IDENTIFICATION OF AXON TERMINALS AND SYNAPSES OF DIFFERENT FIBER SYSTEMS IN THE BRAIN. EM AUTORADIOGRAPHY AND EM DEGENERATION TECHNIQUES COMPARED

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CONTENTS

PREFACE

This thesis is based largely on the following articles

DEKKER, J.J., and KUYPERS, H.G.J.M., 1975. Electron microscopy study of forebrain connections by means of the radioactive labeled amino acid tracer technique. Brain Research 85, 229-235.

DEKKER, J.J., and KUYPERS, H.G.J.M., 1976. Morphology of rat's AV thalamic nucleus in light and electron microscopy. Brain Research 117, 387-398.

DEKKER, J.J., and KUYPERS, H.G.J.M., 1976. Quantitative EM study of projection terminals in the rat's AV thalamic nucleus. Autoradiographic and degeneration techniques compared. Brain Research 117, 399-422.

CHAPTER I

INTRODUCTION

GENERAL INTRODUCTION

The brain consists of a complex network of interconnected neurons. Over the past 100 years neuroanatomical studies have tried to clarify the fiberconnections between the various neurons which make up this network. In the past these fiber connections have been studied mainly by means of the lesion and degeneration technique. In such studies a group of neuronal cell bodies is destroyed and the degenerating nerve fibers as well as their terminals are visualized selectively by means of silver impregnation techniques (Nauta, 1957; Fink & Heimer, 1967). Thus the trajectores of many groups of axons have been demonstrated and their termination areas approximated. In these investigations the light microscope (LM) was generally used. Since 1956 (Palay, 1956) the neuronal connections in the brain have also been studied by means of the electron microscope (EM). In many respects this microscope is a particulary powerful tool because it combines a high resolution with a high magnification. The high magnification provided by this microscope makes it possible to examen in great detail the fine structure of the synapses which represent the functional contact places between neurons. Such EM studies have shown that these synapses consist of three different parts, i.e., the axon terminal of the presynaptic neuron, the receiving neuronal surface of the postsynaptic neuron and the intervening synaptic junction formed by the opposed membranes of both neurons. In the different synapses the morphology of these three elements may show considerable variations. Knowledge of their ultra-

structural variations is of importance for the understanding of the anatomy of the brain, but is also of importance for other types of neurological disciples, such as neuropharmacology, neurophysiology and neurochemistry, which deal more directly with the functional characteristics of the brain. For example the synaptic morphology may be correlated with the synaptic function since morphological different synapses may subserve different types of synaptic transmission (see for instance, Eccles, 1973; Pappas & Waxman, 1972). Many EM studies have therefore been aimed at clarifying the morphology of the synapses provided by the different fiber systems. Such EM studies have also been of importance because they complement some LM studies by further defining the precise termination areas of the various fiber systems.

Axon terminals of the various fiber systems may be labeled by means of the degenerative changes which occur in the terminals after destruction of their parent cell bodies or transsection of their axons which changes can be observed by means of the electron microscope (Gray & Hamlyn, 1962; Colonnier, 1964 and Alksne et al., 1966). Thus the type of terminal which are carried by the various fiber systems can be identified. However, this lesion and degeneration technique has three disadvantages. First, the morphological changes in degenerating terminals are sometimes so bizarre that the affected structures are difficult to recognize as terminals. Second, the lesions which destroy a given group of neurons often also destroy passing fibers, the terminals of which also degenerate. If these terminals are also located on the same neurons as the terminals of the cell group which has been destroyed, the two groups of terminals on these neurons can not be distinguished. Third, at a given moment after the lesion only a limited number of terminals can be observed to degenerate. This is probably due to the rapid and asynchronous nature of the degeneration (Raisman & Matthews, 1972). Therefore at a given moment only a certain number of terminals are degenerating since

the others either still show a normal morphology or are already phagocytosed by glial cells (Garey & Powell, 1971; Jones & Powell, 1970b; Kemp & Powell, 1971; Raisman & Matthews, 1972).

The above difficulties are in part overcome by utilizing the intra-axonal transport of substances and particles which transport has been discovered by Weiss and Hiscoe in 1948. Such axonal transport of different substances and particles occurs in both directions, i.e., from the cellbody to the terminals and from the terminals to the cellbody. This axonal transport can be demonstrated by using isotopes and tracing the radioactivity by means of autoradiography. Lasek et al. (1968) and Cowan et al. (1972) showed that the transport of triated aminoacids from cellbody to terminals when studied by means of autoradiography can be used to trace fiber connections at light microscopic levels. In addition, Hendrickson (1972) showed that axonal transport of such labeled substances can also be used to identify the optic fiber terminals in the lateral geniculate body with the electron microscope. However such an identification of terminals at EM levels requires a refined electron microscopic autoradiographic technique.

The labeled amino acid axonal transport technique combined with electron microscopic autoradiography seems to be a promising method. Therefore, in the present study an attempt has been made to determine whether this technique can be used to identify the axon terminals and synapses of the different afferent fiber systems to a given nucleus in the brain. Moreover the effectiveness of this technique in demonstrating a large number of terminals has been compared with that of the lesion and degeneration technique. For these purposes the antero-ventral (AV) thalamic nucleus of the thalamus was used as a model and the morphology of the synapses of the different fibers from the mammillary bodies, cingulate cortex and hippocampus,

respectively to the AV neurons was studied. The AV thalamic nucleus was chosen because its afferents from the mammillary bodies, cingulate cortex and hippocampus have been described as being rather numerous in LM studies, which would facilitate the EM study of the different afferent axonal terminals on the AV thalamic neurons. In this framework first the normal morphology of the AV thalamic nucleus was studied and the different types of terminals and synapses present in the nucleus were determined. The results of this first study will be reported in chapter II. Subsequently the different types of terminals provided by the three afferent fiber systems were identified both by means of the labeled amino acid axonal transport technique followed by autoradiography and by means of the lesion and degeneration technique. The results of these two studies will be reported in chapter III and IV respectively.

In order to place the results of these two studies in their proper prospectives the normal morphology of terminals as well as the changes in their morphology which occur after axotomy will be described in detail. In addition our present knowledge concerning the antegrade intra axonal transport of substances will be reviewed.

TERMINALS AND SYNAPSES

In the mammalian brain the axon terminal of a neuron in general establishes contact with the dendrite or the cell body of another neuron to form a synapse. In the synapse the membrane of the axon terminal of the first neuron and the membrane of the dendrite or cell body of the second neuron are opposed and establish the synaptic junction. At this synaptic junction the transmission takes place from the first to the second neuron. Thus in synapses three different morphological elements can be distinguished, a, the axonal terminal of the first neuron, b, the receiving surface of the second neuron and c, the intervening synaptic junction formed by the opposed membranes of both neurons. In view of the direction of the transmission the axon terminal of the first neuron is regarded as the presynaptic element of the synapse, while the receiving surface of the second neuron is regarded as the postsynaptic element.

a) The *axon terminal*, which constitutes the presynaptic element, represents the terminal protrusion or enlargement of an axon. The axon terminal is also often called bouton. Terminals of various fiber systems may differ considerably in size, but within one fiber system these differences are limited. This is exemplified by the fact that in the thalamic relay nuclei the specific afferent fibers, such as the fibers of the medial lemniscus or the optic tract all carry large terminals, while the fibers from the other sources such as the cerebral cortex all carry small terminals.

Axon terminals in general contain clear vesicles as well as mitochondria but may also contain dense core vesicles, neurofilaments, multivesicular bodies and glycogen particles. Yet, the small electron-lucent or clear vesicles are the most characteristic feature of axon terminals. These vesicles, which are of the order of 20 nm in diameter are scattered throughout the terminal but are clumped to-

gether at the synaptic junction. It is assumed that these vesicles contain the chemical mediator for synaptic transmission. In the central nervous system many chemical substances have been found which may act as neurotransmitter, such as acetylcholine, norepinephrine, dopamine, serotonine, γ-aminobutyric acid (GABA) and glycine (Cooper et al., 1974).

The clear vesicles in the terminals may be spheric, flattened or pleomorphic in shape, respectively. Several studies have drawn attention to the fact that these differences in vesicular shape may be correlated with functional differences. Thus, Uchizono (1965) demonstrated that axon terminals on the dendritic spines of the Purkinje cells (see later), which terminals are known to be excitatory in nature (Andersen et al., 1963) contain spherical vesicles, while the axon terminals on Purkinje cell bodies, which terminals have been shown to be inhibitory (Andersen et al., 1963) contain flattened vesicles. These findings have been confirmed in mammals (Gray, 1969; Hirata, 1966; Larramedi & Victor, 1967; Uchizono, 1968). The existence of such a correlation between vesicle shape and function of the synapse has also been stressed in respect to the spinal cord (Bodian, 1966) and hippocampus and dentate gyrus (Gottlieb & Cowan, 1972). In respect to the correlation between the morphology and function of synapses it would be of interest to know whether the spherical vesicles contain an excitatory transmitter and the flattened vesicles an inhibitory transmitter. Sofar this appears indeed to be true for the inhibitory, flattened vesicles containing spinal interneurons and cerebellar Golgi II cells which contain the inhibitory transmitters glycine (Matus and Dennison, 1971) and γ-aminobutyric acid (McLaughlin et al., 1974), respectively.

Many mitochondria may be present in axon terminals. They probably do not play an immediate role in transmission but provide the energy for processes such as the sodium pump (Peters et al. 1976).

Several axon terminals in the mammalian central nervous system may contain dense core vesicles in addition to clear vesicles and mitochondria. These dense core vesicles measure 80-90 nm with a dense core of 50 nm and are not present at the synaptic junction (Peters et al., 1976). In some axonal terminal neurofilaments and tubules are present. In the cat these filaments have been found to be arranged in loops surrounding mitochondria (Gray & Guillery, 1966).

b) The *postsynaptic elements* of synapses in general may consist of proximal or distal dendritic shafts of dendritic bulbous protrusions and of cell bodies or their protrusions. Proximal and distal dendritic shafts have in common that they both contain a hexagonal arrangement of tubuli (Peters et al., 1976). However, they can be differentiated by the fact that only proximal dendrites also contain ribosomes. On the other hand bulbous dendritic protrusions do not contain tubuli but have a fuzzy fround substance. Dendritic protrusions in the cerebral cortex and hippocampus have a thin neck and are called spines. They usually also contain a spine apparatus, which consist of two or three sacs alternating with thin laminae of dense material (Peters et al., 1976).

The synaptic input to different regions of the postsynaptic neuronal surface is reflected in differences in physiological parameters (Pappas & Waxman, 1972; Colonnier, 1974). In synapses of axon terminals with cell bodies the postsynaptic potentials recorded from the cell body are frequently larger and have faster rise and fall times than the potentials of synapses between axon terminals and distal dendrites (Fadiga & Brookhart, 1960; Rall, 1967). Synapses of axon terminals with distal dendrites may allow linear summation as well as non-linear summation of axo-dendritic and axo-somatic potentials (Rall, 1967).

c) The *synaptic junction* is formed by the opposed membranes of the pre- and postsynaptic elements. These synaptic membranes are separated by a cleft of 20-40 nm in

which no material seems to be present (DeRobertis, 1956). At the presynaptic membrane hexagonally placed particles are present, which form the 'presynaptic vesicular grid', the holes of which are filled by presynaptic vesicles (Akert et al., 1972). When the depolarization wave arrives at the synapse these vesicles presumably release their chemical transmitter content into the synaptic cleft which effects the transmission across the synaptic junction (Katz, 1966). This type of synapse at which transmitters are released, is called a chemical synapse and most of the synapses in the mammalian brain appeared to be of this type. Moreover, these synapses are characterized physiologically by a transmission delay. In these so-called chemical synapses dense material is associated with the cytoplasmic surface of both the presynaptic and the postsynaptic membrane. On the basis of the relative distribution of this dense cytoplasmic material Gray (1959) distinguished a type I and type II synaptic junction. In type I the postsynaptic density is thicker than the presynaptic one, while in type II the synaptic densities have the same thickness. In OsO_4 fixed, phosphotungstic acid (PTA) stained cerebral cortex Gray (1959) found the type I synaptic junctions mainly on bulbous protrusions and distal dendrites, but type II exclusively on cell bodies. However, in formalin fixed material of the same and other parts of the central nervous system Gray's type I and II terminals were found to be two extremes of a morphological continum (Colonnier, 1968; Peters et al., 1976). In addition, Colonnier (1968) found in other areas that the distribution of Gray's type I and II was slightly different from that in the cerebral cortex. He therefore generalized the terminology in asymmetrical and symmetrical synaptic junctions, respectively. The Colonnier's terminology will also be used in this study.

In the hippocampus as well as in the cerebellar cortex excitatory synapses were found to possess asymmetrical sy-

naptic junctions, while inhibitory synapses were found to possess symmetrical synaptic junctions (Walberg, 1968). As a consequence, excitatory synapses tend to comprise presynaptic terminals with spherical vesicles and asymmetrical synaptic junctions, while inhibitory synapses tend to comprise presynaptic axon terminals with flattened vesicles and symmetrical synaptic junctions. Such correlations have indeed been shown in the cerebellar cortex (Uchizono, 1965), the olfactory cerebral cortex and superior colliculus (Lund & Westrum, 1966). In many areas of the nervous system spherical presynaptic vesicles occur together with asymmetrical synaptic junctions while flattened vesicles occur together with symmetrical synaptic junctions (see later). However, the physiological properties of all these synapses have not been studied as yet. Moreover the above correlation between synaptic morphology and function does not exist in all areas, since Palay (1967) showed that in the cerebellar cortex of the rat climbing fibers which are known to have a powerful postsynaptic excitation (Eccles, 1964), establish symmetrical synaptic junctions.

Although probably most of the synapses are formed between axon terminals and dendrites or cell bodies, there also exist *axo-axonal synapses* between axons and either initial axonal segments or other axon terminals. In synapses with initial axonal segments the presynaptic axon terminals generally contain flattened vesicles and symmetrical synaptic junctions. These initial segments appear to be a favourable location for inhibitory contacts (Eccles, 1964). The synapses between terminals may be distinguished into 3 different types on the basis of the synaptic junction and on the basis of the vesicle shape in the pre- and postsynaptic terminal. In these synapses the presynaptic terminal can be distinguished from the postsynaptic terminal because the vesicles of the former are clumped at the synaptic junction. Type a) axo-axonal synapses have asymmetrical synaptic junctions and presynaptic and postsynap-

tic terminals which both contain spherical vesicles. Physiologically this type of synapse was found to subserve presynaptic inhibition (Eccles, 1964; Gray, 1962; Walberg, 1965b), such that excitiation of the postsynaptic terminal by the presynaptic one results in a decrease or abolition of its release of excitatory transmitter (Eccles, 1973). This type of axo-axonal synapses have been found for instance in the cuneate nucleus (Walberg, 1965b), in the spinal ventral horn (Khattab, 1968) and in the spinal dorsal horn (Ralston, 1968). Type b) axo-axonal synapses have asymmetrical synaptic junctions, presynaptic terminals containing spherical vesicles but postsynaptic terminals which contain flattened vesicles. This type has been found in the ventro-basal complex (Ralston, 1969), the lateral geniculate nucleus (LGN) (Ralston & Chow, 1974; Guillery, 1971; Guillery & Colonnier, 1970; LeVay, 1971; Wong-Riley, 1972a and Robson & Hall, 1977) and the pulvinar (Campos-Ortega & Hayhow, 1973) of the thalamus. Type c) axo-axonal synapses have symmetrical synaptic junctions, presynaptic terminals containing flattened vesicles and postsynaptic terminals with spherical vesicles. These axo-axonal synapses have been found for instance in the main sensory (Gobel & Dubner, 1969) and spinal trigeminal (Gobel, 1974) nuclei, the lateral cuneate (O'Neal & Westrum, 1973) and gracile (Rustioni & Sotelo, 1974) nuclei and in the motoneuronal area (Bodian, 1975) and the substantia gelatinosa (Coimbra et al., 1974) of the spinal cord.

Further studies showed that in many synapses which at first sight seem to consist of two axon terminals, the postsynaptic element contains not only clear vesicles but also ribosomes and multivesicular bodies. In some cases these postsynaptic elements could be traced back to stem dendrites and therefore must be regarded as dendrites instead of axons (Lieberman & Webster, 1974). Moreover it was found (see for instance Ralston & Herman, 1969) that

such vesicle-containing dendrites frequently represent the presynaptic element in synapses with other dendrites. Such vesicle containing dendrites are therefore called *presynaptic dendrites*. Thus, these presynaptic dendrites may represent the presynaptic element in synapses with conventional dendrites, but may also represent the postsynaptic element in synapses with axon terminals. After their discovery in the olfactory bulb (Rall et al., 1966), such presynaptic dendrites have been described in many other places of the central nervous system, e.g., the thalamus (Rinvik & Grofova, 1974a; Harding, 1973a; Ralston & Herman, 1969; Famiglietti, 1970; Lieberman, 1973; Lieberman & Webster, 1974; Wong, 1970; LeVay, 1971; Pasik et al., 1973; Hamori et al., 1974), the hypothalamus (Güldner & Wolff, 1974), the brain stem (Gobel, 1974) and spinal cord (Ralston, 1968; Coimbra et al., 1974). In several thalamic nuclei, the presynaptic dendrites have been identified as processes of interneurons, while the postsynaptic dendrites which were of the conventional type could be identified as processes of relay neurons (see for instance, Rinvik & Grofova, 1974a; Harding, 1973a; Lieberman, 1973; Pasik et al., 1973).

In several places in the central nervous system synaptic complexes exist which are sometimes separated from the surrounding neuropil by glial lamellae. These complexes are called *glomeruli*. Two types of glomeruli may be distinguished. In the one type a centrally localized axon terminal makes synapses with several structures which surround the terminal and may include conventional dendrites, presynaptic dendrites and axon terminals. In the other type of glomeruli a dendrite is located centrally, around which axon terminals and presynaptic dendrites are grouped. Glomeruli have been found for instance in the thalamus (Rinvik & Grofova, 1974a; Harding, 1973a; Jones & Powell, 1969a; Spacek & Lieberman, 1974a; Wong-Riley, 1972a; Guillery & Colonnier, 1970 and LeVay, 1971), the

sensory trigeminal nuclei (Gobel & Dubner, 1969; Gobel, 1974), the cerebellum (Palay & Chan-Palay, 1974) and in the dorsal horn of the spinal cord (Ralston, 1968 and Coimbra, 1974).

Finally, in many areas reports have been made of the so-called *serial* and *triadic synaptic arrangements*, which occur in many glomeruli but also in the interglomerular neuropil. In a *serial* synaptic arrangement a neuronal process is presynaptic to a second neuronal process which in turn is presynaptic to a third one. The previous mentioned axo-axonal synapses form part of serial synapses since their postsynaptic terminal (the second neuronal process) is presynaptic to a third neuronal process. In a *triadic* synaptic arrangement a neuronal process (usually an axon terminal) is presynaptic to both a second (presynaptic dendrite or axon terminal) and a third neuronal process (conventional dendrite), of which the second neuronal process is also presynaptic to the third one. Triadic synaptic arrangements have been described in the thalamus: the VA nucleus (Rinvik & Grofova, 1974a), the VL nucleus (Rinvik & Grofova, 1974a; Harding, 1973a), the paracentral nucleus (Partlow et al., 1977), the ventro-basal complex (Ralston, 1969), the medial geniculate nucleus (MGN) (Jones & Rockel, 1971), the lateral geniculate nucleus (LGN) (Lieberman & Webster, 1974; LeVay, 1971; Hamori et al., 1974; Pasik et al., 1973 and Robson & Hall, 1977) and the pulvinar (Campos-Ortega & Hayhow, 1973; Partlow et al., 1977 and Robson & Hall, 1977); in the brainstem: the main sensory (Gobel & Dubner, 1969) and spinal trigeminal (Gobel, 1974) nuclei and the lateral cuneate (O'Neal & Westrum, 1973) and gracile (Rustioni & Sotelo, 1974) nuclei, and in the substantia gelatinosa of the dorsal horn (Coimbra et al., 1974).

The synapses described so far all are characterized by a synaptic cleft of 20 nm between the membranes of the presynaptic and the postsynaptic element. These sy-

napses are the most common type and appear to function by releasing transmitter substances. However, there also exist an other type of synapse in which the outer lamellae of the opposed pre- and postsynaptic membranes are separated from their inner lamellae and come into very close contact with one an other, being separated by a gap of only 2 nm. These synapses are called *gap junctions* and have been found in some places of the mammalian nervous system such as the vestibular nucleus (Sotelo & Palay, 1967). They are regarded as electrotonic synapses and in contrast to the chemical synapses are physiologically characterized by transmission without delay (Bennett, 1972).

THE IDENTIFICATION OF TERMINALS AND SYNAPSES OF DIFFERENT AXON SYSTEMS BY MEANS OF THE EM DEGENERATION TECHNIQUE

Many electron microscopic studies have tried to determine from which fiber systems the various terminals and synapses in a given neuronal cell group are derived. Yet, in general the afferent sources of the various terminals, which occur on the cells and their dendrites are difficult to determine in normal light and electron microscopic material with the possible exception of some of the terminals in the cerebellar cortex (Palay & Chan-Palay, 1974) and the deep cerebellar nuclei (Chan-Palay, 1973). Several authors (Colonnier, 1964; Walberg, 1965a; Alksne et al., 1966) drew the attention to the fact that the lesion and degeneration method may be used in identifying the terminals and synapse of different fiber systems.

Gray & Hamlyn (1962) were one of the first to study degenerating axons and terminals with the electron microscope. They studied the degenerating terminals in the chick's optic tectum after selective interruption of the optic nerve. After this operation some of the terminals in the optic tectum showed a *hypertrophy of* their *terminal neurofilaments*. These terminals with filamentous hypertrophy were regarded to be derived from the optic nerve fibers, while the other terminals which did not show any changes, were regarded to be derived from other sources. Colonnier and Gray (1962) and Colonnier (1964) studied the degenerating terminals in the cerebral cortex after interruption of the subcortical afferents. They reported a completely different type of degenerative change in terminals in the cerebral cortex than had been observed in the optic tectum. Thus, the degenerating terminals in the cortex showed shrinkage, while the axoplasm and mitochondria became *electron-dense* followed by a rapid phagocytotic destruction of the terminals by glial cells. The

findings in subsequent studies (see Table I for references) suggested that in the central nervous system, most of the terminals exhibit either the hypertrophic filamentous type as showed in the optic tectum or the electron-dense type observed in the cerebral cortex.

The hypertrophic filamentous type of degeneration was displayed by a substantial number of afferents in different cell groups in the central nervous system, some of which are listed below; in the *thalamus*, the cerebellar afferents in the ventro-anterior (VA) nucleus (Rinvik & Grofova, 1974b) and the ventro-lateral (VL) nucleus (Rinvik & Grofova, 1974b; Harding, 1973b), the dorsal column nuclei afferents in the ventro-basal (VB) complex (Ralston, 1969), the inferior colliculus afferents in the medial geniculate nucleus (Jones & Rockel, 1971), the retinal afferents in the lateral geniculate nucleus (Lieberman & Webster, 1974; Szentagothai et al., 1966; Wong-Riley, 1972b; Pecci Saavedra et al., 1968; Guillery & Colonnier, 1970 and LeVay, 1971), occipital cortical afferents in the inferior pulvinar (Campos-Ortega & Hayhow, 1973); in the *cerebellum*, the mossy fibers (Palay & Chan-Palay, 1974); in the *brainstem*, the cerebellar afferents in the red nucleus (King et al., 1973; Nakamura & Mizuno, 1971), the pontine nuclei (Mihailoff & King, 1975) and in the lateral vestibular nucleus (Mugnaini et al., 1967), retinal afferents in the superior colliculus (Valverde, 1973; Lund, 1969), the AVCN afferents in the superior olive (Perkins 1973), the dorsal root afferents in the cuneate nucleus (Walberg, 1966) and in the *spinal cord* for dorsal root afferents in laminae IV, V and VI of the dorsal horn (Ralston 1968). Thus when the above fiber systems are interrupted all their axon terminals show filamentous hypertrophy which is accompanied by accumulation of glycogen, while the other terminals in these nuclei after transection of their parent fibers generally displayed the rapid electron-dense type of degeneration (see Table I for references).

However, the filamentous degeneration which occurs in the above type of terminals apparently represents only the first stage of degeneration because after a slightly longer degeneration time they developed the electron-dense type of degeneration before they were engulfed and phagocytosed by glial processes. The terminals of the cortical fibers to the inferior pulvinar apparently form an exception to this rule since they show only the filamentous type of degeneration up to the moment that they were phagocytosed (Campos-Ortega & Hayhow, 1973).

In addition to these types of degeneration, DeRobertis (1956) and Gentschev and Sotelo (1973) have described a third type of degeneration. They found that after cutting the cochlear nerve many terminals in the anterior ventral cochlear nucleus were swollen and completely empty except for aggregations of vescicles close to the synaptic junctions. This *electron-lucent* type of degeneration however, sometimes also developes into the electron-dense type. The electron-lucent type of degeneration does not only occur in cochlear nerve terminals but has also been found in terminals in the ventrolateral thalamic nucleus after cutting the pallidal afferents (Grofova & Rinvik, 1974), in terminals in the spinal trigeminal nucleus after cutting the primary trigeminal afferents (Westrum & Black, 1971), in the dorsal root terminals in the gracile nucleus as well as in the lateral cuneate nuclei after hemisection of the spinal cord (Rustioni & Sotelo, 1974; O'Neal & Westrum, 1973) and in the perforate pathway terminals from the entorrhinal area to the dentate fascia (Fifkova, 1975).

