mature Purkinje soma in culture is equal to the mature soma plus the main dendrite in vivo, that the perisomatic spines are equivalent to the main dendritic spines, and that the perisomatic dendrites are the primary dendrites. This concept is shown schematically in Fig. 34. In culture, the perisomatic dendrites (primary dendrites) continue to grow, branch and develop spines. The first division of them is equal to the secondary dendrites in vivo, the second division is equal to the tertiary dendrites, and the third division is equal to the spiny branchlets.

In the theory, it has been postulated that for the development of an elaborate dendritic tree, a continuous presence and an increase in the number of parallel fibers is needed. Since a large number of granule cells are continuously destroyed in culture, therefore it is possible to predict that dendrites in culture will be less elaborate with fewer branches. Again, this prediction is confirmed in culture. There is absence of an elaborate dendritic tree, the branching of the dendrites is far less, and the third division of the perisomatic dendrites, i.e. the spiny branchlets, are not present (compare Fig. 19L in vivo with Figs. 18i, j, k, 19l, m, n, ~~in culture~~). However, at times the tertiary dendrites may remain small and give the appearance of the

spiny branchlets. Similarly, Privat and Drian (1976) also did not find spiny branchlets in their cerebellar cultures.

In conclusion, the results of the development of the Purkinje neuron in culture can be explained on the basis of the theory.

According to Cajal (1911) and the Golgi results presented here, the primary, the secondary and the tertiary dendrites in the intact animal are smooth i.e. they have few spines. However, such is not the case in culture; the Golgi results demonstrate that all dendrites are loaded with spines. Why does this modification occur in culture ? This remains to be explained.

(d) Evidences from the literature in support of the theory.

Similar results, i.e. the absence of the lamination of the cerebellar cortex, the absence of the alignement of the Purkinje neurons, the modified shape of the Purkinje neurons, the presence of many dendrites emerging from the soma, and the dendrites loaded with spines have been reported by several investigators. The experimental conditions resulted in selective loss or destruction of the granule cell population: Weaver mutant mouse (Rakic and Sidman 1973: Hirano and Dembitzer 1973), X-irradiated rats (Altman and Anderson 1972), Panleukopenia virus (P.L.V.) infected ferrets (Herndon et al., 1971: Llinas et al., 1973), Methylazoxymethanol (M.A.M.) injected rats (Haddad et al., 1975: Woodward et al., 1975). In all these studies, the Purkinje cell retains its perisomatic dendrites, and the cells are randomly arranged. The proposed theory accounts for the results obtained. In addition, the theory would predict that the Purkinje soma in these experiments would have the perisomatic spines. This observation has not been reported.

In 1972, Kornguth and Scott postulated that "in no species can one find a Purkinje cell with a characteristically elaborate dendritic tree in the absence of the climbing fibers". According to the postulate of Korunguth and Scott (1972), the modified shape of the Purkinje neuron, in culture could be due to the absence of the climbing fibers. Sotelo and Arsenio-Nunes (1976) conducted experiments in which climbing fibers were selectively destroyed in newborn rats. Using the Golgi-Rio Hortega method, they found that the mature deafferented Purkinje neuron had the classical Purkinje cell configuration. It was characterized by the presence of the smooth surfaced soma, main dendrite, primary dendrites and their branches. Therefore, it can be concluded that the climbing fibers are not needed for the development of the classical Purkinje cell configuration; and the postulate of Kornguth and Scott (1972) is not valid.

According to the theory, for the development of classical Purkinje cell configuration, an early formation of parallel fiber-dendritic spine contacts is an essential pre-requisite, and they should begin to form at day 1 - 2 postnatally. This pre-requisite can be confirmed from the investigations of Del Cerro and his collaborators (Del Cerro and Snider 1972: Del Cerro and West 1974). Light microscopically, they found a thin molecular layer and an ill defined internal granular layer, in the cerebellar cortex of the 20 day embryonic rat. Electron microscopically, they found parallel fibers, axo-dendritic and axo-somatic synapses in the molecular layer. However, according to Altman (1972), in the rat, parallel fibers do not begin to form synaptic contacts with Purkinje dendritic spines, before day 13 - 14 postnatally. Therefore, in order to resolve the issue, it is necessary to reinvestigate the question. It has also

been proposed that the synaptic contacts are permanent. Mugnaini (1969) investigated the development of the Purkinje neurons in chicken embryo. From the investigations, he concluded that the parallel fiber-dendritic spine contacts were permanent.