Table I lists several studies in which the axonal terminals and synapses of various fiber connections have been identified after selective interruption of the parent fibers. The terminals could be identified as such because after axotomy they showed clearly distinguishable morphological changes which usually correspond with one of the three above mentioned types of degeneration. The findings

in these studies suggest that the afferent terminals in these various nuclei are of two sizes and are either small (on the left) or large (on the right). In most of the nuclei, especially in the thalamus, the large terminals degenerate after interruption of the *specific* extrinsic afferents, i.e., the cerebellar, collicular and retinal afferents in the thalamus. The same appears to be true for the mossy fibers in the cerebellum; the cerebellar, retinal, primary trigeminal, primary cochlear and dorsal root afferents in the brainstem, as well as for the dorsal root afferents in laminae IV, V and VI in the dorsal horn and the motoneuronal areas in the spinal cord. In contrast, after interruption of the other, so called *non-specific*, extrinsic afferents to the cell groups in the thalamus, brainstem and spinal cord, small terminals are generally found to degenerate (see tables for references). Further, in almost all the extrinsic *specific* and *non-specific* afferent synapses, which are established with dendrites or cell bodies, the presynaptic vesicles are spherical in shape and the synaptic junctions resemble the asymmetrical type (Colonnier, 1968). In most of the thalamic nuclei, in the sensory trigeminal nuclei as well as in the dorsal horn, the large terminals are incorporated in glomeruli while the small terminals are in general dispersed over the interglomerular neuropil. Finally, the extrinsic *specific* and *non-specific* terminals not only establish synapses with conventional dendrites but also with presynaptic dendrites, which themselves in turn form synapses with conventional dendrites. This arrangement occurs in most of the thalamic nuclei, the superior colliculus, the sensory trigeminal nuclei and substantia gelatinosa in the dorsal horn (see table I for references).

Relatively few studies have focused on the morphology of the axon terminals of the *intrinsic* afferents, i.e., those terminals which are derived from interneurons. Kemp & Powell (1971) studied such terminals in the caudate

nucleus of the cat. They selectively destroyed a part of this nucleus and found that in the other part small terminals degenerate. However, in contrast with the small terminals which are derived from the non-specific afferents, the small terminals of the intrinsic afferents contained flattened vesicles, established synapses with cell bodies and with initial segments of axons and exhibited symmetrical synaptic junctions. The same arrangement seems to occur in many other nuclei in the thalamus, brainstem and spinal cord (see Table I for references). In these nuclei also small or medium-sized terminals have been found which in contrast to the extrinsic afferent terminals contain flattened vesicles, establish synapses with dendrites, cell bodies and initial segments and posses asymmetrical synaptic junctions. Most of these terminals probably also belong to interneurons because they remain unaltered after interruption of the extrinsic afferents. Yet this might not be true in all cell groups for Fox et al. (1967), Hamori & Szentagothai (1966) and Mugnaini (1972) showed that the terminals of the fibers from the intrinsic Golgi cells in the cerebellum contain spherical vesicles and establish synapses with asymmetrical synaptic junctions. Lieberman & Webster (1974) in their studies of the afferents in the lateral geniculate nucleus argue that it is unlikely that the terminals with flattened vesicles and symmetrical synaptic junctions belong to interneurons because these interneurons also posses presynaptic dendrites with pleomorphic vesicles. They as well as Harding (1973a) suggest that two types of interneurons might exist, one with terminals with flattened vesicles, and the other with presynaptic dendrites.

The above data have largely been obtained by means of the lesion and degeneration techniques. Yet, many investigators have pointed out that in using this technique several difficulties may arise. There are three different kind of difficulties to be distinguished.

TABLE I, EM DEGENERATION STUDIES

CEREBRAL CORTEX	
motor cortex, laminae I, III & VI	VL thalamic afferents small terminals, spherical vesicles presynaptic in synapses with dendritic spines and shafts asymmetrical synaptic junctions in cat (Strick and Sterling, 1974)
somato-sensory cortex	commissural afferents (in laminae I, III & IV), association afferents (in laminae III, IV & V) and thalamic afferents (in lamina IV) small terminals, spherical vesicles presynaptic in synapses with dendritic spines and shafts asymmetrical synaptic junctions in cat (Jones and Powell, 1970)
visual sensory cortex	thalamic afferents (in lamina IV) small terminals, spherical vesicles presynaptic in synapses with dendritic spines & shafts & soma's asymmetrical synaptic junctions in cat and monkey (Garey & Powell, 1971)
pre-pyriform cortex	olfactory bulb afferents small (?) term., spherical vesicles presynaptic in synapses with dendritic spines and shafts asymmetrical synaptic junctions in rat (Westrum, 1969)
STRIATUM	cortical afferents small terminals, spherical vesicles presynaptic in synapses with dendritic spines & shafts & soma's asymmetrical synaptic junctions in rat (Bak et al., 1975) in cat (caudate nucleus, Kemp and Powell, 1971) substantia nigra afferents small terminals, spherical vesicles presynaptic in synapses with dendritic spines and cell bodies asymmetrical synaptic junctions in rat (Bak et al., 1975) in cat (caudate nucleus, Kemp and Powell, 1971) intrinsic afferents in the caudate nucleus small terminals, flattened vesicles presynaptic in synapses with cell bodies, dendrites and initial segments of axons symmetrical synaptic junctions in cat (Kemp & Powell, 1971)
	afferents from the center median and parafascicular complex large terminals, spherical vesicles presynaptic in synapses with dendritic spines & shafts & soma's asymmetrical synaptic junctions in cat (fundus striati & putamen, Chung et al., 1976 & 1977) in cat (caudate nucleus, Chung et al., 1977; Kemp & Powell, 1971 (?))

TABLE I (continued)

THALAMUS		
ventro-anterior nucleus	cortical (precruciate) afferents small terminals, spherical vesicles presynaptic in synapses with dendrites and presynaptic dendrites asymmetrical synaptic junctions within the interglomerular neuropil in cat (Grofova and Rinvik, 1974)	cerebellar afferents large terminals, spherical vesicles presynaptic in synapses with dendrites and presynaptic dendrites asymmetrical synaptic junctions within glomeruli triadic synaptic arrangements in cat (Rinvik and Grofova, 1974)
ventro-lateral nucleus	cortical (motor) afferents small terminals, spherical vesicles presynaptic in synapses with dendrites and presynaptic dendrites asymmetrical synaptic junctions within the interglomerular neuropil in cat (Grofova and Rinvik, 1974) in monkey (Harding, 1973b) pallidal afferents small terminals, flattened vesicles presynaptic in synapses with dendrites and cell bodies symmetrical synaptic junctions within the interglomerular neuropil in cat (Grofova and Rinvik, 1974) small terminals, spherical vesicles presynaptic in synapses with dendrites and presynaptic dendrites asymmetrical synaptic junctions within the interglomerular neuropil in monkey (Harding, 1973b)	cerebellar afferents large terminals, spherical vesicles presynaptic in synapses with dendrites and presynaptic dendrites asymmetrical synaptic junctions within glomeruli triadic synaptic arrangements in cat (Rinvik and Grofova, 1974) in monkey (Harding, 1973b)
centre median	cortical (motor and pallidal afferents small terminals, spherical vesicles presynaptic in synapses with dendrites and presynaptic dendrites asymmetrical synaptic junctions within the interglomerular neuropil in monkey (Harding, 1973b)	
para-central nucleus		superior colliculus afferents large terminals, spherical vesicles presynaptic in synapses with dendrites and presynaptic dendrites asymmetrical synaptic junctions within glomeruli serial & triadic synap arrangements in monkey (Partlow et al., 1977)
ventro-basal complex	cortical (sensory) afferents small terminals, spherical vesicles presynaptic in synapses with dendrites & dendritic excrescences asymmetrical synaptic junctions within the interglomerular neuropil in rat (Spacek and Lieberman, 1974) small terminals, spherical vesicles presynaptic in synapses with dendrites & presynap (?) dendrites asymmetrical synaptic junctions within the interglomerular neuropil in cat (Jones and Powell, 1969)	dorsal column nuclei afferents large terminals, spherical vesicles presynaptic in synapses with dendritic & somatic excrescences asymmetrical synaptic junctions within glomeruli in rat (Spacek and Lieberman, 1974) large terminals, spherical vesicles presynaptic in synapses with dendrites, presynaptic dendrites & other axonal terminals asymmetrical synaptic junctions within glomeruli serial & triadic synap arrangements in cat (Ralston, 1969)

TABLE I (continued)

medial geniculate nucleus	cortical (temporal) afferents small terminals, spherical vesicles presynaptic in synapses with dendrites & presynap (?) dendrites asymmetrical synaptic junctions within the interglomerular neuropil in cat (Jones and Powell, 1969)	inferior colliculus afferents large terminals, spherical vesicles presynaptic in synapses with dendrites & presynap (?) dendrites asymmetrical synaptic junctions within glomeruli serial & triadic synap arrangements in cat (Jones & Rockel, 1971)
lateral geniculate nucleus	cortical (visual) afferents small terminals, spherical vesicles presynaptic in synapses with dendrites asymmetrical synaptic junctions within the interglomerular neuropil in rat (Lund & Cunningham, 1972; Lieberman & Webster, 1974) in grey squirrel (Robson & Hall, 1977) small terminals, spherical vesicles presynaptic in synapses with dendrites and presynaptic dendrites within the interglomerular neuropil in cat (Jones and Powell, 1969b; Guillery, 1971 and Szentagothai et al., 1966) in monkey (Wong-Riley, 1972c; Pasik et al., 1973; Hámori et al., 1974) superior colliculus afferents small terminals, spherical vesicles presynaptic in synapses with dendrites within the interglomerular neuropil asymmetrical synaptic junctions in grey squirrel (Robson & Hall, 1977)	retinal afferents large terminals, spherical vesicles presynaptic in synapses with dendrites and presynaptic dendrites asymmetrical synaptic junctions within glomeruli serial & triadic synap arrangements in rat (Lieberman and Webster, 1974 in cat (Szentagothai et al., 1966) in monkey (Guillery & Colonnier, 1970; Guillery, 1971; LeVay, 1971; Pasik et al., 1973; Hámori et al., 1974) large terminals, spherical vesicles presynaptic in synapses with dendrites, cell bodies, axonal terminals and presynaptic dendrites (rabbit & sq. monkey) asymmetrical synaptic junctions within glomeruli serial & triadic synap arrangements in rabbit (Ralston & Chow, 1974) in grey squirrel (Robson & Hall, 1977) in squirrel monkey (Wong-Riley, 1972)
pulvinar	cortical (temporal) and superior colliculus afferents small terminals, spherical vesicles presynaptic in synapses with dendrites asymmetrical synaptic junctions within interglomerular neuropil in grey squirrel (caudal & rostro-lateral pulvinar, Robson & Hall, 1977) in squirrel monkey (inferior pulvinar, Mathers, 1971 and 1972) occipital (extrastriate) cortical afferents small terminals, spherical vesicles presynaptic in synapses with dendrites asymmetrical synaptic junctions within the interglomerular neuropil in grey squirrel (caudal & rostro-lateral pulvinar, Robson & Hall, 1977) in squirrel monkey (lateral pulvinar, Mathers, 1972)	superior colliculus afferents medium-sized and large (rhesus monkey) term., spherical vesicles presynaptic in synapses with dendrites , axonal terminals and presynaptic dendrites (rh. monkey) asymmetrical synaptic junctions within glomeruli serial & triadic synap arrangements in grey squirrel (caudal & rostro-lateral pulvinar, Robson & Hall, 1977) in rhesus monkey (inferior pulvinar, Partlow et al., 1977) occipital (striate) cortical afferents large terminals, spherical vesicles presynaptic in synapses with dendrites and axonal terminals asymmetrical synaptic junctions within glomeruli serial & triadic synap arrangements in grey squirrel (rostromedial pulvinar, Robson & Hall, 1977) in rhesus monkey (inferior pulvinar Campos-Ortega & Hayhow, 1973)

TABLE I (continued)

PRE-OPTIC AREA	stria terminalis afferents small (?) term., spher.(?) vesicles presynaptic in synapses with dendritic shafts and spines asymmetrical synaptic junctions in rat (Field, 1972)	
VENTRO-MEDIAL HYPO-THALAMUS	stria terminalis afferents small (?) term., spher. (?) vesicles presynaptic in synapses with dendritic spines asymmetrical synaptic junctions in rat (Field, 1972)	
DENTATE GYRUS	entorrhinal afferents small terminals, spher (?) vesicles presynaptic in synapses with dendritic spines asymmetrical synaptic junctions in rat (Fifkova, 1975; Nafstad, 1967) commissural afferents small (?) term., spher., vesicles presynaptic in synapses with dendritic spines and shafts asymmetrical synaptic junctions in rat (Alksne et al., 1966; Hjorth-Simonsen and Laurberg, 1977; Laatsch & Cowan, 1967)	
SEPTUM	hippocampal afferents small (?) term., spher.(?) vesicles presynaptic in synapses with dendritic spines and shafts asymmetrical synaptic junctions in rat (Raisman, 1969; Raisman & Field, 1973)	
CEREBELLUM		
granule cell layer	Golgi cell afferents small terminals, spherical vesicles presynaptic in synapses with dendrites of granule cells asymmetrical synaptic junctions within glomeruli in monkey (Fox et al., 1967; Hamori & Szentagothai, 1966; Mugnaini, 1972)	mossy fiber afferents large terminals, spherical vesicles presynaptic in synapses with dendrites of granule cells and with cell bodies of Golgi cells asymmetrical synaptic junctions in rat (Chan-Palay & Palay, 1973)

TABLE I (continued)

BRAINSTEM

red nucleus	cortical (sensory-motor) afferents small terminals, spherical vesicles presynaptic in synapses with dendrites asymmetrical synaptic junctions in rat (Flumerfelt & Gwyn, 1973; Brown, 1974a) small terminals, spher. & flat ves. in opposum (King et al., 1972) cerebellar (dentate) afferents small terminals, spherical vesicles presynaptic in synapses with dendrites and cell bodies asymmetrical synaptic junctions in opposum (King et al., 1973)	cerebellar (interpositus) afferents large terminals, spherical vesicles presynaptic in synapses with dendrites and cell bodies asymmetrical synaptic junctions in opposum (King et al., 1973) in cat and rabbit (Nakamura and Mizuno, 1971)
sub-stantia nigra	striatal afferents medium-sized term., pleom. vesicles presynaptic in synapses with dendrites, spines & cell bodies symmetrical synaptic junctions in rat (Hadju et al., 1973) in cat (Grofova & Rinvik, 1970; Kemp & Powell, 1970)	
superior colliculus	cortical (visual) afferents small terminals, spherical vesicles presynaptic in synapses with dendrites & spines asymmetrical synaptic junctions in mouse (Valverde, 1973) in rat (Lund, 1969)	retinal afferents large terminals, spherical vesicles presynaptic in synapses with dendrites, spines & presyn. dendr. asymmetrical synaptic junctions in mouse (Valverde, 1973) in rat (Lund, 1969)
pontine nuclei	cortical (sensory-motor) afferents small terminals, spherical vesicles presynaptic in synapses with dendrites and cell bodies asymmetrical synaptic junctions in opposum (Mihailoff & King, 1975) in cat (Holländer et al., 1969)	cerebellar (interpositus) and cortical (visual) afferents large terminals, spherical vesicles presynaptic in synapses with dendrites and cell bodies asymmetrical synaptic junctions in opposum (Mihailoff & King, 1975)
main sensory tri-geminal nucleus		trigeminal primary afferents large terminals, spher (?) vesicles presynaptic in synapses with dendrites and presynaptic dendrites asymmetrical synaptic junctions postsynaptic in synapses with axon terminals with flat vesicles within glomeruli serial & triadic synap arrangements in cat (Gobel & Dubner, 1969)
lateral vestibu-lar nucleus	primary afferents small (?) term., spherical vesicles presynaptic in synapses with dendrites and spines asymmetrical synaptic junctions in cat (Mugnaini et al., 1967)	cerebellar afferents large (?) term., spherical vesicles presynaptic in synapses with dendrites, spines and cell bodies asymmetrical synaptic junctions in cat (Mugnaini et al., 1967)
superior olive		AVCN afferents large terminals, spherical vesicles presynaptic in synapses with dendrites asymmetrical synaptic junctions in chinchillas (Perkins, 1973)

TABLE I (continued)

ventral cochlear nucleus		cochlear nerve afferents large terminals, spherical vesicles presynaptic in synapses with dendrites and cell bodies asymmetrical synaptic junctions in rat (Reese, 1966 Gentschev and Sotelo, 1973)
inferior olive	cortical afferents small (?) term., spherical vesicles presynaptic in synapses with dendrites asymmetrical synaptic junctions in cat (Walberg, 1965) spinal afferents small terminals, spherical vesicles presynaptic in synapses with dendrites and spines asymmetrical synaptic junctions in opposum (King et al., 1975)	
lateral reticular & facial nucleus	rubral afferents small terminals, spherical vesicles presynaptic in synapses with dendrites and cell bodies asymmetrical synaptic junctions in rabbit (Mizuno et al., 1973)	
spinal trigeminal nucleus (interpolaris)		primary trigeminal nerve afferents large (?) term., spherical vesicles presynaptic in synapses with dendrites asymmetrical synaptic junctions in cat (Westrum & Black, 1971) large terminals, spherical vesicles presynaptic in synapses with dendrites and presynaptic dendrites asymmetrical synaptic junctions postsynaptic in synapses with axon terminals with flat vesicles within glomeruli serial & triadic synap arrangements in cat (Gobel, 1974)
cuneate nucleus	cortical afferents small terminals, spherical vesicles presyanaptic in synapses with dendrites asymmetrical synaptic junctions in cat (Walberg, 1966)	dorsal root afferents large terminals, spherical vesicles presynaptic in synapses with dendrites, cell bodies and axonal terminals asymmetrical synaptic junctions in cat (Walberg, 1966)
lateral cuneate & gracile nuclei	cortical afferents small terminals, spherical vesicles presynaptic in synapses with dendrites asymmetrical synaptic junctions in cat (Rustioni & Sotelo, 1974)	dorsal root afferents large terminals, spherical vesicles presynaptic in synapses with dendrites and cell bodies asymmetrical synaptic junctions postsynaptic in synapses with axonal terminals with flat vesicles triadic synaptic arrangements in cat (O'Neal & Westrum, 1973; Rustioni and Sotelo, 1974)
lateral cervical nucleus		spinal afferents small (?) term., spherical vesicles presynaptic in synapses with dendrites and cell bodies asymm & symm synaptic junctions in cat (Westman, 1969)

TABLE I (continued)

SPINAL CORD		
moto-neuronal area	cortical (motor) afferents small terminals, spherical vesicles presynaptic in synapses with dendrites and cell bodies asymmetrical synaptic junctions in monkey (Bodian, 1975)	dorsal root afferents large terminals, spherical vesicles presynaptic in synapses with dendrites asymmetrical synaptic junctions in cat (Conradi, 1969 ; McLaughlin, 1972c)
	propiospinal afferents small terminals, spherical vesicles presynaptic in synapses with dendrites asymmetrical synaptic junctions small terminals, flattened vesicles presynaptic in synapses with cell bodies asymmetrical synaptic junctions in cat (Matsushita & Ikeda, 1973; McLaughlin, 1972c) in monkey (Bodian, 1975)	large terminals, spherical vesicles presynaptic in synapses with dendrites asymmetrical synaptic junctions postsynaptic in synapses with axonal terminals with flat vesicles serial synaptic arrangements in monkey (Bodian, 1975)
inter-mediate area	rubral afferents small terminals, spherical vesicles presynaptic in synapses with dendrites asymmetrical synaptic junctions in rat (Brown, 1974b)	
dorsal horn lamina I	dorsal root afferents small (?) term., spherical vesicles presynaptic in synapses with dendrites asymmetrical synaptic junctions in cat (Ralston, 1968)	
sub-stantia gelati-nosa		dorsal root afferents large terminals, spherical vesicles presynaptic in synapses with dendrites and presynaptic dendrites asymmetrical synaptic junctions postsynaptic in synapses with axonal terminals with flat vesicles within glomeruli serial & triadic synap arrangements in rat (Coimbra et al., 1974)
lamina III	dorsal root afferents small (?) term., spherical vesicles presynaptic in synapses with dendrites and axonal terminals asymmetrical synaptic junctions serial synaptic arrangements in cat (Ralston, 1968)	
laminae IV, V & VI	dorsal root afferents small (?) term., spher (?) term. presynaptic in synapses with dendrites & axonal terminals asymmetrical synaptic junctions in cat (Ralston, 1968)	dorsal root afferents large (?) term., spherical vesicles presynaptic in synapses with dendrites & cell bodies asymmetrical (?) synapt. junctions in cat (Ralston, 1968)

a) One group of difficulties concerns the *problem of recognition*, because the process of degeneration seriously disrupts the normal architecture of axonal terminals which may make it difficult to recognize degenerating structures as terminals. This is especially true for axonal terminals which exhibit the rapid electron-dense type of degeneration. In this case the shape of the terminal changes considerably and the vesicles become so extremely distorted such that the degenerating structures can not be distinguished reliably as a terminal (Westrum, 1973). In addition in the direct electron-dense degeneration the type of synaptic junction is difficult to determine (Jones & Powell, 1970b; Westrum & Black, 1970). Terminals which exhibit the electron-lucent type of degeneration may also be difficult to recognize because in degenerating they may show a very bizarre morphology (Grofova & Rinvik, 1974).

In identifying the degenerating terminals an other difficulty arises from the fact that in some places in the brain the terminals with filamentous hypertrophic degeneration are difficult to distinguish from normal terminals which may also contain filaments. This difficulty especially arises in the spinal motoneuronal cell pools (McLaughlin, 1972b), in the granule cell layer of the cerebellum (Palay & Chan-Palay, 1974), and in the cuneate nucleus (Walberg, 1966) where many normal terminals contain filaments. Moreover filamentous degenerating terminals, which in their normal state also contain filaments are especially difficult to recognize as degenerating ones (McLaughlin, 1972b).

In the above description it has been pointed out that the filamentous type of degeneration usually proceeds into an electron-dense type of degeneration. In some places however, these two different stages of degeneration may appear simultaneously the one next to the other. This has been reported to occur in the lateral cervical nucleus (Westman, 1969). The author suggests that these two different types of degene-

ration might actually reflect the existence of two different types of axon terminals which normally can not be differentiated but become distinguishable on the basis of their morphological changes while degenerating.

In contrast Conradi (1969) reported that after dorsal root sectioning no degenerating terminals were observed in the motoneuronal pools of the spinal cord. However he noticed that after longer survival times a certain type of terminal diminished in number and therefore suggested that these vanished terminals were actually derived from the dorsal root fibers.

Finally, Sotelo & Palay (1971) described the occurrence of spontaneous degeneration which also has been found in several other places (see for instance Rustioni & Sotelo, 1974).

Despite the various difficulties the studies published so far demonstrate that by a careful study of the normal morphology of the terminals and a careful follow-up the sequence of degenerative changes it is generally possible to show that degenerating terminals are either large terminals or small terminals (Grofova & Rinvik, 1974; Wong-Riley, 1972b). However it is in general impossible to distinguish by means of degeneration the terminals which are derived from different afferents but have a similar morphology (McLaughlin, 1972b; Palay & Chan-Palay, 1974; Parlow et al., 1977). For example even by looking at the very early stages of the degeneration Palay & Chan-Palay (1974) have not been able to distinguish the various mossy fiber terminals which are derived from e.g., the spinal cord, the brainstem nuclei and the pontine grey.

b) The second difficulty in degeneration studies derives from the fact that in destroying a group of cells inevitably some *fibers of passage are also destroyed*. These passing fibers might contribute terminals to the neurons in the area under study and these degenerating terminals can not be distinguished from the terminals of the destroyed

cell bodies. In their study of the pallidal afferents to the ventrolateral nucleus and the centre median nucleus of the thalamus, Grofova & Rinvik (1974) and Harding (1973b) inevitably also encountered degenerating terminals of cortical afferents which were interrupted by the lesion of the globus pallidus. According to Grofova & Rinvik (1974), this might have led Harding (1973b) to the erroneus interpretation that the pallidal afferents are of the same type as the cortical afferents in the ventro-lateral and centre median nuclei of the thalamus.

c) The third, major disadvantage of degeneration studies is the fact that at a given moment only a *limited number of degenerating terminals* can be recognized as such. This is exemplified by the fact that Rustioni & Sotelo (1974) found only a limited number of degenerating terminals in the dorsal column nuclei after cortical lesions and that Garey & Powell (1971) observed only a small number of degenerating terminals in the visual cortex after lesions of the lateral geniculate nucleus. This is most probably due to the rapid and asynchronous nature which the degeneration of terminals exhibit in most places, which implies that at any given moment some of the terminals are degenerating while others still look normal or show already advanced stages of degeneration, in which they either have become electron-dense structures or are phagocytosed (Jones & Powell, 1970b; Garey & Powell, 1971; Grofova & Rinvik, 1970; Raisman & Matthews, 1972). After long survival times ultimately most terminals are degenerating, however at that time the vast majority is in the advanced degenerative stages or are phagocytosed and therefore can not be distinguished as terminal any longer (see for instance Kemp & Powell, 1971; Fifkova, 1975).