It has been proposed that the immature parallel fibers must grow parallel to the pia, for alignement and fixation of the developinc Purkinje neuron. In 1956, Tsang reported that there were wavy T-fibers in the cerebellar cortex of young as well as adult animals. It is known that mature parallel fibers are straight and "stretched". It is possible to explain this observation by assuming that wavy T-fibers represent immature parallel fibers. During the maturation period, they grow parallel to the pia, become straight and form synaptic contacts with as many dendritic spines as possible.

In 1969, Larramendi described intracellular evidence which might support the concept of the nuclear descent toward the initial axonal segment. While studying the development of the Purkinje neuron electron microscopically, he found accumulation of ribosomes between the nucleus and the initial axonal segment at day 7. This accumulation of ribosomes gave a "funnel-like" appearance to the Purkinje cell. The initial axonal segment at this stage was conical in shape. With the maturation of the Purkinje neuron, ribosomes gradually decreased and at day 14, they were minimum. This resulted in the disappearance of the "funnel shape" and the conical formation of the initial axonal segment. It was now originating abruptly from the Purkinje soma. The presence of ribosomes between the initial axonal segment and the nucleus at day 7, and their disappearance at day 14, convinced Larramendi that some descent of the nucleus towards the axon occurs during the development of the Purkinje neuron (Page 830: Larramendi 1969).

Mugnaini (1969) also suggested that due to the growth pattern of the Purkinje dendrites and the permanence of connections of the parallel fibers with early dendrites, the maturing Purkinje neuron would either move upwards or downwards. The Purkinje perikarya cannot move upwards as suggested by Mugnaini, because it would result in an upside down dendritic tree i.e. the tree facing towards the white matter, and the Purkinje soma sitting on top of it. Since the dendritic tree of the Purkinje neuron is towards the pia, and the soma is towards the white matter, therefore the only possible way the developing Purkinje neuron could move is downwards, towards the white matter.

In the rat cerebellar cortex, Smolyaninove (1971) reported that there were approximately 250 granule cells for every Purkinje cell. Further it is known that during development all granule cell migrate from the external granular layer towards the internal granular layer i.e. the granule cell nuclei descends towards the white matter within a process destined to become the axon of the cell. The direction of the granule cell migration is exactly the same which has been proposed for the migration of the Purkinje cell nucleus. Therefore, it is possible to conceive that if the nucleus of the granule cells can migrate then the nucleus of the Purkinje cell can also migrate.

This phenomenon of movement of the nuclear region has been termed perikaryal translocation (Morest, 1970a). Movement of the nucleus has been shown to occur during the development of the retina (Morest 1970b), and the ganglion cells in the chick optic tectum (Domesick 1974).

(e) Ultrastructure and synaptology of the mature Purkinje neuron in culture.

The ultrastructure and synaptology of a mature Purkinje neuron in vivo has been analyzed (Palay and Chan-Palay 1974). The excitatory and inhibitory synaptic connections are known, and have been drawn schematically in Fig. 34. In vivo, the maturation of the Purkinje neuron proceeds under known afferent influences. However, such is not the case in a cerebellar culture, because granule cells are reduced (Allerand 1971: Hendelman et al., 1972) and climbing fibers are absent. Therefore due to such altered afferents, it is reasonable to anticipate that there could be some modifications in the synaptology of the Purkinje neuron, in culture.

Electron microscopically, in culture, the ultrastructural characteristics of the mature Purkinje soma and the dendrite were found to be similar to those described in vivo (Palay and Chan-Palay 1974). The hypolemmal cisternae have been considered a specific characteristic of the Purkinje neuron in vivo. Their presence, beneath and parallel to the plasmalemma has been very useful in identifying the Purkinje neuron in culture. Therefore, it can be concluded that in culture, the Purkinje neuron has been identified correctly.

In Golgi and semithin sections, it has been demonstrated that more than one dendrite emerges from the Purkinje soma. Similarly, in ultrathin sections, two dendrites were found emerging from the Purkinje soma (Figs. 26a and b) in one plane of section. Therefore results confirm that many dendrites emerge from the Purkinje soma. Similar electron microscopic results were reported by Privat and Drian (1976).

In the Golgi material, it has been demonstrated that dendrites are loaded with spines. Electron microscopically, the spines are long with a narrow neck and a rounded head, similar to the spiny branchlet type spines

seen in vivo. It is known that such spines in vivo receive specific contacts from parallel fibers only. In culture, the spines were found to receive asymmetrical type synaptic contacts from boutons which resemble parallel fibers (Figs. 27a, b, 28c and d). In addition, a parallel fiber was seen forming a synapse en-passant in the neuropil (Fig. 31a). Therefore, it can be concluded that in culture, normal parallel fiber-dendritic spine contacts are formed, and synaptic specificity has been retained. Similar conclusions have been reported earlier (Hendelman 1967: Wolf and Dubois-Dalcq 1970: Seil and Herndon 1970: Hendelman 1972, 1975: Aggerwal and Hendelman 1975, 1975, 1976: Privat and Drian 1976: Hendelman et al., 1977).

In culture, clusters of 8 - 12 Purkinje dendritic spines were found (Figs. 30a, b and c). These were contacted by a single large terminal which contained round vesicles and which formed asymmetrical type synaptic contacts. Ultrastructurally, the terminal resembles varicosities of parallel fibers seen in vivo, except for its large size. The interpretation of these findings is that these enlarged terminals are hypertrophic parallel fiber boutons. It is known that the granule cells are reduced in culture. Therefore, it is reasonable to postulate that the parallel fibers have become hypertrophic in culture, because their number has been reduced in proportion to the number of Purkinje dendritic spines. Generally, it is known that in vivo parallel fiber varicosities form synaptic contacts with 1 spines only. Rarely, the contacts are made with 2 - 3 spines, but never more than 4. Herndon et al., (1971) reported terminals contacting 2 - 3 spines in agranular cerebella. In this study it was common to see 1 terminal contacting 10 - 12 spines. This would represent an example of morphological plasticity.

Other types of Purkinje dendritic spines were also found, in the neuropil. These were without a presynaptic terminal, and were either surrounded by a sheath of glia or were without a sheath of glia. Some of those surrounded by a sheath of glia had a post-synaptic density (Figs. 29 b, c and d). The formation of such spines has been reported earlier, in experiments in which the granule cell population has been severely reduced, e.g. Weaver mutant mouse (Rakic and Sidman 1973: Hirano and Dembitzer 1973), X-irradiated rats (Altman and Anderson 1972), P.L.V. infected ferrets (Herndon et al., 1971: Llinas et al., 1973). In the normal animal, it is known that parallel fiber-Purkinje dendritic spine contacts are specific, and spines always have a pre-synaptic terminal (parallel fiber). Therefore in experimental conditions which have reduced granule cell population, the presence of unwed spines indicates that Purkinje dendritic spines can form in the absence of their specific pre-synaptic terminal. Other investigators have reported similar results in cerebellar culture (Seil and Herndon 1970: Aggerwal and Hendelman 1975, 1975: Privat and Drian 1976).

In the Golgi material of Purkinje neuron in culture, a large number of spines was seen on the primary and the secondary dendrites. It is known that in vivo, the primary and the secondary dendrites have few spines, and these are contacted by climbing fibers. Sotelo et al., (1975) and Sotelo and Arsenio-Nunes (1976) conducted experiments in which climbing fibers were destroyed in mature and neonatal rats, respectively. The neonatal rats were studies at maturity. Using the Golgi method and electron microscopy, they found that the number of spines on the primary and the secondary dendrites of Purkinje neurons was increased. This was

thought to be due to the absence of climbing fibers. Similarly in culture, climbing fibers are absent. Therefore it is possible that excessive formation of spines on the primary and the secondary dendrites in culture is due to the absence of climbing fibers.