Many of the above difficulties in identifying the axon terminals of different fiber systems by means of the degeneration techniques are overcome by using the recently developed labeled amino acid axonal transport technique

followed by autoradiography as applied by Hendrickson (Hendrickson, 1969). This technique is based on the axonal transport of radioactive amino acids which are taken up by the cell body and after being incorporated into proteins and particles are transported down to the axonal terminals where the radioactivity may be detected by means of autoradiography. This labeled amino acid axonal transport technique carries the enormous advantages that it leaves the structure of the terminal intact which makes it possible to determine more accurately the precise morphology. Moreover, the radioactive amino acids are only taken up and transported by cell bodies and not by fibers. As a consequence no labeling of terminals of passing fibers occurs. Finally, as will be shown in chapter III and IV the labeled amino acid axonal transport technique has the additional advantage that after sufficient long exposure times this technique labels a much greater number of terminals as compared with the degeneration technique. The next section deals in more detail with some of the aspects of this technique.

THE IDENTIFICATION OF TERMINALS AND SYNAPSES OF DIFFERENT AXON SYSTEMS BY MEANS OF EM AUTORADIOGRAPHY

Neurons have the capacity to take up (exogeneous) substances such as amino acids, but also proteins such as the enzyme horseradish peroxidase (HRP) and albumin. In addition transmitters of amino acid origin are taken up by their transmitter-specific neurons. The non-transmitter amino acids are only taken up by the neuronal cell body, while HRP, albumin and the transmitters may be taken up by any part of a neuron (Fibiger and McGeer, 1974). Most of the substances taken up are transported intra-axonally i.e., the amino acids and the transmitters are transported intra-axonally with the *anterograde* transport to the terminals, while albumin is transported with the *retrograde* transport to the neuronal cell bodies. HRP is transported in both directions (Kristensson et al., 1971, LaVail & LaVail, 1972).

The existence of the *anterograde* intra-axonal transport was demonstrated in 1948 by Weiss and Hiscoe. In their original experiments they ligated a nerve and observed a swelling of the fibers immediately proximal to the ligature. After loosening the ligature they found that the swelling moved along the axon towards the terminals. In subsequent years much of the nature of the anterograde axonal transport has been clarified. With gel electrophoresis, subcellular fractionation and isotope techniques it was shown that this transport involves many substances such as amino acids, proteins, phospholipids, monosaccharides and glycoproteins, but in addition involves mitochondria and possible vesicles and in the transmitter-specific neurons also the specific transmitters (for reference see Grafstein, 1975). When utilizing the transport of labeled substances with the scintillation-counting technique it could be shown that the axonal transport consisted of two main components, i.e., a fast component which proceded with a speed of 230-330 mm/day

Droz et al., 1973; Lasek, 1970; Ochs, 1972). Proteins, glycoproteins, phospholipids, transmitters are transported with the fast transport. They are primarily associated with particulate fractions and are destinated for the terminal membranes which play a role in synaptic transmission (Cuénod et al., 1972; Grafstein, 1975). On the other hand, the slow transport involves mainly soluble amino acids and proteins which are necessary for the maintenance and growth of the axonal trunk (Grafstein, 1967; Schönbach et al., 1973). Furthermore, in tritiated amino acid experiments it could be shown autoradiographically that transported proteins are first manifactured from the amino acids by the rough endoplasmic reticulum, are then delivered to the Golgi apparatus and subsequently are transported along the axon with the fast or slow axoplasmic transport (Droz and Koening, 1970). The neurotubular network of the axon most probably subserves the fast transport of proteins as indicated by electron microscopy autoradiography (Droz and Koening, 1970; Droz et al., 1973). Moreover colchicine which blocks the fast axonal transport has been found to causes also a disassembly of the neurotubules (for references see Ochs, 1975). On the other hand, the slow transport of soluble proteins occurs primarily through the axoplasm (Droz, 1975).

Lasek et al. (1968) were one of the first to show that the antegrade axonal transport of labeled amino acids can be utilized in tracing fiberconnections at *light microscopic* levels. They injected tritiated leucine in the dorsal ganglia of the toad and demonstrated that the ganglion took up the radioactivity and transported it through their axons. Lasek et al. (1968) demonstrated this transport by means of light microscopy autoradiography. Thus the sections of the toad spinal cord and brainstem were coated with a photographic emulsion of silver bromide crystals and stored in the dark for 2-4 weeks. During this period the radiation of the disintegrated isotope affected the silver bromide cry-

stals in the emulsion layer located on top of the section which crystals turned into black grains after photographic development. Thus black silver grains were found above the dorsal root fibers in the dorsal horn, dorsal column nuclei and cerebellum. These dorsal root projection areas as visualized by means of the autoradiography were identical with the areas containing degenerating structures after destruction of the dorsal ganglia (Joseph and Whitlock, 1966).

Before the advent of the labeled amino acid tracing technique, fiber connections had been traced almost exclusively by means of the lesion and degeneration techniques in which cell groups were destroyed and their degenerating fibers were traced by silver impregnation methods (see for instance Nauta, 1957 and Fink and Heimer, 1967). In detailed paper on autoradiography Cowan et al. (1972) drew the attention to several advantages of the autoradiographic method over the degeneration method. Thus with the autoradiography the precise origin of a pathway can be identified; no masking effects occur due to labeled passing fibers because the radioactive amino acids are only incorporated in the cell bodies and not by fibers or terminals. Furthermore it is possible to label preferentially either the terminal field or the fibers by utilizing the fast or the slow axonal transport respectively. However, the authors pointed out that in such autoradiographic studies an accurate estimation of the background is necessary in order to interpretate reliably the results.

Many studies with the labeled amino acid tracing technique have been undertaken since the experiments of Lasek et al. (1968) and Cowan et al. (1972). Tririated leucine was used in many of these studies and tritiated proline in some of them. All neuronal cell bodies in the brain seem to take up and to transport leucine, however not all of them take up the amino acid proline (Künzle and Cuénod, 1973).

Specht and Grafstein (1973) found that labeled pro-

line and the labeled monosaccharide fucose were transported not only antegradely to the corresponding terminals but also transneuronally, to the postsynaptic neuron. They demonstrated this transneuronal transport in the retinal-colliculus-striate system: by injecting tritiated proline or fucose into the mouse's eye and tracing the radioactivity not only in the superior colliculus but also in the striate cortex. The transneuronal transport of fucose and proline has also been reported for the retino-geniculate-striate pathway in the monkey (Wiesel et al., 1974) and in the tecto-parabigeminal pathway (Dräger, 1974).

In 1969 Hendrickson showed that the axonal transport of labeled amino acids can also be used to identify the axon terminals and synapses of fiber system connections at *electron microscopic* levels. She injected radioactive leucine into the vitreous body of the monkey's eye and after processing the lateral geniculate nucleus for electron microscopy the ultrathin sections were exposed for a relative long time, after which she observed short chain-like grains above the lateral geniculate's large terminals which are known to degenerate after optic tract destruction (Colonnier & Guillery, 1964; Glees et al., 1966). These terminals were therefore identified as derived from retinal afferent axons. The type of terminal of retinal afferents could also be identified in the avian optic tectum with this method (Schönbach et al., 1971).

Prior to the publication of our results (Dekker & Kuypers, 1975) a few authors had used the labeled amino acid transport technique in determining the terminals and synapses of fiber systems within the brain. Thus, Hattori et al. (1973) identified the terminals and synapses of nigral afferents in the caudate-putamen of the rat and a little later Rose et al. (1976) identified the terminals of septal afferents in the hippocampus and LeVay & Gilbert (1976) identified the terminals of lateral geniculate nucleus afferents in the visual cortex. Other authors demon-

strated that the intra-axonal transport of labeled specific transmitters can be used to identify the terminals and synapses of the so-called transmitter-specific neurons which act by the release of a particular chemical transmitter. Thus, McGeer et al. (1975) identified the terminals of dopaminergic nigral afferents to the putamen after injection of labeled norepinephrine in the substantia nigra. In a more general way Chan-Palay (1975) identified in the cerebellar cortex the terminals of serotoninergic periventricular gray neurons after intra-ventricular injection of labeled serotonin. Of course transmitter-specific terminals can also be identified after direct application of the labeled transmitter into the terminal field, because these terminals take up the labeled transmitter directly. Thus, Ljungdahl and Hökfelt (1973a, 1973b) could identify the terminals of GABA neurons in the cerebellum after intracerebellar injection of tritiated GABA, and the GABA and glycine terminals of interneurons in the ventral horn of the spinal cord after intraspinal injection of tritiated GABA and tritiated glycine respectively. However, those are different approaches since they are not truly based on anterograde axonal transport of these substances.

THE MORPHOLOGY OF THE RAT'S ANTERO-VENTRAL THALAMIC NUCLEUS IN LIGHT AND ELECTRON MICROSCOPY

INTRODUCTION

The rat's antero-ventral (AV) thalamic nucleus and its afferent projections have been of interest in many light microscopy (LM) studies. These studies show that this nucleus receives numerous fiber projections from the mammillary bodies (Cruce, 1975; Guillery, 1956; Powell et al., 1956; Yamadori, 1973) and rather distinct fiber projections from the cingulate cortex (Domesick, 1969) and hippocampus (Guillery, 1956; Nauta, 1956; Powell et al., 1956; Raisman et al., 1966; Valenstein and Nauta, 1959 and Swanson and Cowan, 1977). Based on the assumption that electron microscopy (EM) studies of fiber projections will be facilitated when these projections are numerous, the rat's AV thalamic nucleus was used as a model in an attempt to identify the terminals and synapses of the different afferent fibers to a cell group by means of both the EM autoradiography (see chapter III) and the EM degeneration technique (see chapter IV).

Because the AV thalamic ultrastructural morphology is as yet unknown it was described in this chapter. In this description special emphasis was given to the AV synapses and synaptic patterns which were compared with those of other thalamic relay nuclei.

MATERIAL AND METHODS

In two adult rats the general structure of the AV thalamic nucleus and its neuronal configuration were studied light microscopically. The animals were deeply anesthetized with pento-barbital (Nembutal^{R}) and perfused with 4% formaldehyde. The brains were kept for five days in this fixative and the forebrains were then cut transversely in 40 micrometers thick celloidin sections were stained with cresyl violet.

In four rats, 4-6 weeks of age, the somatic and dendritic configuration of the AV thalamic neurons were studied with the aid of the Golgi impregnation technique of Rámon-Moliner (1970). The rats were anesthetized and intracardially perfused with saline. The brains were then taken out and impregnated for 20-30 days in a solution of 5% mercurichloride and 5% potassium dichromate. After embedding in celloidin the brains were cut transversely in 150 micrometers sections and treated according to the slow procedure of Rámon-Moliner (1970).

The ultrastructural morphology of the AV thalamic nucleus was studied in 5 adult rats.The animals were anesthetized with pento-barbital (Nembutal^{R}) and subsequently intracardially perfused with a 0.5% gluteraldehyde and 4% paraformaldehyde solution in 0.4 M phosphate buffer (osmolarity of fixative = 1300-1400 mOsm., temp. = 22^{o}C). The animals were artificial respirated with 95% oxygen untill the fixative started to run through the intracardially placed canule. Two liters of fixative were used for each rat. The first liter was run through very rapidly but the second one very slowly. The total perfusion lasted 3-4 hours, after which the heads were kept in the fixative overnight. The next day the brains were taken out of the skull and the rostral thalamus was cut transversely into 4 blocks of 0.5 mm thickness with the aid of a tissue sectioner. The blocks were rinsed in the phosphate buffer

(osmolarity of phosphate buffer = 650-700 mOsm) for 15 minutes and then postfixed for 2 hours in 2% osmium tetroxide and then embedded in Araldite (Durcupan^{R}). Ine micron thick semithin sections taken from the caudal surface of these blocks were stained with toluidine blue or p-phenylenediamine (Höllander and Vaaland, 1968). The middle 2 of the 4 blocks were studied, since in general only these 2 blocks contained the AV thalamic nucleus. On the basis of the findings in the semithin sections the blocks were trimmed to pyramids which contained the antero-ventral (AV) thalamic nucleus as well as the antero-dorsal (AD) thalamic nucleus and the stria medullaris (SM). Ultrathin sections of a pale gold interference colour were cut from the pyramids and mounted on 300 mesh grids (1 grid square equals 2025 square micrometers).

An analysis was made of the distribution of the different terminals in the rostral and in the caudal AV thalamus. For this purpose electron micrographs covering a total area of 560 square micrometers were taken of each of the 9 rostral and 9 caudal EM autoradiographic blocks (see material and methods, chapter III) and subsequently the different terminals were counted. Table II will show the total numbers of terminals counted and the percentage values for the different terminals. In addition the average numbers of terminals per grid square were calculated. Since the area of one grid square equals 2025 square micrometers the average number of terminals in one grid square equals the number found in 560 square micrometers X 2025/560 (see results). In order to determine whether the AV thalamic nucleus was made up of more than one type of neuron, in 2 ultrathin AV thalamic sections all the 53 neuronal cell bodies which were cut through the nucleus were photographed and their morphological characteristics compared. In some cases serial sections through the AV thalamic nucleus were made. For this purpose the blocks were trimmed first to very small pyramids containing only a very small portion

of the nucleus. Nine ribbons of 15 pale gold (± 60 nm thick) sections were cut from the pyramids and 4 ribbons of 40 sections. The sections were mounted on slotted grids and stained with alcoholic uranyl acetate and lead citrate. All the ultrathin material was viewed with a Philips 300 electron microscope.

RESULTS

A, LIGHT MICROSCOPY OF THE ANTERO-VENTRAL THALAMIC NUCLEUS

The antero-ventral (AV) thalamic nucleus in the rat is located in the rostral half of the thalamus. In accordance with earlier descriptions (Gurdjian, 1927) the AV thalamic nucleus was found to have the shape of a mediolaterally flattened pear, with the base located rostrally and the stalk caudally. In the rostral part of the thalamus the AV thalamic nucleus is located immediately under the floor of the lateral ventricle (Fig. 1). On the dorsomedial side the AV thalamic nucleus borders on the antero-dorsal (AD) thalamic nucleus, on the ventromedial side it borders on the antero-medial (AM) thalamic nucleus, and on the ventral and lateral sides it borders on the reticular (R) nucleus (Fig. 1). At more caudal levels the AV thalamic nu-

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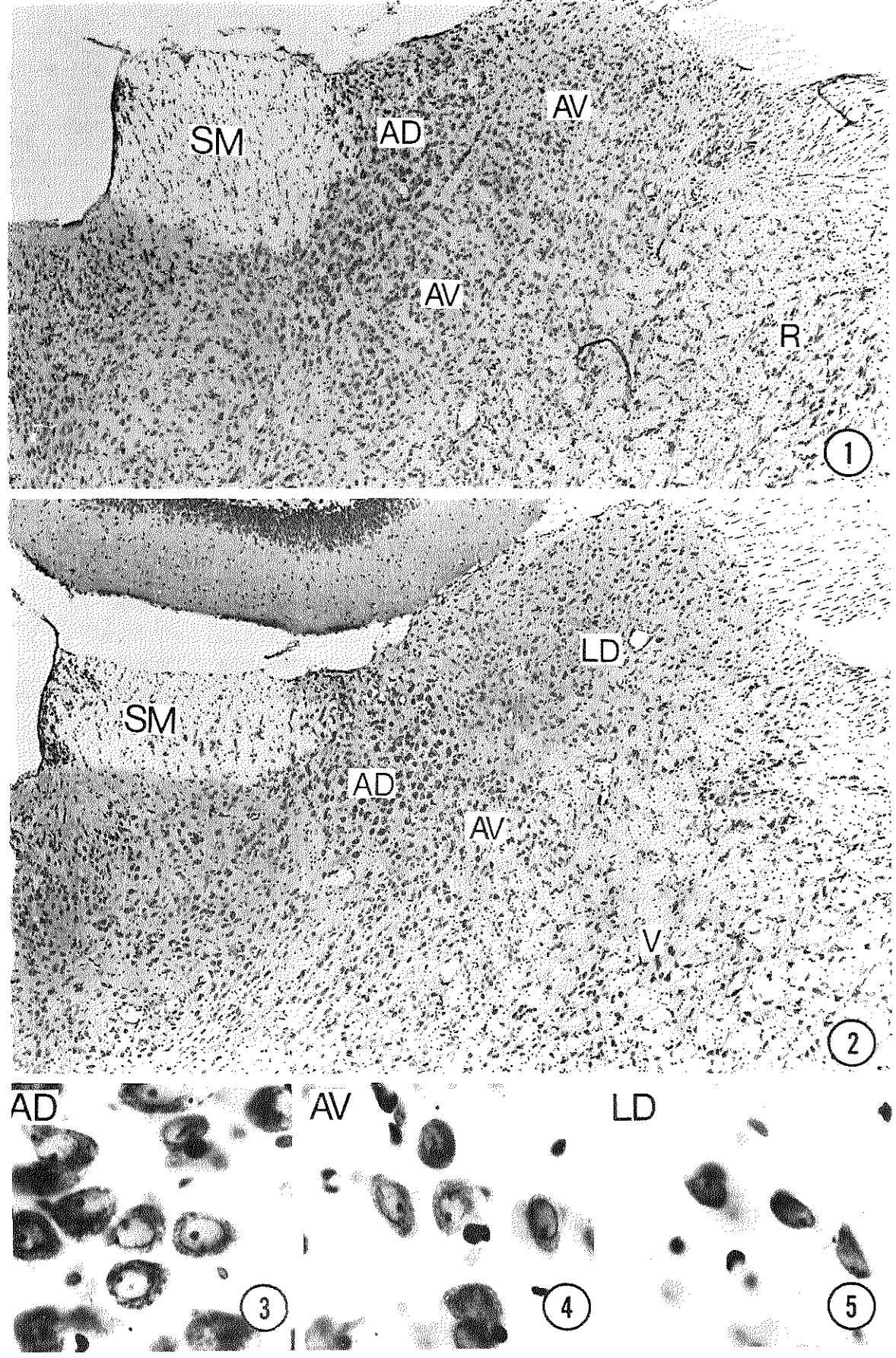
Figs. 1-5. Photomicrographs 1 and 2 are taken from Nissl stained sections through the rostral (Fig. 1) and the caudal (Fig. 2) part of the rat's AV thalamic nucleus. Magnification, x 46. Figs. 3-5 show neurons of the AD, AV and LD thalamic nucleus, respectively. Magnification, x 580. SM, stria medullaris; AD, antero-dorsal thalamic nucleus; AV, antero-ventral thalamic nucleus; LD, lateral-dorsal thalamic nucleus; V, ventral thalamic nuclei; R, reticular nucleus.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cleus still borders dorsomedially on the AD thalamic nucleus and ventromedially on the AM thalamic nucleus (Fig. 2). However, dorsally and laterally the nucleus borders on the lateral-dorsal (LD) thalamic nucleus and ventrally and laterally on the ventral (V) thalamic nuclei.

The AV thalamic nucleus is rather densely packed with round to ovoid neuronal cell bodies which contain little Nissl substance (Fig. 4). On the side of the AD thalamic nucleus a single row of darkly stained large round AD cells (Figs. 1 and 3). Marks the border between the AV and AD thalamic nucleus (see also Cruce, 1975). The border between the rostral part of the AV nucleus and the reticular nucleus is somewhat vague but the reticular nucleus can be distinguished on the basis of the vertical orientation of its small spindle shaped neurons. Rostrally the border between the AV and AM thalamic nuclei is also vague. However, caudally this distinction is facilitated by the presence of the mammillothalamic tract (TMT). The border between the caudal part of the AV and LD thalamic nucleus is rather distinct because the LD thalamic nucleus consists of small neurons which contain more Nissl substance and are less densely packed than the AV thalamic neurons (Fig. 5) (cf. Gurdjian, 1927).

In the Golgi impregnated material of the thalamus many glial cells and only a limited number of neurons could be identified. In this material the AV thalamic nucleus could be easily recognized. The row of large, round AD thalamic cells along the AV thalamic border could also be identified as well as the spindle shaped cells of the reticular nucleus and the relatively small multipolar LD neurons. The cell bodies of the well-impregnated AV thalamic neurons measured 10-15 micrometers in diameter and carried tufted dendrites. In general 3-6 thick stem dendrites extended from the cell bodies. Five to 20 micrometers from the cell body the stem dendrites branched into 2 or 3 thinner second order dendrites which extended for distances of 40-100 mi-

crometers and then in turn ramified into rapidly tapering thin dendrites which were 10-40 micrometers long. In some instances a thin, smooth process resembling an axon arose from the neuronal cell bodies.

The neuronal cell bodies had a smooth surface but the dendrites exhibited short appendages. Some of these appendages resembled the spines found on the dendrites of cortical neurons, but the majority were bulbous protrusions lacking the narrow neck of the classical spines. In our Golgi material all the AV thalamic neurons showed roughly the same configuration, and in this material therefore only one type of AV thalamic neurons could be distinguished.

B, THE ULTRASTRUCTURAL MORPHOLOGY OF THE ANTERO-VENTRAL THALAMIC NUCLEUS

The ultrastructure of the rostral and the caudal parts of the antero-ventral (AV) thalamic nucleus as seen in the ultrathin sections cut from the two thalamic blocks was virtually the same (see below). The nucleus contained neuronal cell bodies, dendrites, fibers and fiber terminals as well as glial cells and their processes. The neuronal cell bodies could be distinguished from the glial cell bodies by the large size of the former, the relative sparsity of chromatin particles in their karyoplasm, the presence of a nucleolus and the abundance of cytoplasmic organelles. In the electron micrographs of the 53 photographed AV thalamic neurons the cell bodies of 51 of them were round to ovoid in shape ($\pm$ 12 micrometers to $\pm$ 7.7 micrometers in diameter) and contained a large nucleus with an extensively folded membrane. These neurons possessed a large amount of cytoplasm which contained ribosomes, multivesicular bodies, microtubules, lysosomes, etc. However, the amount of rough endoplasmic reticulum in

the AV thalamic neurons was much less than in the neighbouring AD thalamic neurons which accounted for the relatively darker staining of the AD thalamic neurons in the Nissl sections and facilitated the distinction between the two nuclei in the electron micrographs. The cytoplasmic membrane of the AV neuronal cell bodies was smooth and carried no spines or other appendages which is in keeping with the findings in our Golgi material. Occasionally a short, thick stem dendrite was seen to extend from the cell body and this dendrite usually contained the same types of cytoplasmic organelle as the cell body. Only two of the 53 AV thalamic neuronal cell bodies were round and smaller than the others. Their nuclear membrane was relatively less folded and they contained relatively less cytoplasm which was less densely packed with organelles than that of the other neurons.