Terminals have been described in culture which resemble mossy fibers (Fig. 31d). In cerebellar cultures, the presence of mossy fibers has been reported repeatedly (Hendelman 1967: Wolf and Dubois-Dalcq 1970: Kim 1970: Hendelman 1975: Privat and Drian 1975: Aggerwal and Hendelman 1976: Hendelman et al., 1977). It is known that in vivo all mossy fibers originate from sources which are located outside the cerebellum. Therefore, the source of the mossy fiber-like terminal in cerebellar culture remained unknown. Recently, the existence of a pathway from Deep nuclear neurons (D.N.) to cortex in cerebellar culture has been demonstrated electrophysiologically (Wojtowicz et al., 1975). Using neuroanatomical and neurophysiological techniques, this pathway from D.N. to cortex has been demonstrated in vivo (Tolbert et al., 1976: Bloedel et al., 1976: Gould and Graybriel 1976). These studies have indicated that the D.N. axons terminate in the internal granular layer as mossy fibers. If this fact is proven, then mossy fiber-like terminals in cerebellar culture would represent an ultrastructural demonstration of D.N. axons in the cortex.

At times, in culture the Purkinje dendritic spines were found to receive asymmetrical-type synaptic contacts from mossy fibers (Fig. 31d). Similar connections have been reported by Llinas et al., in agranular cerebella (1973).

It is known that in vivo, typical mossy fiber rossettes are totally restricted to granule cell dendrites and the Golgi cell (Palay and (Chan-Palay 1974), and they do not form contacts with Purkinje dendritic spines. Therefore, their presence in culture indicates that mossy fiber-

Purkinje dendritic spine contacts can form, in the absence of their target neuron i.e. the granule cell. A similar view was expressed by Llinas et al., (1973) also. This would represent an example of synaptic plasticity.

It has been demonstrated from the Golgi material that in addition to the perisomatic dendrites, the perisomatic spines are also retained by the mature Purkinje soma in culture (Figs. 14c, d, e, 15a, b, c and d). They are called perisomatic spines because of their location on the soma. Electron microscopically, in culture, the perisomatic spines were found to be long with a narrow neck, a rounded head, and they contained extensions of agranular reticulum (Figs. 23a, b, c, 24a, b, c, d, and e). In vivo, such spines have been classified as long (spiny branchlet-type) spines, which receive synaptic contacts from parallel fibers (Palay and Chan-Palay 1974). The perisomatic spines in the immature and the main dendritic spines in the adult animal are short and stubby; they receive synaptic contacts from climbing fibers. Since in culture, the perisomatic spines resemble the long spines, therefore it is concluded that the short and stubby spines do not form in culture.

In vivo, climbing fiber- perisomatic spine contacts are specific. The climbing fibers are absent in culture. Therefore, it is possible to anticipate that in culture, the perisomatic spines would not form, or would be missing pre-synaptic terminal or would be altered by available afferents. It has been demonstrated that most of perisomatic spines receive asymmetrical-type synaptic contacts from terminals which contain round vesicles. Morphologically, these terminals resemble varicosities of parallel fibers. Therefore, it is proposed that in culture, the terminals which form synaptic contacts with the perisomatic spines are the parallel fibers. Since, Sotelo et al., (1975) and Sotelo and Arsenio-Nunes (1976) have postulated that "in normal ontogenesis climbing fibers and parallel fibers compete for the post-synaptic space", therefore, it is not a surprise to find parallel

fibers at a post-synaptic site which normally would be occupied by climbing fibers.

Some of the perisomatic spines seen in culture, lacked a presynaptic terminal. These were either surrounded by a sheath of glia or were without a sheath of glia. Many of them had a post-synaptic density. All such perisomatic spines also resemble spiny branchlet-type (Figs. 23a, b and c). These results also can be interpreted along the lines of the unwed dendritic spines, i.e. the perisomatic spine can form in the absence of its specific pre-synaptic terminal.

The reason for the absence of short and stubby spines in culture is not known. However, it is possible to speculate that during development, initially all spines are spiny branchlet-type. When the spines receive synaptic contacts from climbing fibers, the perisomatic and the main dendritic spines shrink; they become short and stubby. The speculation can account for the presence of the perisomatic spines which are covered by a sheath of glia. These have not been contacted by a pre-synaptic terminal, during development; and are still spiny-branchlet type. Based upon the speculation and the postulate of Sotelo and his collaborators (Sotelo et al., 1975: Sotelo and Arsenio-Nunes 1976), it would seem reasonable for parallel fibers to form synaptic contacts with the perisomatic spines. Therefore, the spines remain long and branchlet type.