The AV thalamic neuropil in the electron micrographs consisted of numerous profiles of dendrites, fibers and fiber terminals. Large as well as small dendritic profiles were present. The large profiles possessed microtubules, mitochondria, multivesicular bodies, smooth endoplasmic reticulum and characteristically contained ribosomes. They could, however, seldom be traced to

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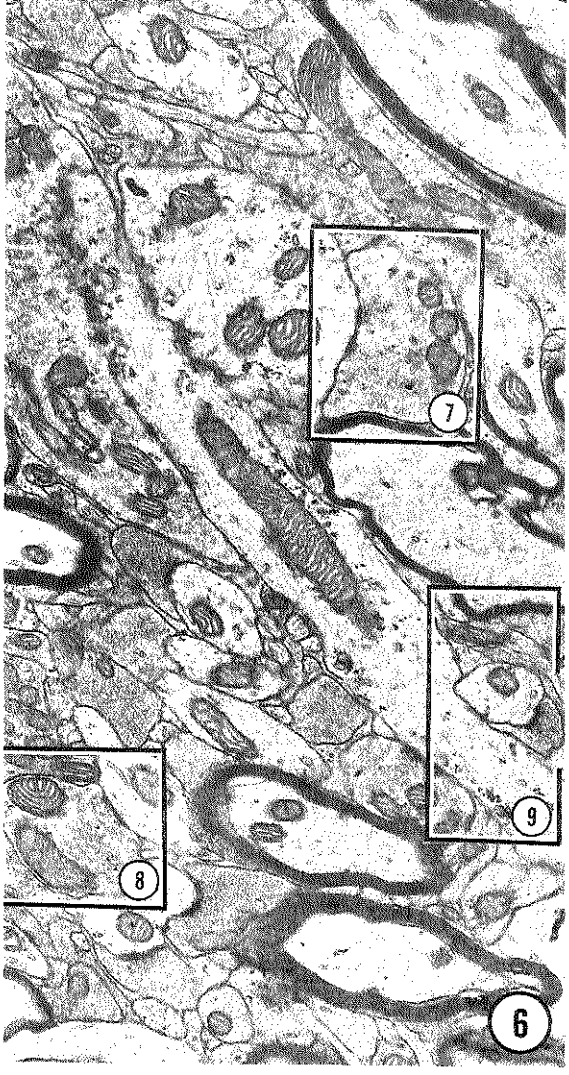
Figs. 6-9. Electron micrographs of axon terminals in the AV thalamic nucleus. Fig. 6 shows the three different types (magnification, x 18,600), which are enlarged in Figs. 7, 8 and 9. Fig. 7: Large terminal (T) loosely packed with spherical vesicles and also containing mitochondria and dense core vesicles (arrow). Arrow heads indicate two asymmetrical synaptic junctions with a large proximal dendrite (D) with ribosomes. Magnification, x 68,400. Fig. 8: Medium-sized terminal (T) containing flattened vesicles. Arrow head indicates a symmetrical synaptic junction with a small dendrite (D). Magnification, x 68,400. Fig. 9: Small terminal (T) densely packed with spherical vesicles. Arrow head indicates an asymmetrical synaptic junction with a small dendrite (D). Magnification, x 68,400.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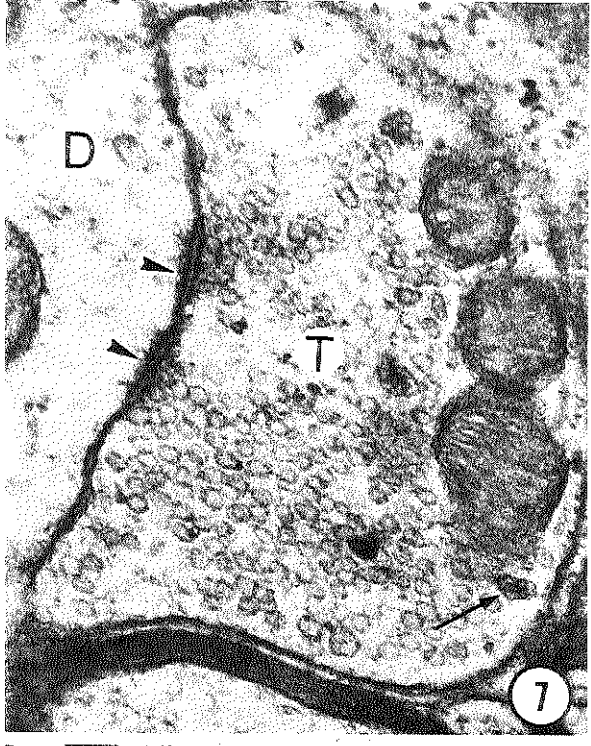
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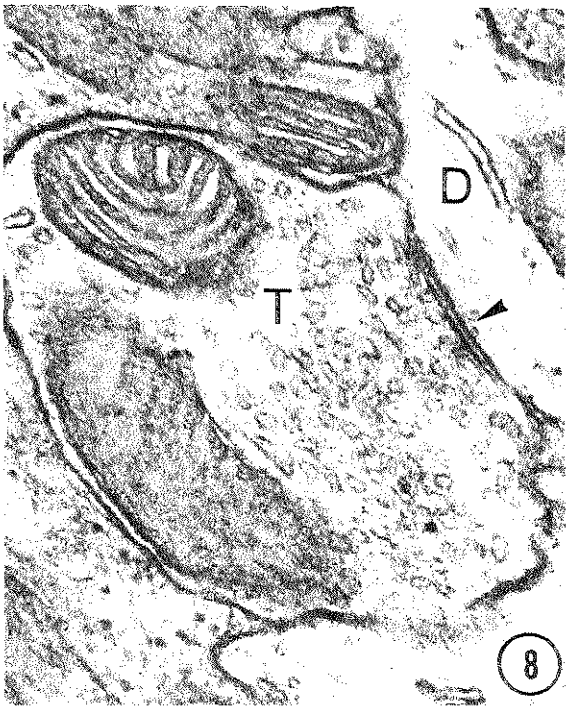
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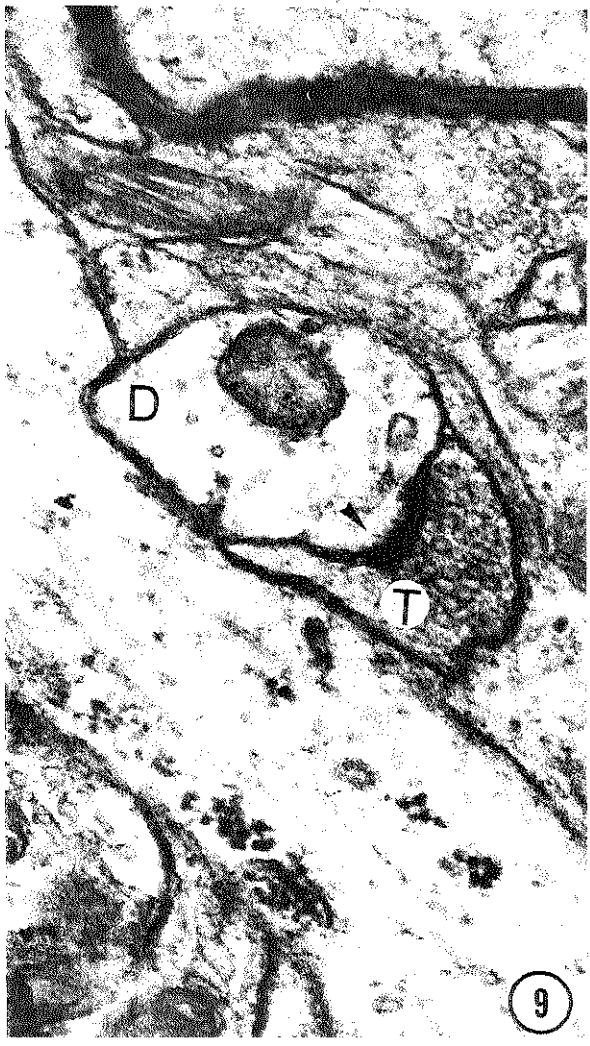
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cell bodies and were therefore regarded to represent mainly second-order dendrites. The small dendritic profiles also contained microtubules and mitochondria but no ribosomes. Several small profiles were also present which resembled small dendrites but lacked microtubules and had a relatively unstructured matrix. In some instances these profiles were continuous with either small (Fig. 12) or large dendritic processes and therefore probably represented the spines and the other appendages seen in the Golgi material. Sometimes dendritic profiles made contact with each other, but these contacts were regarded not to be of a synaptic nature since they were never accompanied by synaptic vesicles.

Fiber terminals in the AV thalamic neuropil were distinguished on the basis of the presence of vesicles and the absence of microtubules and ribosomes. Fiber terminals showing synaptic junctions with other neuronal processes could be subdivided into two types on the basis of the configuration of their synaptic junctions (Colonnier, 1968). One type (A) established symmetrical synaptic junctions, while the other (B) established asymmetrical synaptic junctions. The terminals with symmetrical synaptic junctions (A) were of medium size with a diameter of approximately 1-2 micrometers contained predominantly flattened vesicles and some mitochondria and in a few instances a dense core vesicle. A proportion of these synaptic terminals

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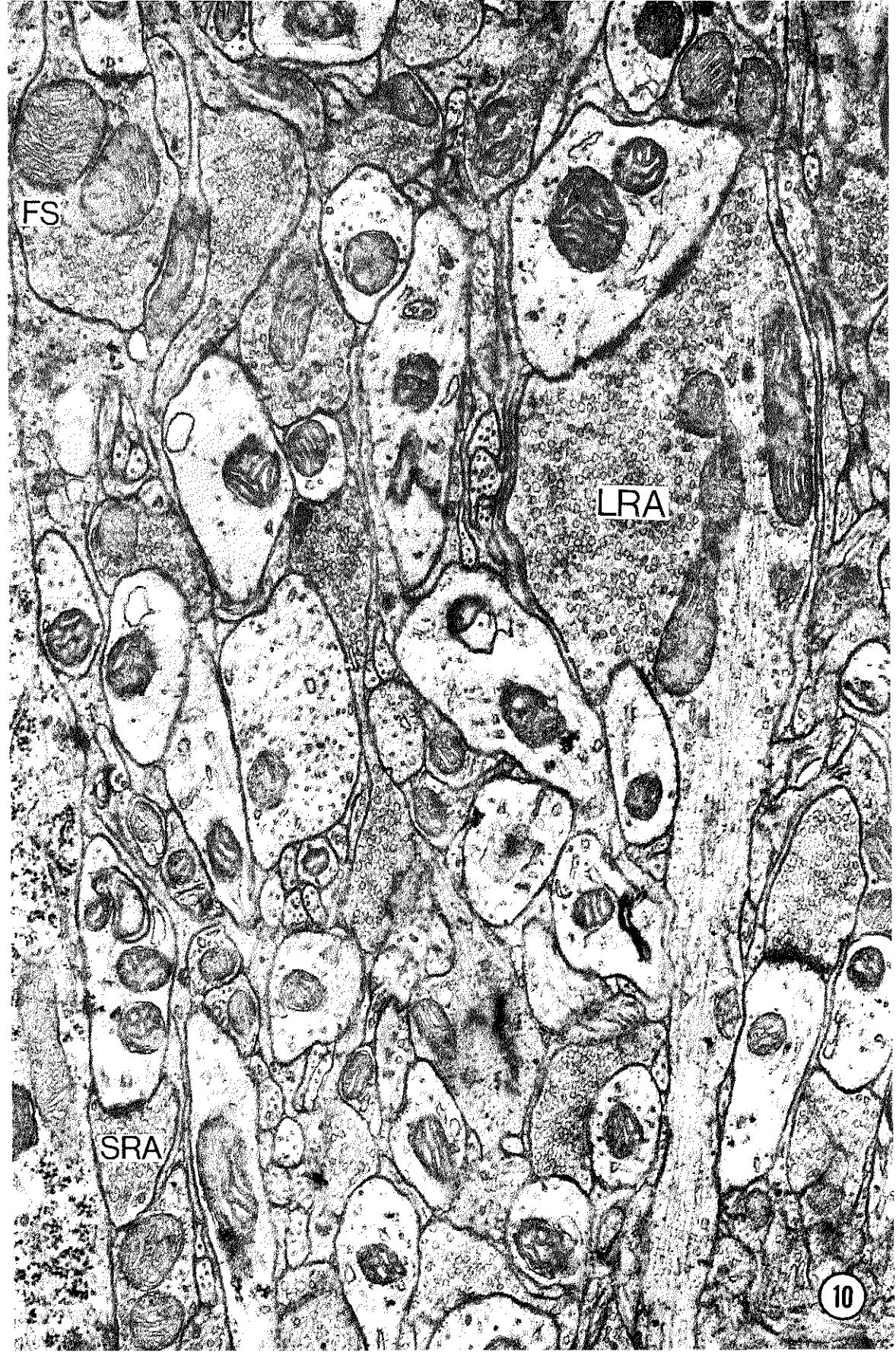
Fig. 10. Electron micrograph through the AV thalamic nucleus. This micrograph shows a large terminal (LRA) in continuation with its unmyelinated axon. This large terminal contains spherical vesicles and establishes asymmetrical synaptic junctions with a proximal dendrite. Also present in this micrograph are several small asymmetrical synaptic terminals (SRA) with spherical vesicles and a symmetrical synaptic terminal (FS) containing flattened vesicles. Magnification, x 35,000.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established synaptic junctions with dendrites (Figs. 6,8, 10) but the majority made synaptic junctions with neuronal cell bodies (Fig. 14). In addition, all the initial axon segments in our material carried this type of terminal exclusively.

The fiber terminals which established asymmetrical synaptic junctions (B) were either small (B1) or rather large (B2). The small terminals (B1) were approximately 0.4 - 0.8 micrometers in diameter and were densely packed with clear spherical vesicles (20 nm) and sometimes contained a mitochrondrion but no dense core vesicles. The majority of these small terminals had synaptic junctions with small dendrites (Figs. 6,9,10) while only a very few had synaptic junctions with large dendrites and none with stem dendrites or cell bodies. Small terminals also made synaptic junctions with spines and other appendages. In those instances in which the origin of these appendages could be determined, they were found to protrude from small dendritic profiles lacking ribosomes (Fig. 12).

The large terminals (B2) were 1.5 - 4.0 micrometers in diameter and contained loosely packed round to pleomorphic vesicles, several mitochondria and some dense core vesicles (90 nm) (Fig. 7). These terminals established single or

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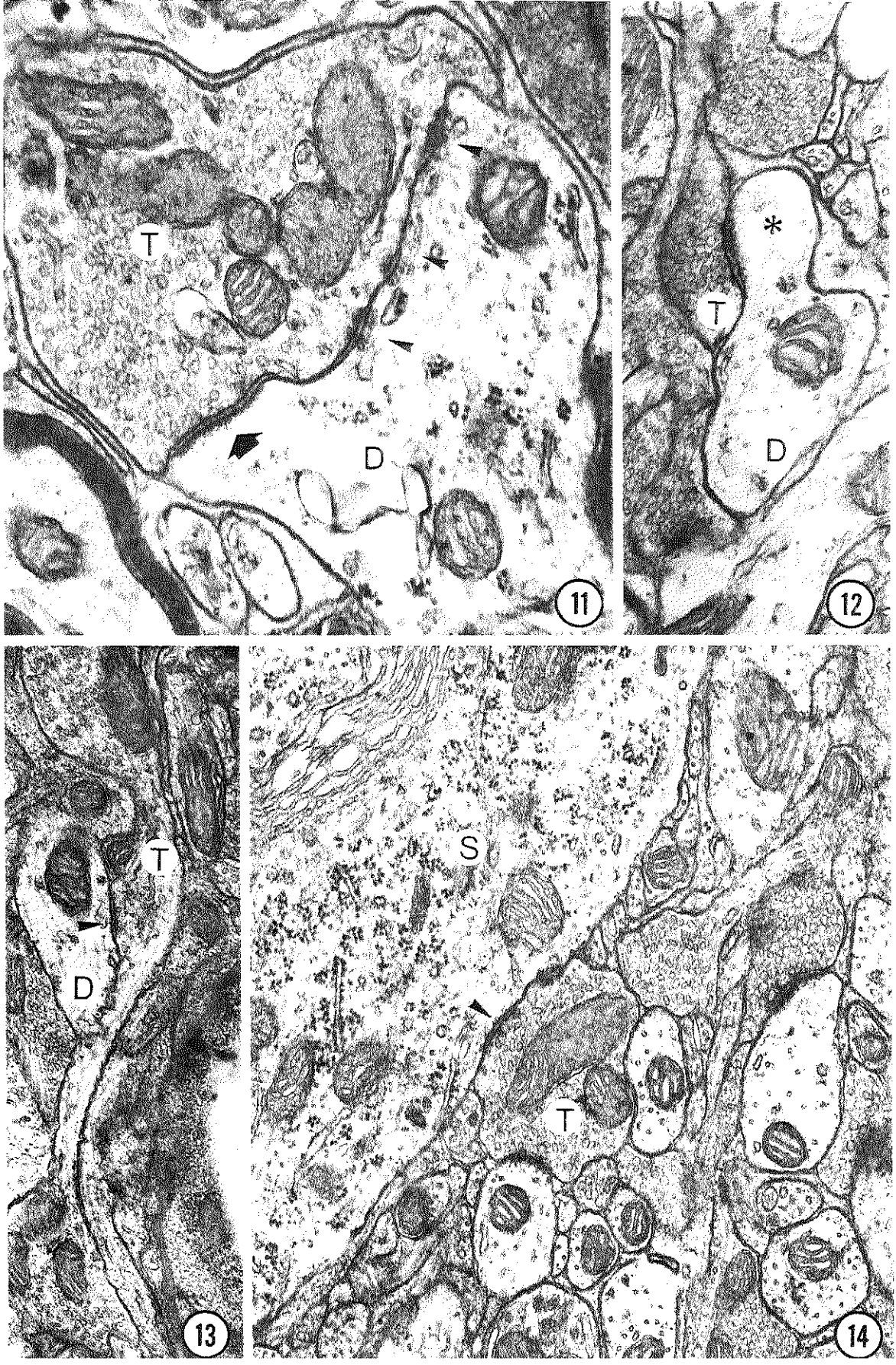
Figs. 11-14. Electron microhraphs of AV thalamic terminals Fig. 11: Large terminal with spherical vesicles establishing an asymmetrical synaptic junction (thick arrow) and three filamentous non-synaptic junctions (thin arrow heads) with a large proximal dendrite (D) containing ribosomes. Magnification, x 59,400. Fig. 12: Small terminal (T) establishing an asymmetrical synaptic junction with a dendritic spine (asterisk); D, dendrite; magnification, x 48,000. Fig. 13: Terminal 'en passage' (T) with flattened vesicles establishing a symmetrical synaptic junction (arrow) with a small dendrite (D). Magnification, x 27,900. Fig. 14: Terminal (T) with flattened vesicles and a symmetrical synaptic junction (arrow) with a nerve cell soma (S). Magnification, x 37,000.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multiple synaptic junctions with stem dendrites, but especially with large, presumably second-order dendrites containing ribosomes (Figs. 6,7,10,11) and with spines and other appendages. However, they did not establish synaptic junctions with small dendrites or cell bodies. In those instances in which the origin of the appendanges carrying large terminals could be ascertained, they protruded from the profiles of large dendrites.

Most of the large terminals established synaptic junctions with only one dendritic profile and only few synaptic junctions with two. The large terminal frequently maintained multiple non-synaptic contacts with large dendrites and stem dendrites but never with dendritic appendages. On both sides of the non-synaptic contacts membrane densities occurred which in general were thicker on the dendritic side than on the terminal side. The dendrites on the dendritic side were frequently accompanied by cisternae of smooth endoplasmic reticulum and by filaments (fig. 11, thin arrow heads).

Table II shows the total number of terminals and the percentage values for the three different types of terminals obtained from 560 square micrometers of each of the 9 rostral and 9 caudal EM autoradiographic blocks (see material and methods chapter III). The total number of terminals in the different blocks showed a great variation: An average (mean $\pm$ SE) of 47 $\pm$ 18.7 was obtained for the rostral blocks and 56 $\pm$ 16.5 for the caudal blocks. The average total number of terminals (mean $\pm$ SD) for all the blocks together was 52 $\pm$ 17.8. Since this value is obtained from 560 square micrometers and the area of one grid square equals 2025 square micrometers, the average total number of terminals per grid square equals 52 X 2025/560 = 188 (see also material and methods). Table II shows that the rostral and caudal blocks were almost equal in respect to the percentage values for the various types of terminals which showed a synaptic junction with other neuronal

TABLE II

Analysis of the percentages of different terminals in the rostral and caudal AV thalamic blocks. For each block a total area of 560 square micra (equals 10 photographs) was analysed. The total area analysed was 5040 square micra for both the rostral and the caudal blocks.

rostral blocks	total number of axon terminals	percentages of small asymmetrical synaptic terminals	percentages of large asymmetrical synaptic terminals	percentages of symmetrical synaptic terminals
A3	22	56	9	0
A4	24	41	12	8
A5	70	52	8	3
A11	42	58	3	2
A12	61	45	10	1
A13	67	53	1	1
A18	51	56	10	1
A19	58	45	5	4
A20	28	42	10	4
total	423			
mean ± SD	47 ± 18.7	50 ± 6.6	8 ± 3.7	3 ± 2.4
caudal blocks				
A6	76	48	9	3
A7	53	51	6	1
A8	47	49	9	2
A11	76	51	4	1
A13	73	54	5	1
A14	48	46	13	3
A18	27	47	8	3
A19	47	50	15	0
A20	59	46	0	4
total	506			
mean + SD	56 ± 16.5	49 ± 2.6	8 ± 4.9	2 ± 1.1

processes. Two percent of these terminals established symmetrical junctions (type A) (average per grid square = 52 X 2025/560 X 0.02 = 4), and 58% established asymmetrical synaptic junctions (types B1 and B2). Of the asymmetrical synaptic junctions 50% were small (type B1) (average number per grid square = 52 X 2025/560 X 0.5 = 94) and 8% were large (type B2) (average number per grid square = 52 X 2025/560 X 0.08 = 15).

Approximately 40% of all terminal profiles did not show a synaptic junction with other neuronal processes. Thirthy five percent (average number per grid square = 52 X 2025/560 X 0.35 = 66) were small, densely packed with spherical

vesicles, 4% (average number per grid square = 52 X 2025/560 X 0.04 = 8) were small, loosely packed with spherical vesicles and 1% (average number per grid square = 52 X 2025/560 X 0.01 = 2) were large, loosely packed with spherical vesicles. Generally the large profiles also contained dense core vesicles and despite the absence of synaptic junctions were therefore classified as parts of large asymmetrical synaptic terminals. However, the small profiles caused some difficulties since they could represent either profiles of small terminals densely packed with vesicles or small profiles of large terminals cut at the level of their high vesicle density. In the absence of synaptic junctions with profiles of small or of large dendrites containing ribosomes these small terminal profiles were difficult to classify. Twenty-five high-density profiles were therefore traced in 9 sets of 15 serial sections and in the 4 sets of 40 sections. In all instances the small densely packed profiles were found to represent parts of small terminals densely packed with vesicles and making asymmetrical synaptic junctions with small dendrites without ribosomes. Six small, low vesicle density profiles were also reconstructed from the serial sections and they were found to represent profiles through peripheral parts of large terminals which established asymmetrical synaptic junctions with large dendrites containing ribosomes. On the basis of these findings, small profiles without synaptic junctions but densely packed with round vesicles and lacking dense-core vesicles were classified as profiles of small terminals with asymmetrical synaptic junctions.

DISCUSSION

Our findings concerning the morphology of the anteroventral (AV) thalamic neuronal cell bodies and their dendrites are in keeping with earlier findings in Golgi studies of the thalamus (Cajal, 1911; Scheibel & Scheibel, 1966a, 1966b). In addition, Cajal's study (1911) which specifically mentioned the AV thalamic nucleus also distinguished only one type of AV thalamic neuron on the basis of the somatic and dendritic morphology.

The present observation in respect to the ultrastructural morphology of the AV thalamic neurons have much in common with the EM findings in other thalamic nuclei of rat, cat and squirrel monkey (Lieberman, 1973; Mathers, 1972a; Morest, 1971; Ralston & Herman, 1969). In many of the thalamic nuclei of these animals large and small neurons have been described. The large neurons were regarded as representing cortical relay neurons and the small ones interneurones. In the present EM material also two types of neurons have been found in the AV thalamic nucleus, which exhibited roughly the same ultrastructural characteristics as the small and large neurons in the other thalamic nuclei, respectively. However, from our findings it appears that in the rat the small AV thalamic neurons are very few in number, 2 out of 53, which may explain why in Golgi material only one type of neuron was found. As a consequence, if interneurons exist in the AV thalamic nucleus, their morphology must be either the same as that of the relay neurons, or more likely they correspond to the small AV neurons, in which case they are very few in number.

In our material, the synaptic terminals in the AV thalamic nucleus were found to be located primarily on dendrites and only a few were found on the cell bodies (Fig. 15). The cell body, axon hillock and stem dendrites carried, almost exclusively, symmetrical synaptic terminals, which were present also in limited numbers dispersed over the

dendritic tree. Large asymmetrical synaptic terminals interspersed with the few symmetrical synaptic terminals populated the surface of the large second-order dendrites, while small asymmetrical synaptic terminals interspersed with a few symmetrical synaptic terminals were crowded onto the distal dendrites. This topical distribution presumably applies to the large AV thalamic neurons since they apparently make up 95% of the AV thalamus population. The observed distribution of synaptic terminals over the receptive surface of the AV thalamic neurons in the rat resembles the distribution reported in the VB thalamic nucleus of this animal (Spacek & Lieberman, 1974a & 1974b). In this nucleus large and small terminals are also present. The large terminals make filamentous as well as synaptic junctions with large dendrites, while the small terminals establish synaptic junctions mainly with distal dendrites in the same way as in the AV thalamic nucleus. However, in the VB thalamic nucleus the synaptic junctions of the large terminals occur only with dendritic and somatic appendages, while in the AV thalamic nucleus they occur mainly with dendritic shafts. Moreover, in the VB nucleus the large terminals possess glial envelopes which were not evident in our AV thalamic material.

The three different types of terminals described in the AV thalamic nucleus are present also in thalamic nuclei of several other species, e.g., the ventro-anterior (VA) and ventro-lateral (VL) nuclei of the cat (Rinvik & Gorfová, 1974a); the ventro-lateral and centre median nuclei of the rhesus monkey (Harding, 1973a); the paracentral nucleus in the rhesus monkey (Partlow et al., 1977); the ventro-basal (VB) complex of the cat (Jones and Powell, 1969a; Ralston and Herman, 1969); the medial geniculate nucleus (MGN) of the cat (Jones and Powell, 1969a; Jones and Rockel, 1971); the lateral geniculate nucleus (LGN) of grey squirrel (Robson and Hall, 1977), cat (Guillery, 1971; Jones and Powell, 1969a), squirrel monkey

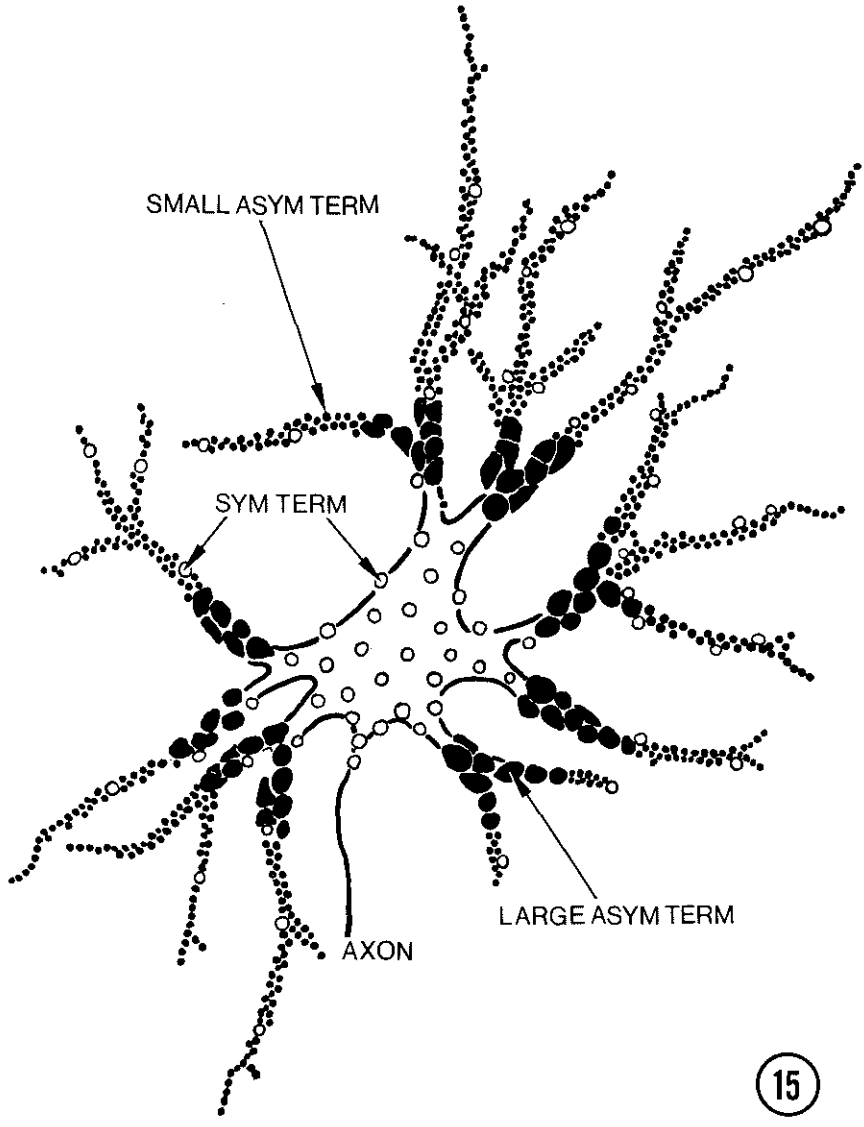

Fig. 15. Schematic representation of the distribution of the various types of terminals over soma, axon hillock and dendrites of an AV thalamic neuron. Symmetrical synaptic terminals (open circles); small asymmetrical synaptic terminals (small dots); large asymmetrical synaptic terminals (large dots).

Wong-Riley, 1972a) and rhesus monkey (Guillery and Colonnier, 1970); the pulvinar of grey squirrel (Robson and Hall, 1977), squirrel monkey (Mathers, 1972a) and rhesus monkey (Campos-Ortega and Hayhow, 1973; Partlow et al., 1977). However, in thalamic nuclei of these animals only the small asymmetrical synaptic terminals as some of the symmetrical synaptic terminals with flattened vesicles display the simple type of synapses observed in the rat's AV nucleus. The remainder of the symmetrical synaptic terminals with flattened vesicles and all the large asymmetrical synaptic terminals are arranged together with dendrites or dendritic appendages into synaptic arrangements which are sometimes enclosed by glial lamellae. In these multiple synaptic arrangements, the large asymmetrical synaptic terminals and the symmetrical synaptic terminals with flattened vesicles establish synaptic junctions with dendrites and dendritic appendages but in addition may establish axo-axonal synaptic junctions with one another. In addition, the dendritic elements within such a complex also may have synaptic junctions with one another. In the rat's thalamus such synaptic arrangements have only been described sofar in the dorsal lateral geniculate nucleus (Lieberman, 1974). In the AV and VB (Spacek & Lieberman, 1974b) thalamic nuclei of this animal such synaptic arrangements are lacking and also no dendro-dendritic synaptic contacts have been found. Since the bulk of such dendro-dendritic contacts apparently occurs between the dendrites of interneurons and those of relay cells (Famiglietti and Peters, 1972; Hamori et al., 1974; Lieberman, 1974; Morest, 1971), the absence of such synapses in the rat's AV and VB thalamic nuclei may be related to their paucity of interneurons (cf. Spacek & Lieberman, 1974b).

Earlier studies have shown that the AV thalamic nucleus in the rat receives a heavy projection from the mammillary bodies as well as from the cingulate cortex and the hippocampus (Cajal, 1911; Cruce, 1975; Domesick, 1969;

Guillery, 1956; Nauta, 1956; Powell et al., 1956; Raisman et al., 1966; Swanson and Cowan, 1977, Valenstein and Nauta, 1959). The findings in other thalamic nuclei (Grofová and Rinvik, 1974; Hendrickson, 1969; Jones and Powell, 1969b; Jones and Rockel, 1971; Ralston, 1969; Rinvik and Grofová, 1974b; Spacek and Lieberman, 1974b) suggest that the large terminals in the AV nucleus belong to the specific afferents, which are probably derived from the mammillary bodies, while the small terminals are presumably derived from the fibers of the cerebral cortex and of other areas. This would be in keeping with the observations of Cajal (1911) who suggested, on the basis of his findings in Golgi material, that the mammillo-thalamic fibers in the AV thalamic nucleus mainly contact the large dendrites. The findings obtained by means of the labeled leucine transport technique followed by EM autoradiography provide further information on these points and will be dealt with in the accompanying chapter.

SUMMARY

In this chapter the structural morphology of the AV thalamic nucleus in LM and EM material has been described. Nissl stained sections showed that the rat's AV thalamic nucleus contains mainly relatively light stained neurons which in Golgi material were found to possess tufted dendrites. In EM material three types of synaptic terminals were found which showed a topical distribution over the neuronal surface. Soma and stem dendrites carry a limited number of terminals with symmetrical synaptic junctions and flattened vesicles. Proximal dendrites carry mainly large asymmetrical synaptic terminals while distal dendrites are crowded with small asymmetrical terminals. No dendro-dendritic synapses and no glomeruli have been found.

THE IDENTIFICATION OF TERMINALS AND SYNAPSES OF DIFFERENT AFFERENTS IN THE AV THALAMIC NUCLEUS BY MEANS OF THE INTRA AXONAL TRANSPORT OF LABELED AMINOACIDS AND EM AUTORADIOGRAPHY

INTRODUCTION

In this study an attempt was made to determine whether the intra-axonal transport of labeled amino acids followed by electron microscopy (EM) autoradiography can be used to identify reliably the terminals and synapses of different fiber systems to a specific cell group in the brain.

The antero-ventral (AV) thalamic nucleus of the rat was used as a model. In the preceding chapter the ultrastructural morphology of its terminals and synapses was described. In this study labeled leucine was injected into the cingulate cortex, the hippocampus and the mammillary bodies respectively, which areas provide afferent fibers to the AV thalamic nucleus (Cruce, 1975; Domesick, 1969; Guillery, 1956; Nauta, 1956; Powell et al., 1956; Raisman et al., 1966; Valenstein & Nauta, 1959; Swanson & Cowan, 1977; Yamadori, 1973). Subsequently the distribution of the radioactivity in the AV thalamic nucleus was studied autoradiographically first with light microscopy and then with electron microscopy after 1 month and 4 month autoradiographic exposure time.

MATERIAL AND METHODS

Nineteen adult rats were studied autoradiographically with light and electron microscopy. In 8 of them (cases A1 to A8) 3-4 injections of 10 microcuries ^{3}H leucine (l-leucine - 4,5 H3, specific activity = 55 Ci/mmol, Radiochemical Centre, Amersham) in 0.5 microliters distilled water were made in the posterior cingulate cortex with the aid of a 22g gauge Hamilton syringe needle. In 6 other rats (cases A9 to A14) a part of the caudo-lateral convexity of one hemisphere was removed and 4 - 5 injections of 8 microcuries ^{3}H leucine in 0.4 microliters distilled water were made in the exposed hippocampus. In the remaining 5 rats (cases A16 to A20) 20 microcuries ^{3}H leucine in 1.0 microliters distilled water were injected unilaterally into the mammillary bodies through the contralateral forebrain. The animals survived the injections for 15 to 20 hours. Two of each 3 groups (A1, A2; A9, A10; A16, A17) were studied light microscopically and the others electron microscopically.

The rats which were to be studied light microscopically were anesthetized with pento-barbital (Nembutal^{R}) and perfused with saline, followed by 4% formaldehyde. After 5 days in 4% formaldehyde the brains were cut transversely in frozen sections of 20 micrometers thickness which were mounted on sides, coated with Ilford G5 emulsion by dipping and stored in the dark at 4^{o}C. After 2 to 4 weeks the material was developed with Kodak D19 fixative and counterstained with cresylviolet.

The animals to be studied electron microscopically were anesthetized with pento-barbital (Nembutal^{R}) and perfused with a 0.5% glutaraldehyde and 4% paraformaldehyde solution in 0.4M phosphate buffer under artificial respiration with 95% oxygen (see for details material and methods preceding chapter). After the heads had been kept in the fixative overnight at 4^{o}C the brains were dissected. The diencephalon was divided into a rostral and a caudal part by a transverse cut

immediately rostral to the mammillary bodies.

The hemispheres as well as the caudal diencephalon which contained the injection sites were fixed for 5 days and then cut transversely in frozen sections of 20 micrometers thickness which were prepared for light microscopy autoradiography (described above). The rostral diencephalon which contains the AV thalamic nucleus was prepared for electron microscopy autoradiography and was cut transversely into 4 blocks of 0.5 millimeters thickness with the aid of a Sorvall^{R} tissue sectioner. After a 15 minute rinse in the phosphate buffer (osmolarity = 650-700 mOsm) the blocks were postfixed for 2 hours in 2% osmium tetroxide and then embedded in Araldite (Durcupan^{R}). From the caudal surface in each block semithin 1 micrometer thick sections were cut which were stained with toluidine blue or p-phenylen-diamine (Höllander & Vaaland, 1968) and studied light microscopically. The AV nucleus was generally found in the sections of only two blocks. Those of one block usually contained the rostral part of the AV thalamic nucleus situated immediately under the lateral ventricle and those of the other block the caudal part situated centrally in the thalamus (see preceding chapter II). However, in several animals the AV thalamic nucleus was found only in one block and its caudal surface sections showed either the rostral or caudal part of the nucleus.

In the 6 animals with cingulate cortex injections (cases A3-A8, see table III), 3 blocks containing the rostral part of the AV thalamic nucleus and 3 blocks containing the caudal part were obtained. In the 4 animals with hippocampus injections (A11-A14) as well as in the 3 animals with mammillary body injections (A18-A20) 3 blocks containing the rostral part of the AV thalamus and 3 blocks containing the caudal part were also obtained. From all the 18 blocks first semithin sections were cut and prepared for light microscopy autoradiography (see above). In these sections the distribution of the silver grains over the AV thalamic nucleus was studied. Subsequently each block was trimmed to a pyramid

containing the AV thalamic nucleus, parts of the anterodorsal (AD) thalamic nucleus and of the stria medullaris (SM). From these pyramids ultrathin sections of a pale gold interference colour were cut, mounted on formvar coated slides and stained with alcoholic uranyl acetate and lead citrate (Reynolds, 1963). A thin carbon layer was evaporated on the slides which were then coated with Ilford L4 emulsion by dipping. This should produce a slightly overlapping monolayer of silver bromide crystals (Vrensen, 1970), with a purple interference colour. Therefore test slides were dipped first and the interference colour of the emulsion layer was checked. In addition the emulsion thickness of some slides was checked directly with the EM. The slides with the sections were stored at $4^{\circ}C$ in light proof plastic boxes which contained a drying agent and in which the air was replaced by carbon dioxide (Rogers, 1973). In each case one series of slides was kept in the dark for 1 month and an other for 4 months. They were then developed with Microdol X and fixed with 30% sodium thiosulfate at exactly $17^{\circ}C$. The formvar films were then floated off in distilled water and mounted on 300 mesh grids (one grid square equals 2025 square micrometers) which were studies with a Philips 300 electron microscope.

ANALYSIS OF EM AUTORADIOGRAPHIC FINDINGS

In 2 or 3 ultrathin autoradiographic sections from each of the 18 blocks the number of silver grains in each grid square after one month exposure was counted. From the averages the quantitative distribution of silver grains over the cut surface of each block was determined (see Fig. 17) The same was done after 4 months exposure. The grains counts over the stria medullaris (SM) in each of the 18 blocks were used as a measurement of the background activity (see Discussion).

The sources of the radioactivity causing the various silver grains were determined from all the grains in the AV grid squares which were randomly selected in 2-3 ultrathin sections from each block (see Table III). In this way an analysis was performed on a total of 2008 grains after cingulate cortex injections, 1364 grains after hippocampal injections and 9522 grains after mammillary body injections. In pale gold ultrathin sections covered with a slightly overlapping monolayer of Ilford L4 emulsion and developed with Microdol X, the source of the tritium isotope radioactivity giving rise to a silver grain can be located with 70% confidence within a 480 nanometers (nm) diameter circle centered on the silver grain (Bachmann et al., 1968; Salpeter & Bachmann, 1965 and Vrensen, 1970). A circle of a diameter corresponding to 480 nm printed on a transparent sheet was therefore centered on each grain in the electron micrographs and the tissue compartment located within the circle was registered and regarded as the source of the radioactivity. If parts of 2 or more tissue compartments were enclosed in the circle, all these compartments were registered and were regarded with equal probability to be the source of the radioactivity. In the analysis therefore it was possible to have items which consist of only one tissue compartment as well as items which consist of a combination of two or more different tissue compartments.

The number of the various radioactive tissue compartments and combinations of tissue compartments found in the random AV grid squares in each of the 18 blocks were expressed as percentages of the total number of such items found in that block. In each of the 3 groups of animals the percentages obtained in the 3 rostral AV thalamic blocks after one month exposure were averaged, and the same was done for 3 caudal blocks. Thus, for each type of injection 2 sets of averages were obtained at one month exposure. The same was done after 4 months exposure. The 4 sets of values for each of the 3 types of injections were represented in the 4

graphs of Figs. 18 and 19 as obliquely shaded, solid black and horizontally shaded bars, respectively.

The grain distribution over the different AV thalamic tissue compartments and combinations of tissue compartments in each block was tested for randomness by means of the Williams' effective area measurement technique (Williams, 1969). For this purpose electron micrographs (56 square micrometers) were taken of the left upper corner of all the random AV thalamic grid squares of each block (the grid squares with one month exposure plus those with 4 months, see table III). Transparent sheets with 78 randomly distributed circles of a diameter corresponding to 480 nm were placed over the electron micrographs and the tissue compartments and combinations of tissue compartments located within the circles were registered (Williams, 1969). The number of these various items in each block were expressed as percentages of the number of such items found in that block. Because the percentage effective area of the various items is independent of the type of injection, the values for the respective items in all the 9 rostral and all the 9 caudal blocks were also averaged. The resulting 2 sets of values for the average percentage effective areas (Williams, 1969) in the rostral and the caudal blocks are represented as open bars in the Figs. 3 and 4 graphs of the rostral and the caudal blocks respectively. The randomness of the distribution of the silver grains was assessed by comparing in each block the percentage effective areas of the various items with the percentages of the silver grains found over these respective items.

The total number of labeled terminals in the random AV grid squares of each block was determined, both after 1 month and 4 months exposure (see table III). From these findings the average number of labeled terminals per AV thalamic grid square was calculated for each group of animals, both after 1 month and 4 months exposure (see table III). The same was done in the caudal blocks. Since the stria

medullaris grain counts indicated a background activity from 10% to 15% after one month exposure and from 5% to 10% after 4 months, the values for the labeled terminals in each section were therefore reduced from 10% to 15%, according to the background activity in that section. This correlation seems to be appropriate in respect to small terminals because they generally carried only one silver grain but it may resulted in underestimating the number of labeled large terminals which generally carried more than one.

In the cases with cingulate cortex and hippocampal injections the ratio between the average number of labeled small terminals (see later) per grid square and the average total number of such terminals per grid square was also calculated for the rostral and caudal AV thalamic blocks, both after 1 month and 4 months exposure time and was expressed in percentages. In the cases with mammillary body injections the same was done for the large terminals.

RESULTS

A, LIGHT MICROSCOPY AUTORADIOGRAPHY

In the autoradiographically treated frozen sections of the animals with posterior cingulate injections, a heavy accumulation of silver grains occurred over its granular cortex and parts of its agranular cortex (Fig. 16A). Densely concentrated rows of silver grains, presumably signaling radiocative fibers could be traced from the injected cortex through the ipsilateral internal capsule into the thalamus where the AV, antero-medial (AM) and the lateral-dorsal (LD) nuclei carried dense accumulations of silver grains. Some grains were present also contralaterally over the internal capsule and the AV and AM nuclei.

In the animals with hippocampal injections dense

accumulations of silver grains were found over the dorsal half of the hippocampal formation (the dorsal parts of the subiculum, presubiculum, parasubiculum, dentate gyrus, C1-C4) (Fig. 16B) and the ipsilateral fornix. In a few animals some grains were also present over the agranular cingulate cortex and in one animal also over the lateral portion of the superior colliculus. In the thalamus silver grains were present over the ipsilateral AV and AM nuclei. The number of silver grains over the AV nucleus in these case was slightly more than half of that found following posterior cingulate cortex injections.

In the animals with mammillary body injections a heavy concentration of silver grains was found over the mammillary complex (fig. 16C) on both sides and dense rows of silver grains could be traced along the mammillothalamic tracts to the AV, AM and AD nuclei on both sides. The silver grains over the AV nuclei were at least 3 times as numerous as after cingulate cortex injections.

B, ELECTRON MICROSCOPY AUTORADIOGRAPHY

In the animals in which the AV thalamic nucleus was studied EM autoradiographically, the distribution of the silver grains in the cortex, hippocampus and mammillary bodies was

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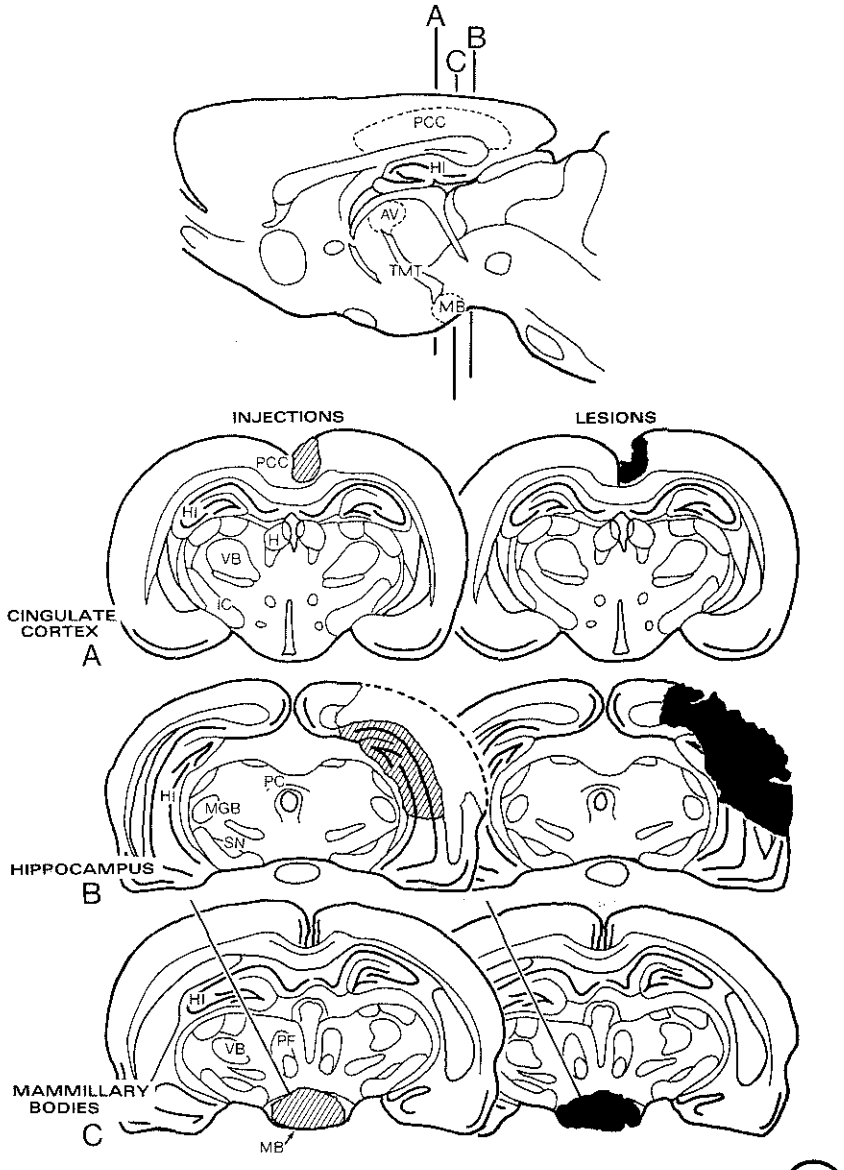
Fig. 16. Semidiagrammatic representation of the location and the extent of the injections (shaded areas) and the lesions (solid black areas) in the cingulate cortex (A), hippocampus (B) and mammillary bodies (C). The lesions are discussed in chapter IV. AV, antero-ventral thalamic nucleus; H, habenular nucleus; HI, hippocampus; IC, internal capsule; MB, mammillary bodies; MGB, medial geniculate body; PC, posterior commissure; PCC, posterior cingulate cortex; PF, parafascicular nucleus; SN, substantia nigra; TMT, mammillothalamic tract.


A
B
C
PCC
HI
AV
TMT
MB
INJECTIONS
LESIONS
PCC
HI
H
VB
IC
CINGULATE CORTEX
A
HI
MGB
PC
SN
HIPPOCAMPUS
B
HI
VB
PF
MAMMILLARY BODIES
C
MB

16

studied light microscopically and was found to be the same as in the animals studied with LM autoradiography exclusively (see preceding paragraph).

In all the animals the semithin as well as the ultrathin autoradiographic sections showed many silver grains over the AV thalamic nucleus and a few over the AD thalamic nucleus and the stria medullaris (SM).

In the animals with the cingulate cortex and those with hippocampal injections, on the average 6-10 grains per grid square were found over the AV thalamic nucleus after 1 month exposure, and 11-20 grains after 4 months (Fig. 17). However, in the animals with mammillary body injections on the average 21-40 grains per grid square were found after 1 month exposure and 41-80 after 4 months (Fig. 17). The average numbers of grains found over the stria medullaris (SM) in the rostral and the caudal AV blocks in each of the 3 groups of animals were used as an indication of the background activity (see Material and Methods and Discussion). After both the cingulate cortex and the hippocampal injections an average of 1.0 grains per SM grid square was found after 1 month exposure and after mammillary body injections an average of 1.9. This represented in all cases from

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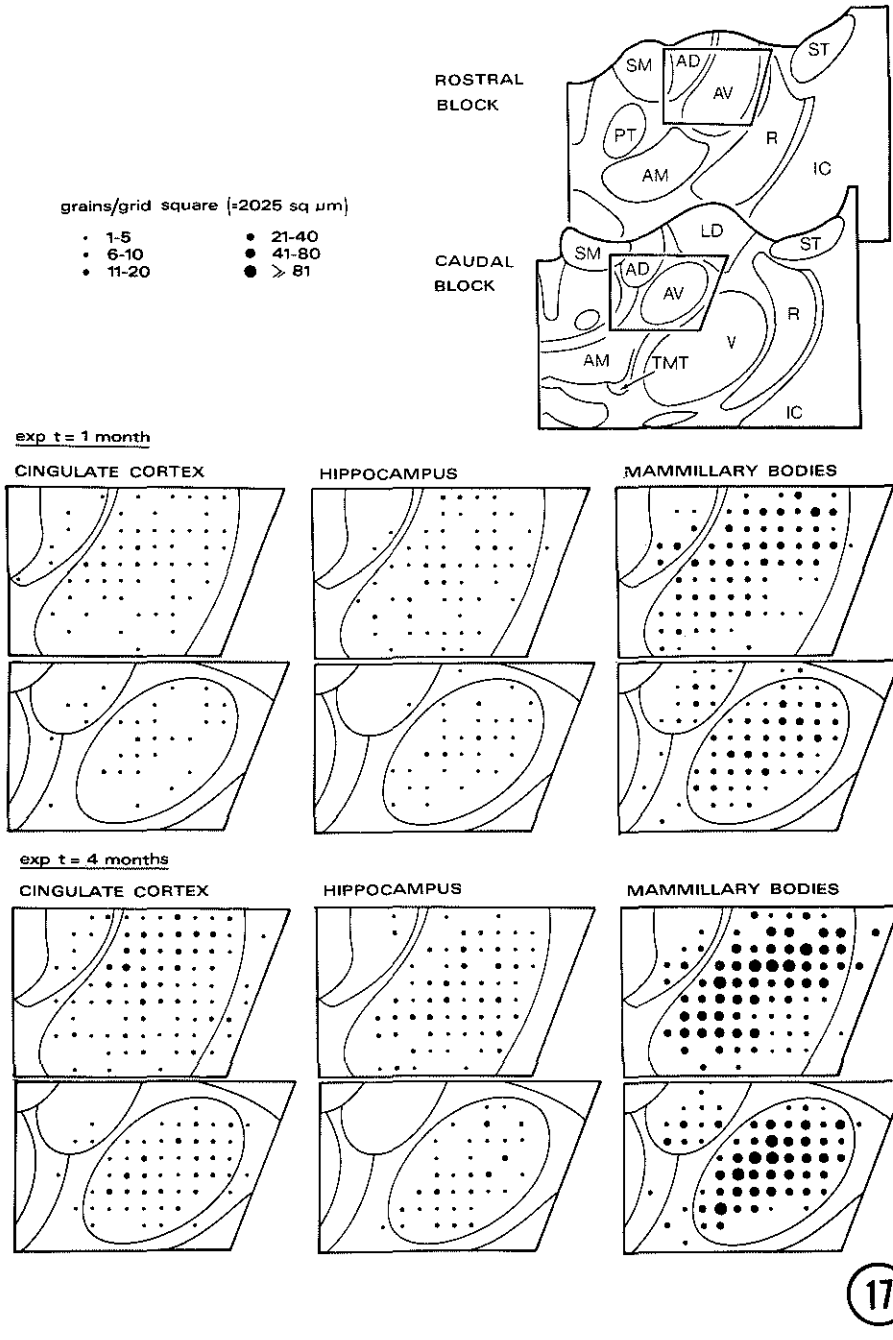
Fig. 17. Diagrammatic representation of the quantitative distribution of silver grains over the autoradiographic ultrathin sections from the rostral and caudal AV thalamic blocks after injections in the cingulate cortex, hippocampus and mammillary bodies respectively, followed by 1 month and 4 months autoradiographic exposure time. Size of dot indicates number of silver grains per grid square. The area of one grid square equals 2025 square micra. AD, antero-dorsal thalamic nucleus; AM, antero-medial thalamic nucleus; AV, antero-ventral thalamic nucleus; IC, internal capsule; LD, lateral-dorsal thalamic nucleus; PT, paratenial nucleus; R, reticular nucleus; SM, stria medullaris; ST, stria terminalis; TMT, mammillothalamic tract, V, ventral thalamic nuclei.


grains/grid square (=2025 sq μm)
1-5
6-10
11-20
21-40
41-80
⩾ 81
ROSTRAL BLOCK
CAUDAL BLOCK
SM
AD
AV
ST
PT
R
IC
AM
LD
V
TMT
exp t = 1 month
CINGULATE CORTEX
HIPPOCAMPUS
MAMMILLARY BODIES
exp t = 4 months
CINGULATE CORTEX
HIPPOCAMPUS
MAMMILLARY BODIES

17

10-16% of the number of grains over the AV thalamic nucleus. After 4 months exposure an average of 1.0 grains per SM grid square was found for the cingulate cortex and hippocampal injections and an average of 2.4 for the mammillary body injections. These values represented in all cases from 5-10% of the number of grains over the AV thalamic nucleus.