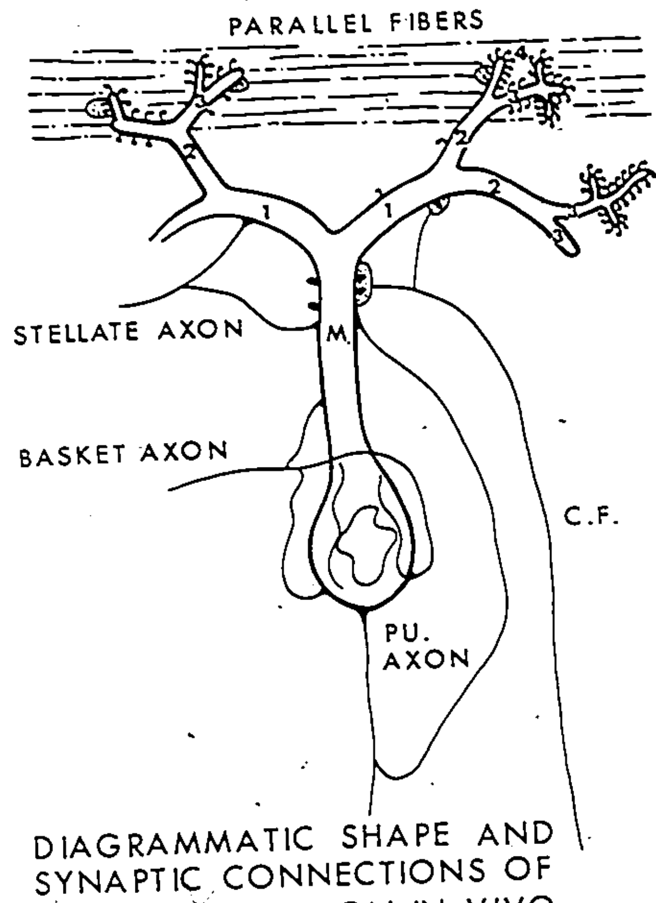
Alternatively, it is possible to conceive that the perisomatic spines form as short and stubby spines. In culture, since the climbing fibers are absent, the perisomatic spines have been contacted by parallel fibers which induce the transformation to spiny branchlet-type. Therefore, the pre-synaptic terminal (Parallel fiber) has modified the shape of the post-synaptic site (the perisomatic spine), in order to suit its need. This would represent an example of synaptic plasticity, and its influence on the post-synaptic site.

Electron microscopically, in culture the Purkinje recurrent collateral type terminals (Figs. 25c, d and 28b), and basket/stellate type terminals (Figs. 25a, b and 28a), were also seen. They form symmetrical type synaptic contacts with the smooth surface of the Purkinje soma and the dendrite; and are comparable with their counterparts in vivo. The formation of such contacts has been reported earlier (Hendelman 1972: Privat and Drian 1974, 1976: Aggerwal and Hendelman 1975, 1975: Hendelman 1975: Hendelman et al., 1977), and they represent another example of synaptic specificity in culture. The results demonstrate that the Purkinje soma in culture is mature, because during development basket/stellate type terminals and Purkinje recurrent collaterals are the last to form synaptic contacts with the smooth surface of the mature Purkinje soma. Therefore in culture, the soma is mature and has perisomatic dendrites, perisomatic spines, basket/stellate type terminals and Purkinje recurrent collaterals. In order to illustrate these results and the known synaptic connections of a mature Purkinje neuron in vivo and in culture have been drawn schematically (Fig. 34). The mature soma in culture possesses the characteristics of the soma plus the main dendrite in vivo.

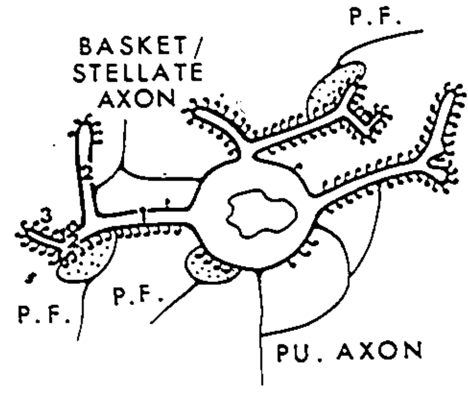
One another type of terminal was seen in these cultures (Figs. 32a and b). The terminal contains a mixed population of vesicles, and contacts the perisomatic spines. The presence of similar terminals in experimental conditions has been reported by others also (Llinas et al., 1973: Privat and Drian 1976: Sotelo and Arsenio-Nunes 1976). The origin of the terminal is not known. However, 3 possibilities can be considered:

1) According to Llinas et al., (1973) and Privat and Drian (1976), the terminals represent boutons of Purkinje recurrent collaterals.
2) According to Sotelo and Arsenio-Nunes (1976), the terminals represent boutons of stellate cell axons.