In the preceding chapter the AV thalamic nucleus was found to contain nerve fibers, cell bodies, dendrites, glial elements and axon terminals. The dendrites could be subdivided into small and large dendrites of which the latter contained ribosomes. The axon terminals could be subdivided into terminals with symmetrical synaptic junctions and with asymmetrical synaptic junctions (Colonnier, 1968). The symmetrical synaptic terminals contained predominantly flattened vesicles and were mainly located on cell bodies and dispersed over dendrites. The asymmetrical synaptic terminals contained predominantly round vesicles and were either small or very large. The small asymmetrical synaptic

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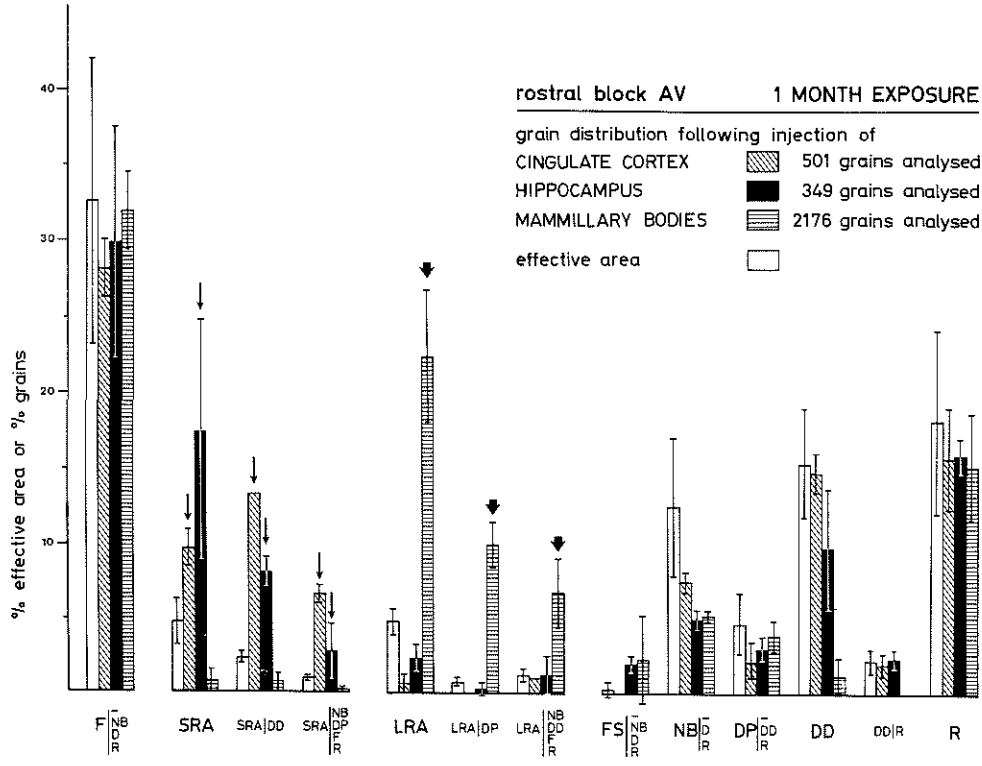
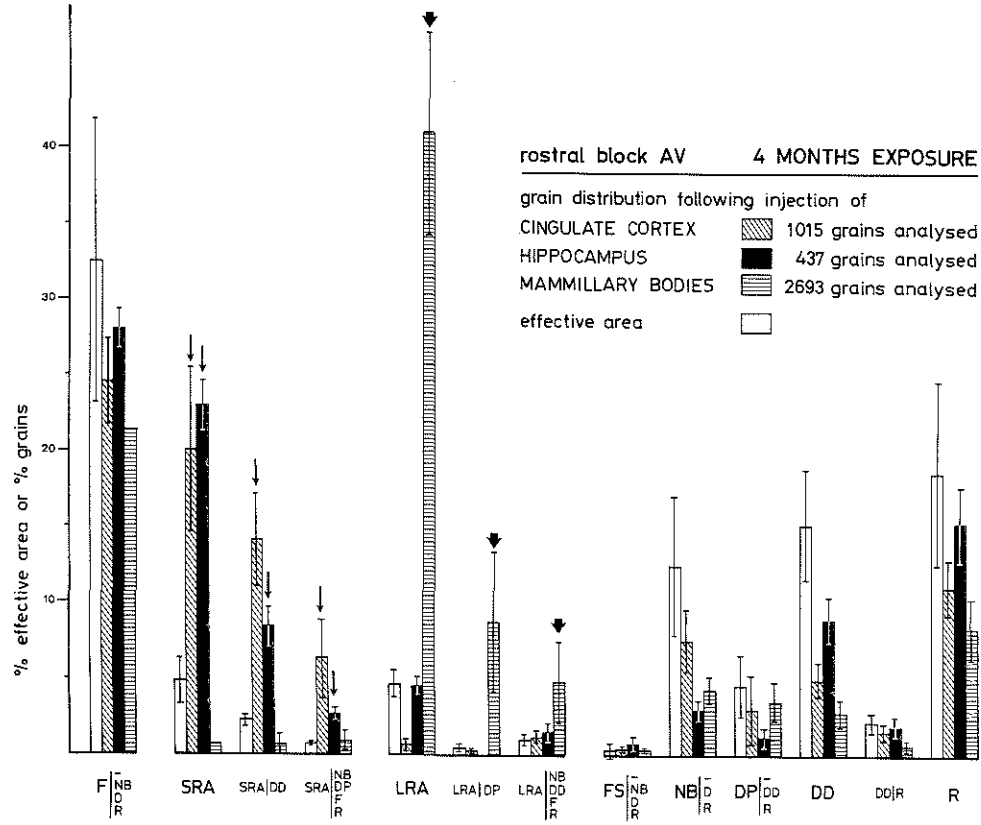
Fig. 18. Upper graph shows the frequency distribution of the mean values (± SD) for the percentages of the silver grains over the different tissue compartments and combinations of tissue compartments obtained from the 3 rostral blocks of the AV thalamic nucleus after 1 month autoradiographic exposure time following injections in cingulate cortex, hippocampus and mammillary bodies. Lower graph shows the same after 4 months exposure time. Open bars indicate percentage effective area (mean ± SD) of the different items calculated from the values obtained in ultrathin sections from each of the 9 rostral AV thalamic blocks examined. Note selective labeling of small (SRA, thin arrows) and large (LRA, thick arrows) terminals respectively, after different injections and the similarities in the frequency distribution after the different exposure times. F, nerve fibers; SRA, small asymmetrical terminals; LRA, large asymmetrical synaptic terminals; FS, symmetrical synaptic terminals with flattened vesicles; NB, neuronal cell bodies; DP, proximal dendrites; DD, distal dendrites; D, proximal and distal dendrites; R, glia, blood vessels and unidentified structures.

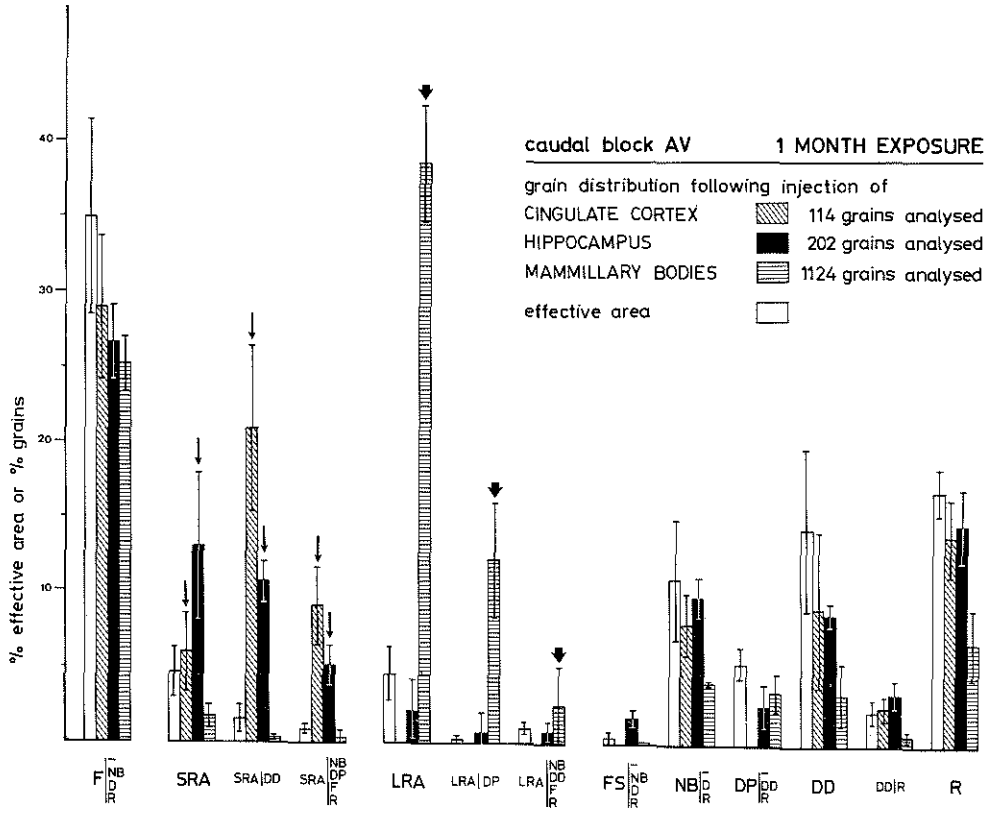

rostral block AV
1 MONTH EXPOSURE
grain distribution following injection of
CINGULATE CORTEX
501 grains analysed
HIPPOCAMPUS
349 grains analysed
MAMMILLARY BODIES
2176 grains analysed
effective area
% effective area or % grains
40
30
20
10
F
NB
D
R
SRA
SRA|DD
SRA
NB
DP
F
R
LRA
LRA|DP
LRA
NB
DD
F
R
FS
NB
D
R
NB
D
R
DP
DD
R
DD
DD|R
R

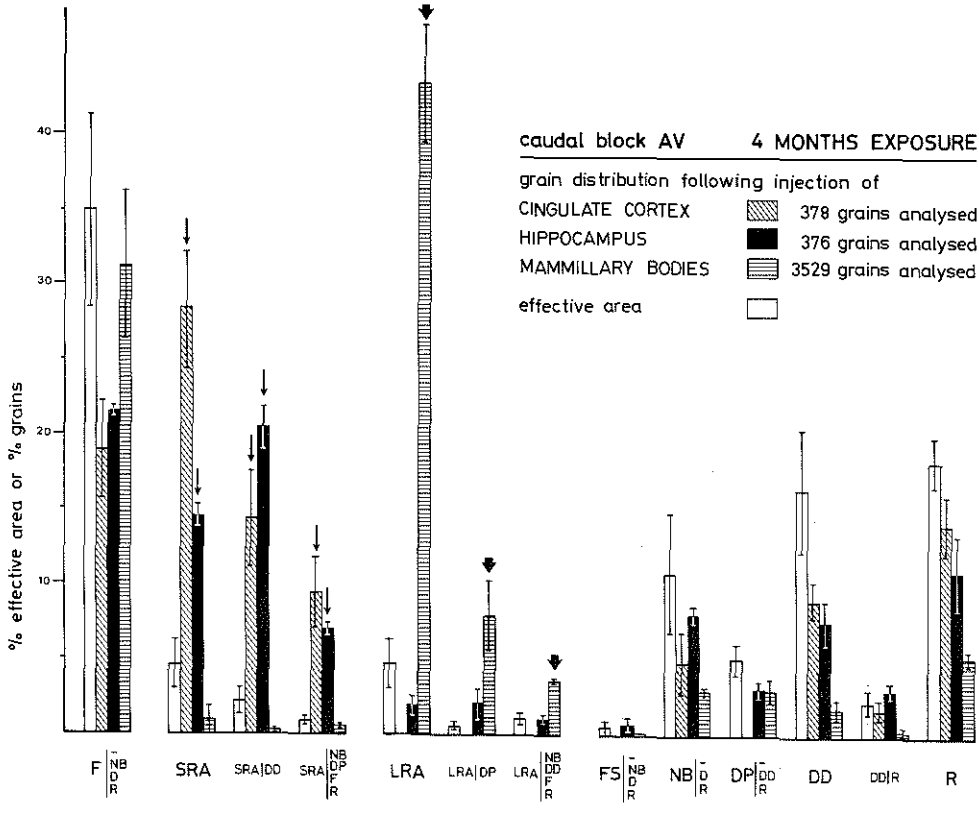

rostral block AV
4 MONTHS EXPOSURE
grain distribution following injection of
CINGULATE CORTEX
1015 grains analysed
HIPPOCAMPUS
437 grains analysed
MAMMILLARY BODIES
2693 grains analysed
effective area
% effective area or % grains
40
30
20
10
F
NB
D
R
SRA
SRA|DD
SRA
NB
DP
F
R
LRA
LRA|DP
LRA
NB
DD
F
R
FS
NB
D
R
NB
D
R
DP
DD
R
DD
DD|R
R

terminals were densely packed with round vesicles, sometimes also contained mitochondria and made synaptic junctions mainly with distal dendrites lacking ribosomes and with their appendages. The large asymmetrical synaptic terminals contained round to pleomorphic vesicles, several mitochondria and some dense core vesicles and established synaptic junctions, mainly with proximal dendrites containing ribosomes, and with their appendages. In the AV electron micrographs terminal profiles without synaptic junctions were also present which terminals are either packed with round vesicles or loosely packed with round to pleomorphic vesicles and contain mitochondria. These profiles were found to represent peripheral parts of small and large asymmetrical synaptic terminals, respectively. In determining the sources of the radioactivity (Material and Methods), most frequently tissue compartments of fibers (F) and synaptic terminals were found to contain the radioactivity with 70% confidence and, less frequently, cell bodies and dendrites. Frequently combinations of tissue compartments were also found to contain radioactivity, especially the synaptic terminal-dendrite combination.

Analysis of the sources of the radioactivity in the AV

Fig. 19. Upper graph shows the frequency distribution of the mean values (± SD) for the percentages of silver grains over the different tissue compartments and combinations of tissue compartments obtained from the 3 caudal blocks of the AV thalamic nucleus after 1 month autoradiographic exposure time following injections in cingulate cortex, hippocampus and mammillary bodies. Lower graph shows the same after 4 months exposure time. Open bars indicate percentage effective area (mean ± SD) of the different items calculated from the values obtained in ultrathin sections from each of the 9 caudal AV thalamic blocks examined. Note selective labeling of small (SRA, thin arrows) and large (LRA, thick arrows) terminals respectively, after different injections and the similarities in the frequency distribution after the different exposure times. See Fig. 18 for abbreviations.


caudal block AV
1 MONTH EXPOSURE
grain distribution following injection of
CINGULATE CORTEX
114 grains analysed
HIPPOCAMPUS
202 grains analysed
MAMMILLARY BODIES
1124 grains analysed
effective area
% effective area or % grains
40
30
20
10
F
SRA
SRA|DD
SRA
LRA
LRA|DP
LRA
FS
NB
DP
DD
DD|R
R


caudal block AV
4 MONTHS EXPOSURE
grain distribution following injection of
CINGULATE CORTEX
378 grains analysed
HIPPOCAMPUS
376 grains analysed
MAMMILLARY BODIES
3529 grains analysed
effective area
% effective area or % grains
40
30
20
10
F
SRA
SRA|DD
SRA
LRA
LRA|DP
LRA
FS
NB
DP
DD
DD|R
R

thalamic nucleus after cingulate cortex and hippocampal injections showed that fibers, synaptic terminals and their various combinations with other tissues compartments contained after 1 month exposure 50% of the radioactivity and after 4 months exposure about 70% (see Figs. 18 and 19). The radioactivity in synaptic terminals in these cases was most frequently located in small asymmetrical synaptic terminals (SRA) (Figs. 25 and 26) and in their combination with distal dendrites (SRA/DD) or with other compartments, i.e., neuronal cell bodies (SRA/NB), proximal dendrites (SRA/DP), fibers (SRA/F), and glia, blood vessels and unidentified structures (SRA/R). In contrast, the large asymmetrical synaptic terminals seldom carried radioactivity. Each of the small terminals generally carried only one silver grain, both after 1 month and after 4 months exposure time. The frequency distributions of silver grains over the different items after 1 and 4 months exposure time were substantially the same and are represented as obliquely shaded and solid black bars respectively in the four graphs of Figs. 18 and 19.

In the cases with mammillary body injections, fibers, synaptic terminals and their various combinations with other tissue compartments were found to contain after 1 month exposure 60% of the radioactivity and after 4 months 70% (see Figs. 18 and 19). The radioactivity in terminals was found most frequently in large asymmetrical synaptic terminals

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Fig. 20. Autoradiographic electron micrograph through the AV thalamic nucleus after injection of tritiated leucine into the mammillary bodies. Autoradiographic silver grains are present above a large terminal which contains spherical vesicles and which establishes an asymmetrical synaptic junction with a proximal dendrite (D). Magnification, x 61,600

D

(LRA) (Figs. 20-24) and in their combinations with other tissue compartments such as proximal dendrites (LRA/DP) and others (LRA/DP, LRA/NB, LRA/DD, LRA/F), while small asymmetrical synaptic terminals seldom carried radioactivity. The labeled large terminals generally carried two silver grains after 1 month exposure (Fig. 21) and 2-6 after 4 months exposure (Fig. 22). The frequency distributions of the silver grains over the different items after mammillary body injections with 1 month and with 4 months exposure time were also substantially the same and are represented as horizontally shaded bars in the 4 graphs of Figs. 18 and 19.

In the cases with cingulate cortex and hippocampal injections, respectively, the percentage of the silver grains carried by the small asymmetrical synaptic terminals (SRA) together with their combinations with distal dendrites (SRA/DD), on which they mainly terminate, and with other tissue compartments (SRA/NB, SRA/DP, SRA/F, SRA/R) was in each block always higher than their percentage effective area in that block both after 1 month and after 4 months exposure time. On the other hand, in these cases the percentages of the silver grains carried by the large asymmetrical synaptic terminals was in each block always lower than their percentage effective area in that block also both after 1 month and after 4 months exposure time. (In Figs. 18 and 19 average values are given). The opposite was found after mammillary body injections in which cases

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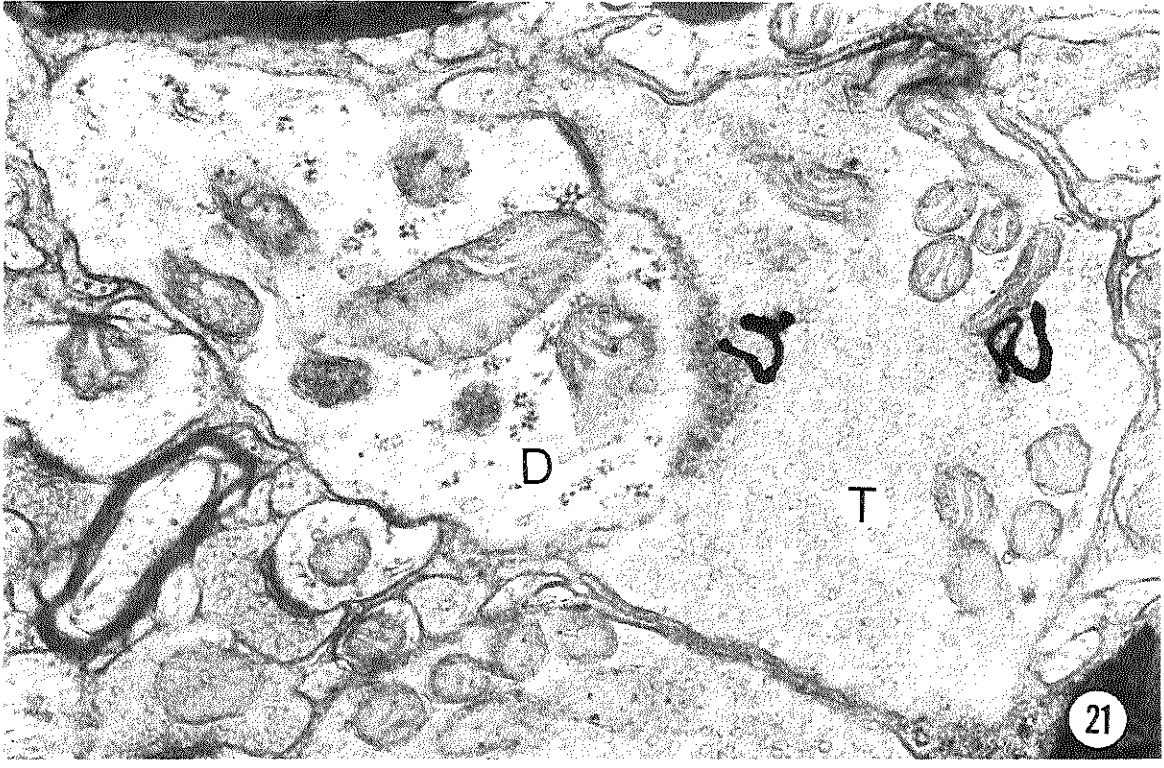
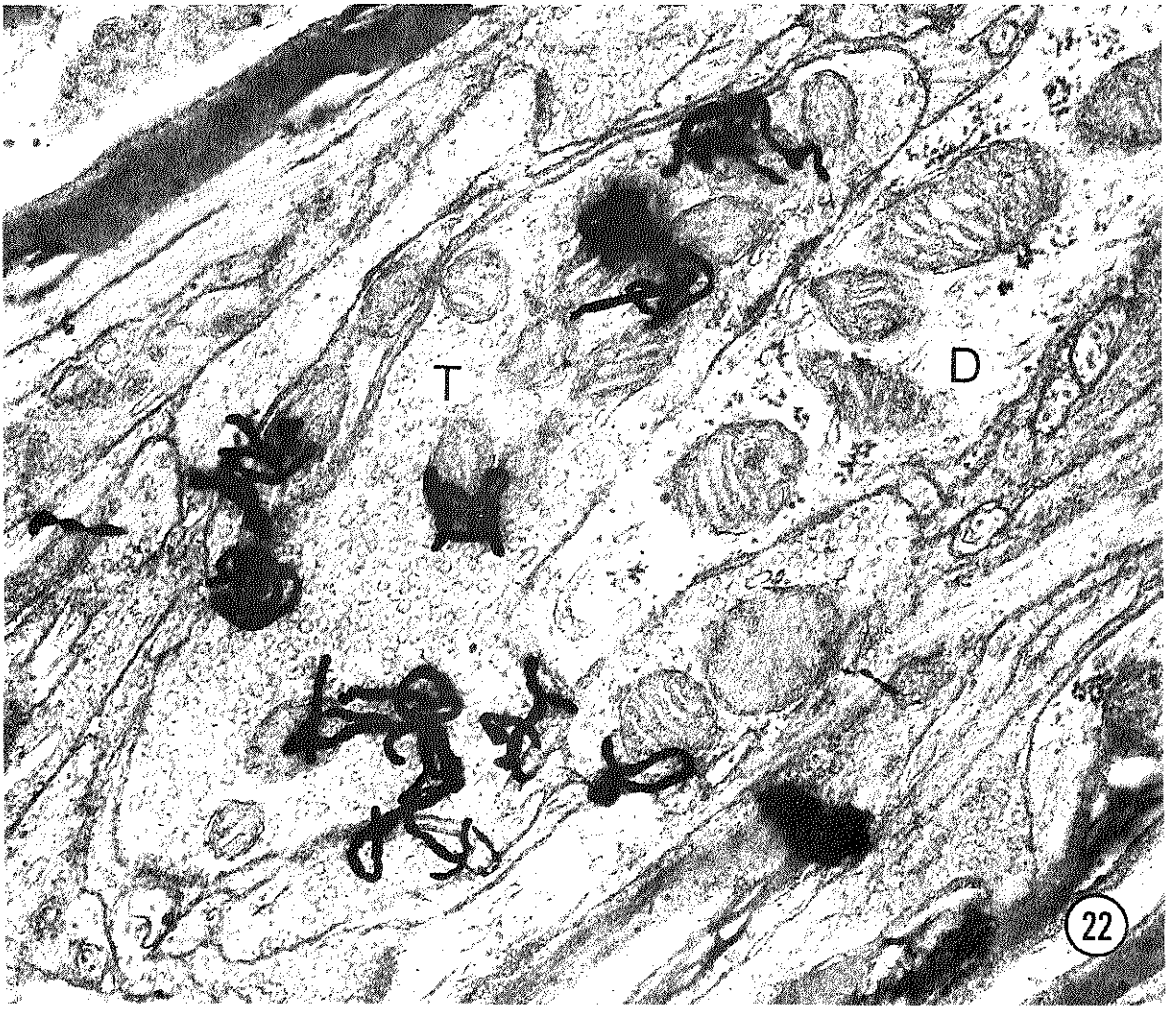
Fig. 21-22. Autoradiographic electron micrographs of two large asymmetrical synaptic AV thalamic terminals (T), labeled after mammillary body injections and establishing synaptic junctions with proximal dendrites (D). Fig. 21: Terminal with 2 silver grains after 1 month exposure. Fig. 22: Terminal with more than 10 silver grains after 4 months exposure. Magnification, x 23,400.


D
T
21


T
D
22

the percentages of the silver grains carried by the large terminals was in each block always higher than their percentage effective area in that block both after 1 month and after 4 months exposure time. On the other hand, in these cases the percentages carried by the small terminals and their combinations with other tissue compartments (SRA, SRA/DD, SRA/NB, SRA/DP, SRA/F and SPA/R) was in each block always lower than their percentage effective area in that block. (In Figs. 18 and 19 average values are given). In addition, in the AV thalamic blocks of the cases with cingulate cortex and hippocampal injections the tissue compartments which were radioactive in combination with the small asymmetrical synaptic terminals contained very little radioactivity by themselves since their percentages of silver grains in each block after 4 month exposure time were in all cases either smaller or the same as their percentage effective areas in that block. In the original description of Williams (1969) the distribution of silver grains carried by the various items was compared with their effective areas by means of the chi square test. However, in our experiments the chi square test was omitted because the values for the percentage of silver grains carried by the various items were in each block always different from their percentage effective areas. The 3 sets of data together indicate that after cingulate cortex and hippocampal injections the radioactivity transported to the AV thalamic ter-

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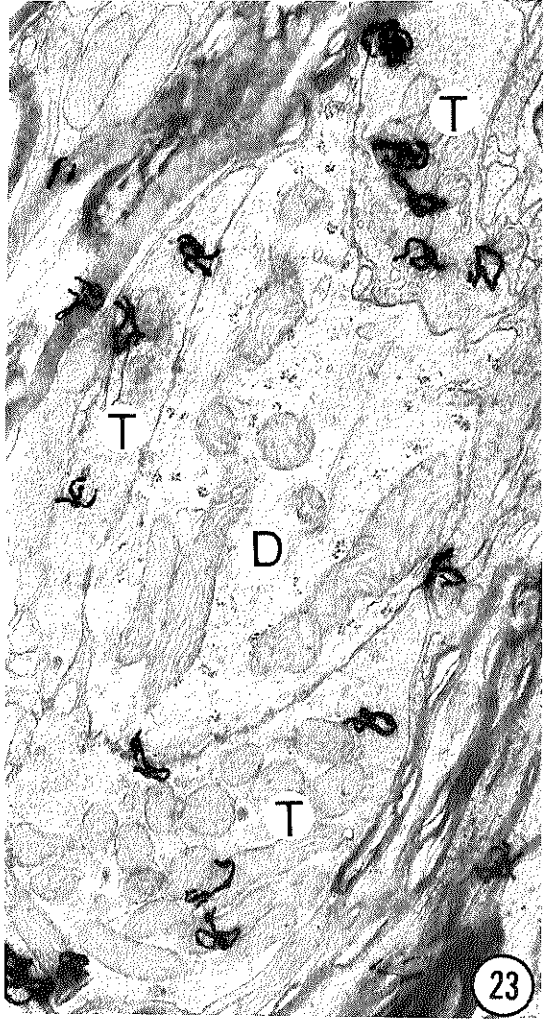
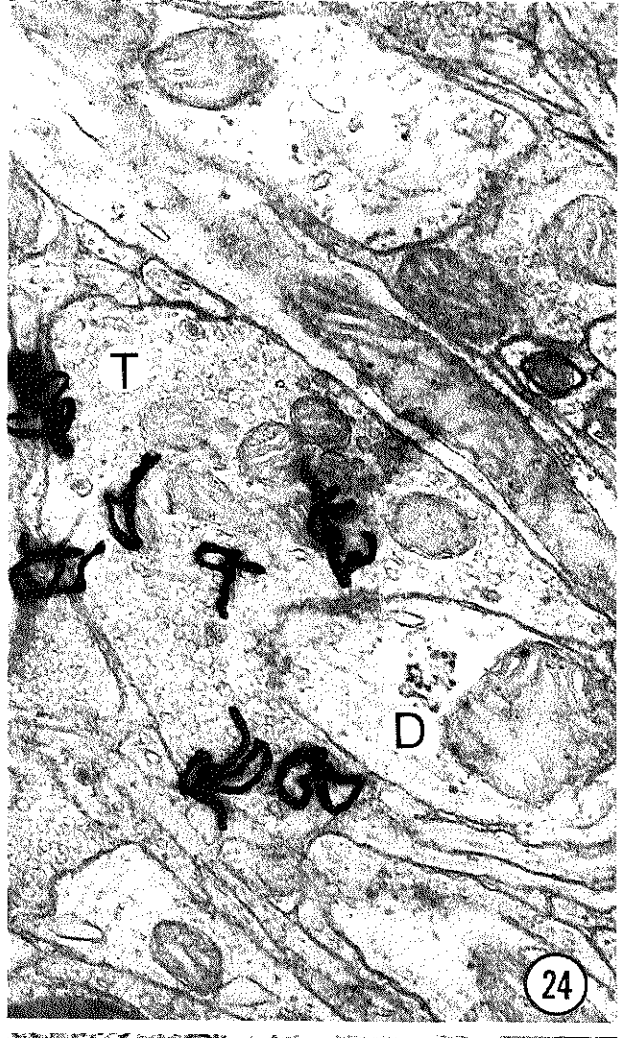
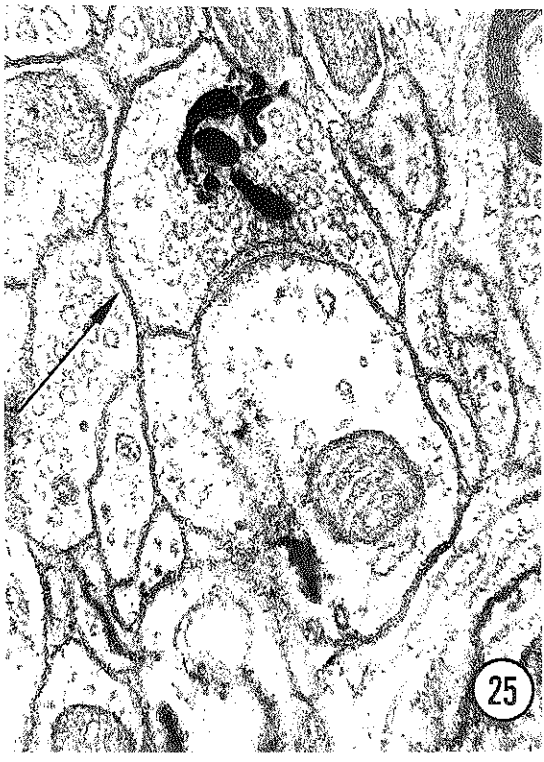
Figs. 23-26. Autoradiographic electron micrographs of labeled terminals in the AV thalamic nucleus. Figs. 23 and 24: Labeled large asymmetrical synaptic terminals (T) after 4 months exposure, following mammillary body injection. Magnification, x 18,900 (Fig. 23) and x 37,800 (Fig. 24) Fig. 25: Labeled small asymmetrical synaptic terminal (arrow) after hippocampal injection. Magnification, x 67,200. Fig. 26: Labeled small asymmetrical synaptic terminal (arrow) after cingulate cortex injection. Magnification, x 23,400.



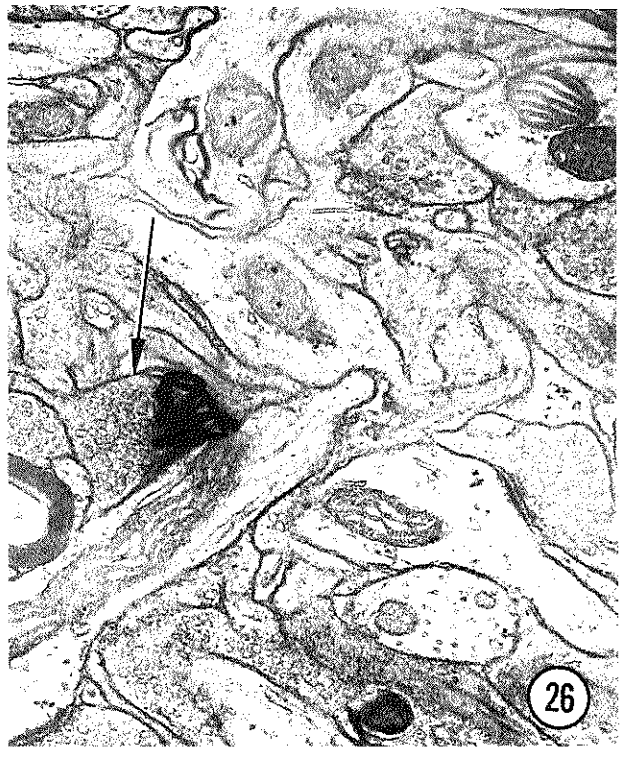
23



24



25



26

TABLE III

Representation of the number of grid squares analysed, total number of grains and labeled terminals and mean values (± SD) for the labeled terminals per grid square for the 3 different types of injections. The mean values were obtained from the average number of labeled small or large terminals per grid square in the 3 rostral and 3 caudal AV thalamic blocks. The numbers are given both after 1 month and 4 months exposure. The area of one grid square equals 2025 square micra.

injection	total number of AV grid squares analysed		total number of grains		total number of labeled small AV terminals		mean value (± SD) for the labeled small AV terminals per grid square	
	1 mth	4 mths	1 mth	4 mths	1 mth	4 mths	1 month	4 months
CINGULATE CORTEX								
rost blocks								
A3	55	46	172	547	40	175		
A4	53	34	164	361	42	143	0.8 ± 0.1	4.0 ± 0.2
A5	50	23	165	108	45	97		
caud blocks								
A6	23	18	45	119	16	64		
A7	23	23	50	132	14	84	0.7 ± 0.2	3.5 ± 0.1
A8	6	21	19	127	6	71		
HIPPOCAMPUS								
rost blocks								
A11	31	25	122	151	31	93		
A12	10	24	147	144	44	91	2.0 ± 0.9	3.7 ± 0.1
A13	32	24	80	142	19	86		
caud blocks								
A11	28	20	69	120	42	69		
A13	18	21	89	124	23	76	1.1 ± 0.4	3.4 ± 0.2
A14	20	23	44	131	14	73		
					total number of labeled large AV terminals		mean value (± SD) for the labeled large AV terminals per grid square	
					1 mth	4 mths	1 month	4 months
MAMMILLARY BODY								
rost blocks								
A18	35	16	732	905	305	182		
A19	32	11	719	895	227	207	7.1 ± 1.9	15.1 ± 3.7
A20	31	14	724	892	159	213		
caud blocks								
A18	15	25	383	1153	168	430		
A19	18	22	385	1174	145	409	8.9 ± 2.0	20.7 ± 4.9
A20	19	16	396	1164	142	421		

minals was selectively transported to small asymmetrical synaptic terminals, while after mammillary body injections it was selectively transported to large asymmetrical synaptic terminals.

Table III shows the average number of labeled terminals per grid square for each type of injection both after 1 month and 4 month exposure. The average number of labeled small asymmetrical synaptic terminals per grid square (see Material & Methods) after cingulate cortex and hippocampal injections with 1 month exposure was found to be 0.7-2.0 which represents lessthan 2% of the population of such terminals. After 4 months exposure an average of 3.4-4.0 labeled small terminals was found per grid square which represents less than 3% of the population (see preceding chapter). After mammillary body injections with 1 month exposure an average of 7.1-8.9 labeled large asymmetrical terminals was found per grid square which represents 30% of the population of such terminals (see preceding chapter). After 4 months exposure an average of 15.1-20.7 was obtained which represents 80% of the population (see preceding chapter). Longer exposure times have been tried but were abandoned since the high concentration of silver grains began to obscure the structure of the terminals.

DISCUSSION

The reliability of the EM autoradiographic findings depends on the degree to which the silver grains over terminals and other structures signal radioactivity transported axonally from the injection sites. Generally some of the silver grains in autoradiographs are brought about by other factors. The bulk of this background probably represents a pressure artefact which occurs during drying of the emulsion after it has been applied to the specimen (Rogers,

1973). This is in keeping with the relative decrease in the background activity found after longer exposure time. Other factors contributing to the background are e.g., chemography, contamination of the emulsion, environmental radiation, and too high temperature of the developer (Rogers, 1973).

In studying purely unilateral fiber connections to a cell group, the actual background activity can be determined by counting the number of silver grains over the same cell group on the other side (Schönbach et al., 1971) However, this was not applicable in the present study because fibers are known to be distributed from some of the injected areas to the AV thalamic nucleus on both sides (Domesick, 1969). The background activity was therefore measured by counting the silver grains over the stria medullaris thalami, which could be examined in the same ultrathin sections as the AV thalamic nucleus and does not contain fibers derived from the injected areas (Guillery, 1956; Nauta, 1956; Powell et al., 1956; Raisman et al., 1966; Valenstein and Nauta, 1959).

The stria medullaris measurements indicated a background activity from 10-15% after one month exposure and from 5-10% after 4 months, which values are intermediate between those quoted by others (Hendrickson, 1972; Schönbach et al., 1971). The values for the grains and the labeled terminals per grid square have been reduced with the percentage background activity found in the section from which they were obtained. The possible distortion in the frequency distribution of the silver grains over the various items caused by the presumably randomly distributed background was minimized by applying the Williams' effective area measurements technique (Williams, 1969). Thus it could be demonstrated that the distribution of the silver grains over the various terminals did not occur randomly and therefore did not reflect background activity.

The reliability of the data obtained by EM autoradio-

graphy is also determined by the resolution of the method, which is a function of the sizes of the undeveloped and the developed silver grains, and the source-detector distance, which in turn depends on the thickness of the section, of the emulsion layer and of the intervening carbon layer, as well as of the type of isotope (Bachmann et al., 1968; Salpeter and Bachmann, 1965; Vrensen, 1970). In the type of autoradiographic material used in the present study the area containing the source of the radioactivity causing a particular silver grain can be delineated with 70% confidence by a 480 nm diameter circle centered on the silver grain (Bachmann et al., 1968; Salpeter & Bachmann, 1965; Vrensen, 1970). When dealing with structures of considerably smaller diameter the radioactivity causing a silver grain over that structure may therefore be located either in that tissue compartment or in parts of adjoining tissue compartments, enclosed within the 480 nm diameter circle. This limitation in the resolution is negligible in respect to the large terminals (diameter: 1500 to 4000 nm) since the 480 nm diameter circle centered on a silver grain over such a terminal seldom contains portions of neighbouring tissue compartments. However, when dealing with small terminals (500 nm diameter) the 480 nm diameter circles enclosed in several instances small terminals together with other tissue compartments, especially small dendrites. In these instances the other compartments of the combination also had to be regarded with equal probability as the source of the radioactivity. Yet, contrary to the small terminals the other compartments of the combinations were found after 4 months exposure to contain little radioactivity. The small terminals were therefore regarded to represent the radioactivity component of the combinations.

The three different types of synaptic terminals in the rat's AV thalamic nucleus possess a topical distribution over the receptive surface of the AV thalamic neurons. The relatively limited number of medium sized symmetrical synaptic terminals is located at the axon hillock, on the cell

body and dispersed along the dendrites. The large asymmetrical synaptic terminals are located interspersed with the few symmetrical synaptic terminals on the large dendrites originating from the stem dendrites and the many small asymmetrical synaptic terminals, interspersed with the few symmetrical synaptic terminals, are located on the distal dendrites. The present findings indicate that both in the caudal and the rostral parts of the AV thalamic nucleus the mammillary body fibers contribute to the large terminals on the proximal dendrites, while the cortical and hippocampal fibers contribute to the small terminals on the distal dendrites (Fig. 27). These conclusions strikingly fit the original findings of Cajal (1911) and are in keeping with other findings in the ventro-basal complex (Jones & Powell, 1969b; (Ralston, 1969; Spacek & Lieberman, 1974a & b) medial (Jones & Rockel, 1971) and lateral geniculate bodies (Hendrickson, 1969) and ventro-anterior and ventrolateral (Grofova and Rinvik, 1974; Harding, 1973b; Rinvik & Grofova, 1974b) nucleus of the thalamus in rats, cats and monkeys. The medial lemniscus, inferior colliculus and optic tract afferents as well as the afferents from the cerebellar nuclei possess large asymmetrical synaptic terminals while the cortical fibers to these cell groups possess small ones.

According to the present findings the transport of radioactive leucine from the mammillary bodies resulted after 4 months autoradiographic exposure time in the labeling of 80% of the large terminals. This makes it likely that virtually all these terminals are derived from mammillary body fibers. However, after the same exposure time only 6% of the small terminals were labeled from the cingulate cortex and the hippocampus together (the actual numbers of labeled terminals obtained after the 3 different injections and as shown in Table III will be compared and discussed with the number of degenerating terminals after the 3 different lesions in the discussion of the proceding chapter). Probably not all cortical and certainly not all hippocampal neurons

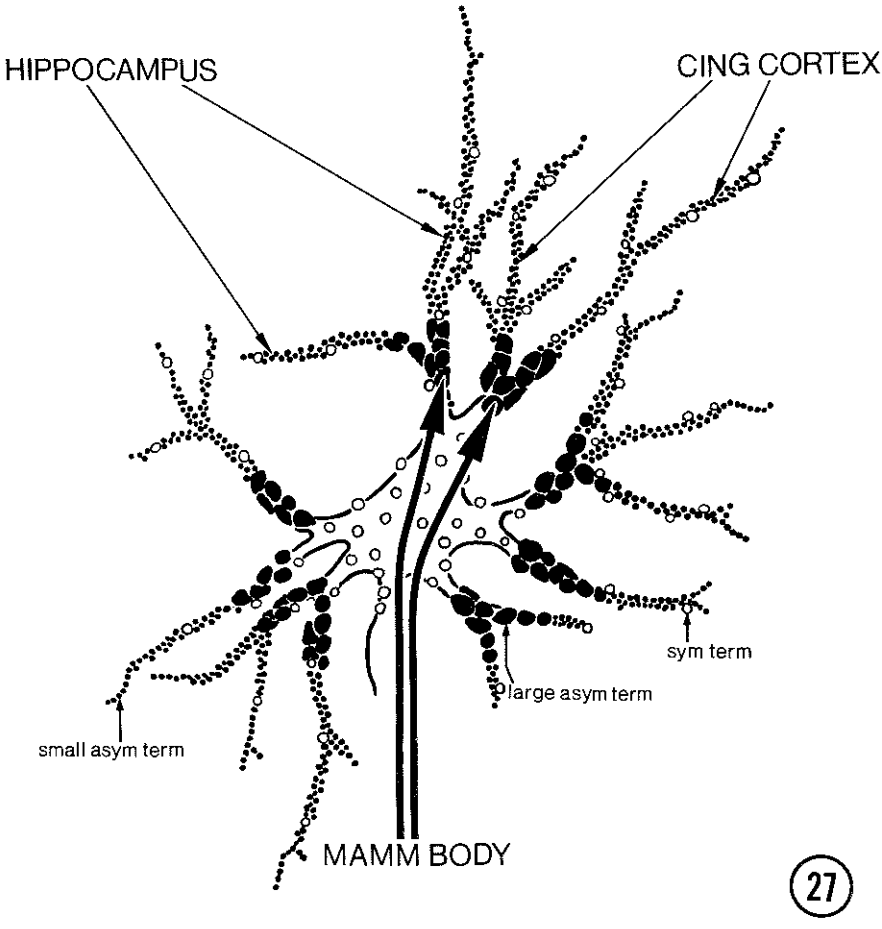


Fig. 27. Schematic representation of the afferent fibers contributing to the population of small and large asymmetrical synaptic terminals on an AV thalamic neuron.

had received labeled leucine and transported it to all their terminals. In the latter cases for instance only the dorsal portion of the hippocampal formation was injected while it is now known from just recent studies of Swanson & Cowan (1977) that also the ventral pre- and para subiculum project to the AV thalamic nucleus. Nonetheless, the relatively small percentage of terminals labeled from these sources suggest that small terminals are also provided by other AV thalamic afferent fibers, e.g., from the contralateral cingulate (Domesick, 1969) and other cortical areas, from the contralateral subiculum of the hippocampal formation (Swanson & Cowan, 1977), from the brain stem cell groups as the locus coeruleus (Pickel et al., 1974) and possible from other nuclei.

One of the major drawbacks of the EM labeled leucine technique is that in order to label a high percentage of the terminals of a certain system by means of the labeled EM autoradiography, the long exposure time of 4 months appears to be necessary. Yet, it has to be kept in mind that this long exposure time is only required for estimating the *number* of terminals provided by a certain system, while for determining their *type* the short exposure time suffices. This is concluded from the fact that the frequency distribution of the silver grains over the various items after one month exposure time are substantially the same as after 4 months exposure time (cf Figs 18 and 19). Moreover, if the number of items labeled from the different sources would be roughly the same, it would be sufficient to analyse a much smaller number of silver grains than in the present study. However, studying the AV thalamic afferents the labeled amino acid transport from the mammillary bodies was much more pronounced than from the other sources. As a consequence in order to analyse e.g. 50 grains per block after cingulate cortex and hippocampal injections 25-30 grid squares had to be studied, which after mammillary body injections carried 300 grains.

SUMMARY

In this study an attempt was made to determine whether the intra axonal transport of labeled amino acids followed by EM autoradiography can be used in determining the terminals and synapses of different fiber systems to a cell group in the brain. The antero-ventral (AV) thalamic nucleus was used as a model and injections of tritiated leucine were made in the mammillary bodies, cingulate cortex and hippocampus which areas provide afferent fibers to this nucleus. In both the sections prepared for LM autoradiography and the ultrathin sections prepared for EM autoradiography many silver grains were present over the AV thalamic nucleus after all these types of injections but especially after mammillary body injections. In the EM autoradiographs the distribution of the silver grains over the different tissue compartments was analysed and by means of the Williams' effective area measurement technique it could be shown not to be at random. With one month autoradiographic exposure time it could be demonstrated that after cortical and hippocampal injections the radioactivity was transported to small terminals on distal dendrites, while after mammillary body injections it was transported to large terminals on proximal dendrites. After 4 months exposure time altogether 6% of the small terminals and 80% of the large ones had been labeled.

THE IDENTIFICATION OF TERMINALS AND SYNAPSES OF DIFFERENT AFFERENTS IN THE AV THALAMIC NUCLEUS BY MEANS OF THE EM LESION AND DEGENERATION TECHNIQUE

INTRODUCTION

In the preceding chapter it has been possible to determine the type of axon terminals and synapses that the cingulate cortex, hippocampus and mammillary bodies make in the AV thalamic nucleus by means of the intra axonal transport of labeled amino acids followed by EM autoradiography. In order to evaluate the usefullness of the EM autoradiography technique an EM lesion and degeneration study of the same afferent fibers to the AV thalamic nucleus was undertaken and described in this chapter in which the results obtained were compared with those of the EM autoradiography. For this purpose lesions were made in the areas which correspond with the injected areas in the EM autoradiography study. After an optimal survival period the type of degenerating terminal and their number per grid square after each lesion were determined.

MATERIAL AND METHODS

Thirty nine adult rats were studied by means of the degeneration method. In 12 rats (cases D1 to D12) the posterior cingulate cortex was removed. Two of them (D1 and D2) were studied light microscopically and survived for 5 days, while the remaining 10 were studied electron microscopically. In 7 animals 60 hours was found to be the optimal survival time for degenerating cortical terminals in the AV thalamic nucleus and the remaining 3 rats (D10-D12) were kept alive for that amount of time.

In 12 rats (D13-D24) the caudo-lateral convexity of one cerebral hemisphere was removed and the exposed hippocampus was ablated. One rat (D13) was studied light microscopically. In 7 animals the optimal survival time for degenerating hippocampal terminals was found to be 36 hours and the remaining 4 rats (D21-D24) were kept alive for that amount of time.