Figure 34: Shows schematically, the shape and synaptic connections of a mature Purkinje neuron in vivo and in culture.



DIAGRAMMATIC SHAPE AND SYNAPTIC CONNECTIONS OF A PURKINJE NEURON IN VIVO



DIAGRAMMATIC SHAPE AND SYNAPTIC CONNECTIONS OF A PURKINJE NEURON IN CULTURE

Figure 34

3) According to Bloom et al., (1971), the terminals resemble boutons of Locus coeruleus neurons. They demonstrated that Locus coeruleus terminals contained pleomorphic vesicles and dense core type vesicles, and formed contacts with the smooth surface of the main dendrite and the main dendritic spines. It is possible to speculate that some of the brain stem neurons which at times become included in cultures are the Locus coeruleus neurons. Therefore, it is possible to consider that the unknown terminals demonstrated in this study are the Locus coeruleus terminals.

CHAPTER - 6

SUMMARY

1) Using the Golgi method, the development of the Purkinje neuron of the mouse has been investigated. The maturation period of the Purkinje neuron has been classified into 5 distinct phases, namely:

1: The immature cell (0 - 3 days)

2: The phase of the perisomatic dendrites (4 - 6 days)

3: The phase of the perisomatic spines (7 - 10 days)

4: The phase of the main dendrite (11 - 14 days)

5: The mature neuron (over 15 days).

2) The perisomatic dendrites have been identified and distinguished from the perisomatic spines. The perisomatic spines become the main dendritic spines. The perisomatic dendrites and the apical dendrites grow laterally and towards the pia, and form the mature dendritic tree.

3) A theory of the development of the Purkinje neuron has been proposed, which postulates the descent of the nucleus. The nuclear descent is responsible for the change of location of the perisomatic dendrites and the perisomatic spines, and the development of the main dendrite.

4) It has been proposed that the Purkinje neuron develops under the influence of parallel fibers.

5) In culture, the mature shape of the Purkinje neuron is modified, and is characterized by:

1: The presence of the perisomatic spines on the soma.

2: The presence of the perisomatic dendrites on the soma.

3: The branching of the perisomatic dendrites.

4: The primary dendrites loaded with spines.

5: The absence of a stout long single main dendrite.

6: The perikaryal size is between 18 - 20 μ.

6) The Purkinje neurons in culture fail to align in a row, and the cerebellar cortex fails to laminate. In addition, there is a lack of granule cells. The modified shape of the Purkinje neuron is explained by these factors and the failure of the nucleus to descent.

7) Electron microscopically, the ultrastructural characteristics of the Purkinje neuron in culture are similar to those in vivo.

8) The development of the parallel fiber-dendritic spine contact has been demonstrated, and it has been concluded that at this level synaptic specificity is maintained in culture. In order to compensate for their reduced number the parallel fibers can become hypertrophic, and this has been termed morphological plasticity.

9) It has been demonstrated that the Purkinje dendritic spines can form in the absence of their pre-synaptic terminal.

10) It has been demonstrated that excessive formation of spines on the primary and secondary dendrites occurs in these cultures, probably due to the absence of climbing fibers.

11) Mossy fibers can form synaptic contacts with the Purkinje dendritic spines. This has been termed as an example of synaptic plasticity.

12) It has been concluded that in culture, the perisomatic spines are not short and stubby, they are spiny branchlet-type, and receive synaptic contacts from the parallel fibers. It has been demonstrated that the perisomatic spines can also form in the absence of their pre-synaptic terminal.

13) The presence of basket/stellate type terminals and the Purkinje recurrent collaterals on the mature Purkinje soma and the dendrite has been demonstrated. The results represent another example of synaptic specificity in culture.

14) The results of the electron microscopic studies have demonstrated that the mature Purkinje soma in culture has perisomatic dendrites, perisomatic spines, basket/stellate type terminals and the Purkinje recurrent collaterals. The presence of all 4 on the mature Purkinje soma in culture has been explained on the basis of the theory of development.

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