In 14 rats (D26-D39) electrolytic lesions (electrode current of 1 mA during a period of 90-120 msec) were made in the mammillary bodies after introducing an electrode through the contralateral forebrain. One rat (D26) was studied light microscopically and survived for 5 days. The other 13 were studied electron microscopically. In 9 of these rats the optimal survival time for degenerating mammillary body terminals was found to be 36 hours and the remaining 4 rats (D36-D39) were kept alive for that amount of time.

The rats which were to be studied light microscopically were deeply anesthetized with pento-barbital (Nembutal^{R}) and perfused with saline, followed by 4% formaldehyde. After 5 days in 10% formaldehyde the brains were cut transversally in 20 micra thick frozen sections, which were impregnated with silver according to the Fink-Heimer (Fink & Heimer, 1967) technique.

The brains of the animals to be studied electron microscopically were treated in roughly the same way as the

material prepared for EM-autoradiography (see Materials and Methods chapter III). After perfusion with 0.5% glutaraldehyde and 4% paraformaldehyde under artificial respiration, the hemispheres and the diencephalon were dissected and the diencephalon was cut transversely into a rostral and a caudal part. The hemispheres and the caudal diencephalon which contained the lesions were cut transversely in 40 micra celloidin sections which were stained with cresyl violet. The rostral diencephalon in which the degeneration was to be studied electron microscopically, was cut into 4 blocks in the same was as in the autoradiographically studied material (see Material and Methods chapter III).

From the material of the 12 animals with the lesions, 18 AV thalamic blocks were available, i.e. 3 rostral and 3 caudal AV thalamic blocks for each 3 types of lesion (see table IV). From these blocks semithin sections were cut, on the basis of which the blocks were trimmed to pyramids containing the AV thalamic nucleus, together with parts of the AD thalamic nucleus and the stria medullaris (SM). From these pyramids ultrathin sections were cut in which the degenerating structures were studied.

In 2 or 3 ultrathin sections from each of the 18 blocks the degenerating axons and terminals in the AV thalamus were counted per grid square. Only those degenerating structures were regarded as terminals which still contained either synaptic vesicles, a synaptic thickening or both (see discussion). From the findings in the 3 rostral and the 3 caudal blocks of each of the 3 groups of animals the average quantitative distribution of degenerating terminals and axons in the rostral and the caudal parts of the AV thalamic nucleus in each of the 3 groups was computed (see fig. 28). The total number of degenerating terminals was determined from all the available AV thalamic grid squares in the 2-3 ultrathin sections of each block (see table IV). From these findings the average number of degenerating terminals per grid square for each block was calculated (see table IV).

RESULTS

A, DEGENERATION IN LIGHT MICROSCOPY

The distribution of degenerating fibers and terminals in the Fink-Heimer impregnated frozen sections after the different lesions were virtually the same as the distribution of the autoradiographic silver grains after injections in these respective areas (see chapter III). However, in the animals with hippocampal lesions combined with cortical ablations (Fig. 15B) degenerating fibers could be traced not only through the fornix but also from the damaged cerebral white matter through the internal capsule towards the AV nucleus of the thalamus. As a consequence it could not be determined whether the degeneration in the AV nucleus was derived from the fornix, or from the internal capsule, or from both.

B, DEGENERATION IN ELECTRON MICROSCOPY

In the cases D10-D12; D21-D24; D36-D39, in which the degenerating AV terminals were studied electron microscopically, the cingulate cortex lesions (D10-D12) involved virtually the entire granular part of the posterior cingulate cortex as well as the adjoining portion of its granular part unilaterally (see fig. 15A). The hippocampal lesions (D21-D24) involved the dorsal portion of the hippocampus as well as the overlying parietal cortex and white matter unilaterally (see Fig. 15B). All mammillary body lesions (D36-D39) were bilateral and destroyed the entire mammillary complex (see fig. 15C) and in case D38 also the adjoining parts of the hypothalamus.

In the electron micrographs after the various lesions

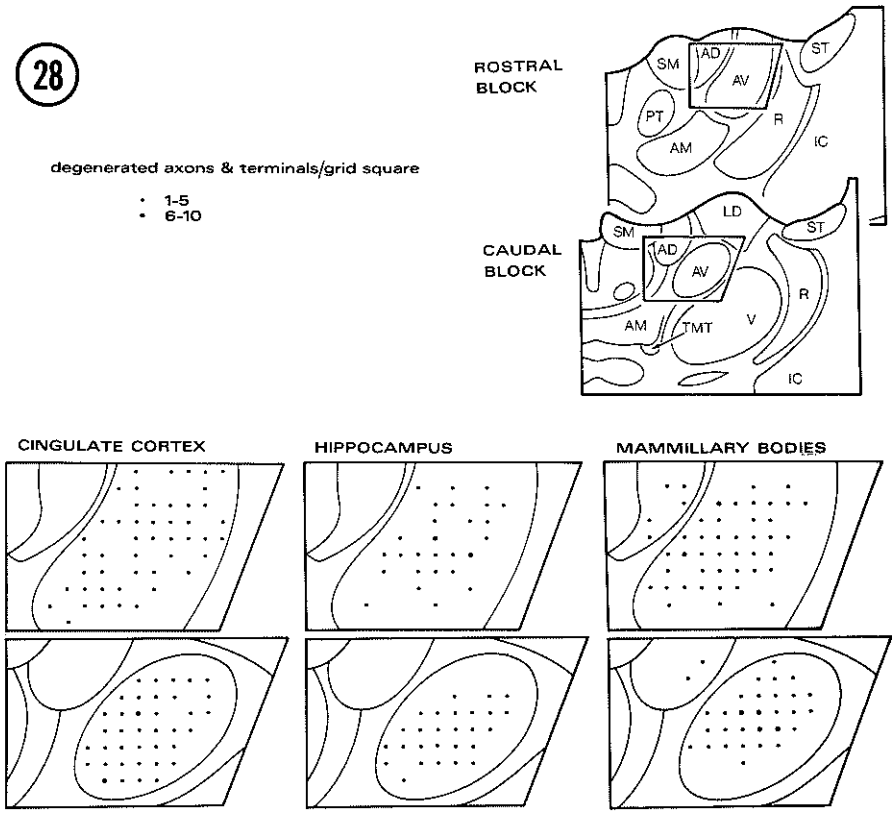


Fig. 28. Semidiagrammatic representation of the average quantitative distribution of degenerating axons and terminals in the different parts of the ultrathin sections from the rostral and the caudal AV thalamic blocks after cingulate cortex, hippocampus and mammillary body lesions. Size of dot indicates the number of degenerating elements per grid square (=2025 square micrometers)

followed by the optimal survival times an average of 1 to 5 degenerating terminals and axons were found per AV thalamic grid square (see Fig. 28). Only those degenerating structures were regarded as terminals which contained synaptic vesicles or synaptic thickenings, or both.

In the animals with cingulate cortex lesions, degenerating terminals in the AV thalamus were most numerous after 2½ days survival (cases D10-D12), in two cases more

than 20 per ultrathin section and in one case even more than 30. This was concluded to be the optimal survival time for these terminals, since after 28 hours less than 10 degenerating terminals were found and after 24 hours or less merely 4. After 72 and 86 hours only degenerating fibers were observed and virtually no degenerating terminals.

In the animals with combined hippocampal and cerebral cortex lesions a bimodal distribution was found. After 24 hours survival less than 10 degenerating AV terminals were present per section (case D14), after 37 to 44 hours (cases D21-D24) more than 20 and after 48 hours (case D16) again less than 10. When the animals survived for 60 hours (cases D17, D18) again more than 20 degenerating terminals were found. This probably reflected degeneration of cortical fiber terminals since they had been found to be most numerous after 60 hours. It was therefore concluded that 37 to 44 hours represented the optimal time for the hippocampal fiber terminals.

In the animals which survived the cingulate cortex and the hippocampal lesions for an optimal time, the degenerating synaptic terminals showed shrinkage, swelling of the vesicles and darkening of the intervesicular space. The vast majority of these terminals in both groups of cases seemed to represent small terminals (see Figs. 29 and 31), i.e. the same type which was labeled preferentially in the EM auto-

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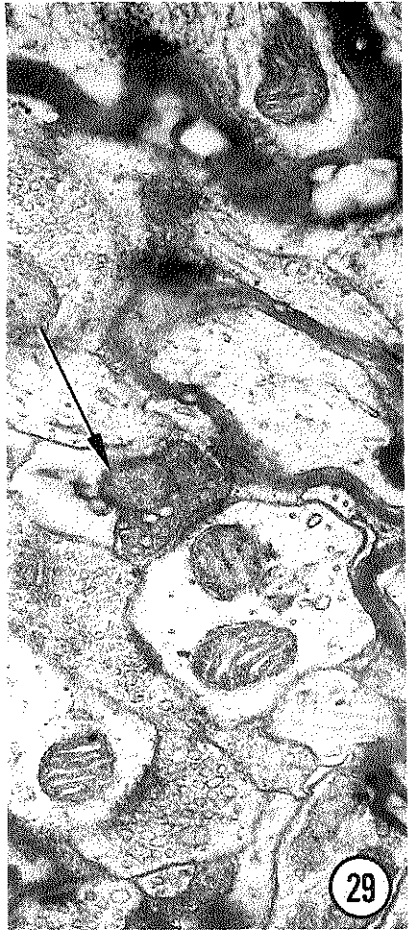
Figs. 29-32. Electron micrographs of degenerating terminals in the AV thalamic nucleus. Figs. 29 and 31: Degenerating small terminals (arrows) after cingulate cortex (Fig. 29) and hippocampal (Fig. 31) lesions. Magnification, x 39,000 Figs. 30 and 32: Degenerating large terminals after mammillary body lesions. Fig. 30: Electron-dense degenerating terminal (T), Magnification, x 39,000. Fig. 32: Electron-lucent degenerating terminal (T). Magnification, x 29,000. D, dendrite: G, glia.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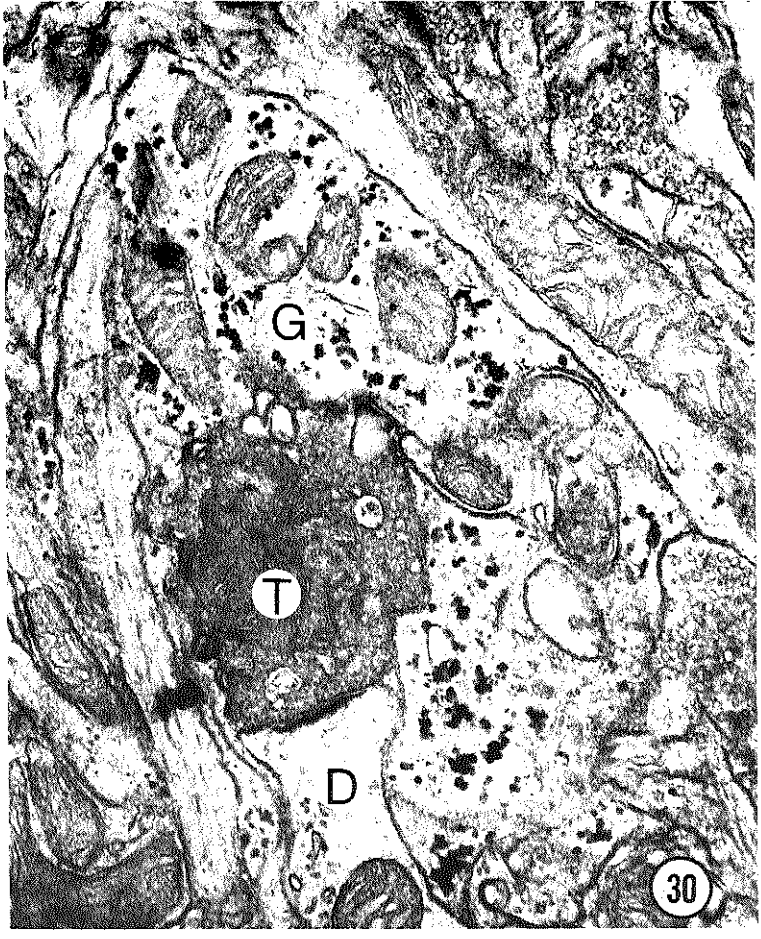
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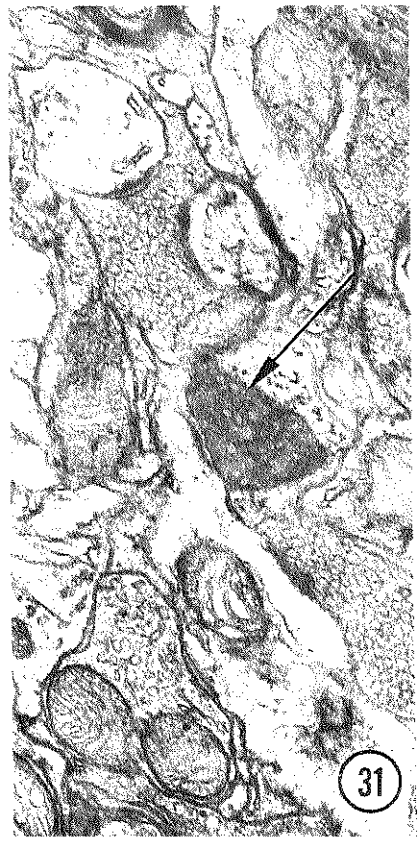
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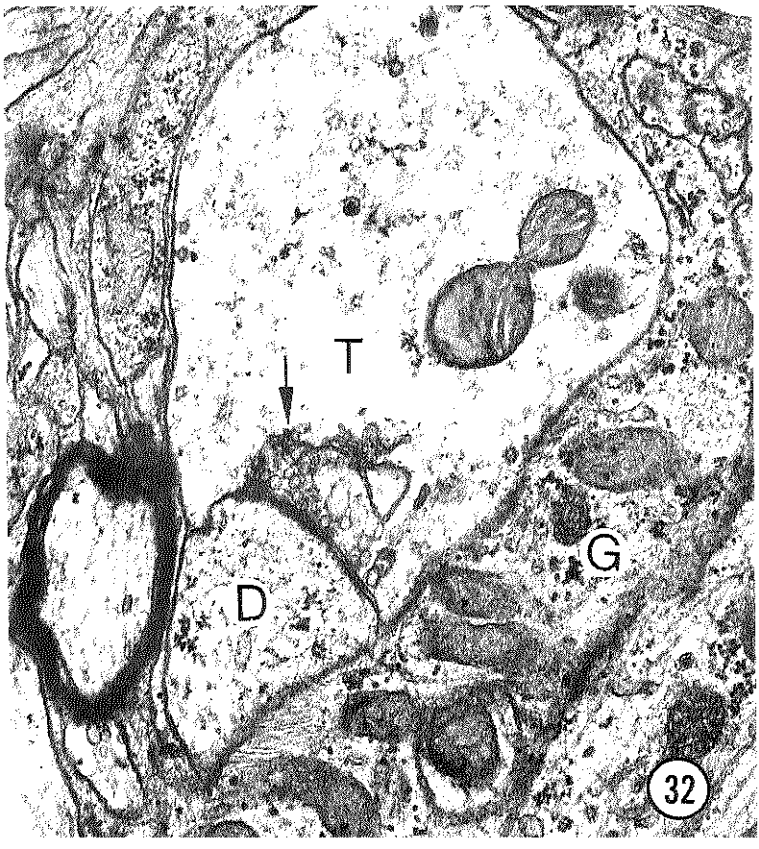
T
G
D
32

radiographs. A certain number of degenerating terminals could be seen to have synapses with distal dendrites.

The degenerating terminals after mammillary body lesions were found to be most numerous, i.e., 20 per section, after survival periods ranging from 36 to 42 hours (cases D36-D39). Fewer degenerating terminals were found after 24 hours (D27 and D28), 48 hours (D29-D31), 60 hours (D32) and 72 hours (D33) survival. A survival time of 36 to 42 hours was therefore concluded to be optimal for the degeneration of mammillary body terminals.

The majority of the degenerating mammillary body terminals showed pronounced shrinkage, swelling of vesicles and mitochondria and darkening of the terminal matrix (Fig. 30). The amount of shrinkage in these terminals was much more pronounced than for the small terminals after cingulate and hippocampal lesions. Many of the degenerating mammillary body terminals were in addition surrounded by glial processes containing glycogen granules. After mammillary body lesions a few terminals however showed an electron-*lucent* type of degeneration. These terminals were swollen and almost empty except for a few synaptic vesicles close to the synaptic thickening and some dense core vesicles (Fig. 32). Both the electron-*dense* and the electron-*lucent* type of degenerating terminals seemed to represent mainly large terminals, i.e. the same type as was labeled preferentially after mammillary body injections. In a few instances both the electron-dense as well as the electron-lucent terminals could be seen to establish synapses with proximal dendrites containing ribosomes (see Figs. 30 and 32).

The number of degenerating terminals in the 3 groups of animals have been determined (see table IV). In these determination degenerating terminals were distinguished by the presence of vesicles or synaptic thickenings or both, while all the other unrecognizable electron-dense profiles were disregarded (see discussion). From these findings

TABLE IV

Representation of the number of grid squares analysed, numbers of degenerating terminals and mean values (± SD) for the degenerating terminals per grid square for the 3 different lesions. The mean values were obtained from the average number of degenerating terminals per grid square in the 3 rostral and 3 caudal AV thalamic blocks. The area of one grid square equals 2025 square micra.

lesion	total number of AV grid squares analysed	total number of degenerating terminals	mean value (± SD) for the degenerating AV terminals per grid square
CINGULATE CORTEX			
rostral blocks			
D10	66	172	
D11	155	186	1.9 ± 0.6
D12	137	329	
caudal blocks			
D10	105	210	
D11	50	20	1.1 ± 0.4
D12	40	52	
HIPPOCAMPUS			
rostral blocks			
D21	79	134	
D23	45	86	1.7 ± 0.2
D24	30	42	
caudal blocks			
D22	102	184	
D23	70	119	1.8 ± 0.1
D24	63	126	
MAMMILLARY BODY			
rostral blocks			
D36	76	91	
D37	120	192	1.4 ± 0.2
D38	40	60	
caudal blocks			
D37	138	124	
D38	61	85	1.4 ± 0.2
D39	103	103	

the average numbers of degenerating terminals per grid square were calculated (see table IV) (one grid square equals 2025 square micrometers). After cingulate cortex and hippocampal lesions an average of 1.1 to 1.9 degenerating (small) terminals per grid square was found in the rostral and caudal parts of the AV thalamic nucleus (see Table IV). After mammillary body lesions an average of 1.1 to 1.4 degenerating (large) synaptic terminals per grid square was found. Thus, after cingulate cortex and hippocampal lesions altogether 3% of all the small terminals could be identified as degenerating small terminals, while after mammillary body lesions 6% of all the large terminals could be identified as degenerating large terminals.

DISCUSSION

All the terminals of cortical and hippocampal and almost all the terminals of mammillary body afferents underwent an electron-dense type of degeneration. Although degenerating large terminals in many other thalamic nuclei do show filamentous changes before becoming electron-dense, no filamentous hyperplasia have been observed for the large terminals in the AV thalamic nucleus. However they are not an exception because direct electron-dense degeneration without early filamentous hyperplasia was also observed for the large terminals of collicular afferents in the paracentral and inferior pulvinar nuclei of the rhesus monkey (Partlow et al., 1977), and for the large terminals in the lateral geniculate nucleus of the rat (McMahan, 1969).

In determining the terminals of the mammillary bodies electron-lucent degenerating terminals were also found. These terminals are large and some can be shown to establish synapses with proximal dendrites. However the source of these terminals could not be determined with certainty. It might be possible that these terminals are derived from the mammillary bodies, which afferents then may degenerate in two different morphological forms. An other possibility is that they belong to fibers of passage running through places destroyed by the electrode tract or the lesion and that they therefore originate from other sources than the mammillary bodies. This question may be answered when looking at the results of the EM labeling tracing method, because with that method (see preceding chapter III) it could be show that at least 80% of the large terminals are derived from the mammillary bodies after 4 months exposure, which makes it likely that all the large terminals are derived from the mammillary bodies and that therefore the degeneration of mammillary body terminals probably may occur in two different morphological forms.

Many studies (see for references Raisman & Matthews,

1972) have reported that the actual numbers of degenerating terminals are difficult to measure. This is probably due to the rapid and asynchronous nature of the degeneration, i.e., not all terminals degenerate simultaneously and therefore at any stage only a certain amount of terminals degenerate, while others are still in the normal state or show advanced stages of degeneration, in which they are electron dense or are already phagocytosed (Jones & Powell, 1970b; Grofova & Rinvik, 1970; Garey & Powell, 1971; Raisman & Matthews, 1972). One would suggest that in order to obtain large numbers of degenerating terminals long survival periods would suffices. This is probably true, however at long survival periods it is difficult to identify the terminals at an advanced stage of degeneration due to the morphological degenerating changes or because the terminal is already completely phagocytosed by glial cells (see for instance Kemp & Powell, 1971; Raisman & Matthews, 1972).

Also in this degeneration study the actual number of degenerating terminals was difficult to measure due to the same underlying phenomenon. In addition a second problem arose because in this study the impression was gained that a number of electron-dense structures were degenerating unmyelinated axons, and that after cingulate cortex lesions some AV dendrites underwent degenerative changes which structures make there profiles resemble electron-dense terminals without synaptic thickenings or vesicles. Therefore for a meningful quantification of the degenerating AV thalamic terminals the optimal survival time was established at which time the largest number of recognizable degenerating terminals was found. Structures were identified as degenerating terminals only if they possessed synaptic thickenings, synaptic vesicles or both. Other degenerating structures were not included in the counts. The largest number of recognizable degenerating terminals was found at 40 hours for the mammillary body and hippocampal terminals and at 60 hours for the cingulate cortex terminals.

The number of affected degenerating terminals per grid

TABLE V

Mean values (± SD) for the labeled and degenerating terminals per grid square in the AV thalamic nucleus after injections and lesions of the different structures. The mean values were calculated from the average number of labeled or degenerating terminals per grid square in the 3 rostral and 3 caudal AV thalamic blocks. The mean values of labeled terminals are given both after 1 month and 4 months autoradiographic exposure time. The area of one grid square equals 2025 square micra. (See also tables III and IV).

injection/lesion	labeled small asymmetrical synaptic terminals		degenerating terminals
	1 month	4 months	
CINGULATE CORTEX			
rostral blocks	0.8 ± 0.1	4.0 ± 0.2	1.9 ± 0.6
caudal blocks	0.7 ± 0.2	3.5 ± 0.1	1.1 ± 0.4
HIPPOCAMPUS			
rostral blocks	2.0 ± 0.9	3.7 ± 0.1	1.7 ± 0.2
caudal blocks	1.1 ± 0.4	3.4 ± 0.2	1.8 ± 0.1
	labeled large asymmetrical synaptic terminals		degenerating terminals
	1 month	4 months	
MAMMILLARY BODY			
rostral blocks	7.1 ± 1.9	15.1 ± 3.7	1.4 ± 0.2
caudal blocks	8.9 ± 2.0	20.7 ± 4.9	1.1 ± 0.2

square (one grid square equals 2025 square micra) obtained in the degeneration material as described above was calculated and compared with the labeled terminals obtained with the labeled amino acid tracing method. The results show that with the degeneration method, after cingulate cortex and hippocampal lesions an average of 1.1 to 1.9 degenerating small terminals per grid square was found in the rostral and caudal parts of the AV thalamic nucleus. This number is either equal, slightly higher or lower than the number of labeled small terminals found with EM autoradiography after cingulate cortex or hippocampal injections with 1 month exposure (0.8 to 2.0 per grid square). However, the number of degenerating terminals was only half of the 3.4 to 4.0 labeled terminals per grid square found after 4 months exposure (see Table V). After mammillary body lesions in the degeneration experiments an average of 1.1 to 1.4 degenerating (large) terminals per grid square was found in the ro-

stral and caudal parts of the AV thalamic nucleus. This is considerably less than the 7.1 to 8.9 labeled terminals per grid square found with the EM autoradiography after mammillary body injections with 1 month exposure time and the 15.1 to 20.7 labeled terminals per grid square after 4 months (see Table V).

The above comparison therefore clearly shows that much more AV thalamic terminals can be labeled by means of the EM autoradiography technique than by means of the EM degeneration technique.

SUMMARY

In this chapter the type of terminals and synapses of the afferent fibers from the cingulate cortex, hippocampus and mammillary bodies to the AV thalamic nucleus was determined by means of the EM lesion and degeneration technique, and the results were compared with those of the EM autoradiography technique. In agreement with the EM autoradiography studies the small asymmetrical synaptic terminals were found to degenerate after both cingulate cortex and hippocampal lesions and the large asymmetrical synaptic terminals after lesions of the mammillary bodies. However, the number of effected terminals per grid square in the EM lesion and degeneration study was much lower than that obtained with the intra axonal transport of labeled amino acids and EM autoradiography after 4 months exposure. The number of degenerating small asymmetrical terminals and of degenerating large asymmetrical terminals were only one half of the number of autoradiographically labeled small asymmetrical synaptic terminals and only one tenth of that of the autoradiographically labeled large asymmetrical synaptic terminals, respectively.

CONCLUSIONS

From the findings in the present study it may be concluded that the labeled amino acid transport technique combined with EM autoradiography is a powerful tool in identifying the various terminals of the afferent systems to a certain cell group in the brain. However, the findings also show that in order to draw reliable conclusions, the EM autoradiography data should be subjected to a quantitative analysis such as the William's effective area measurement technique. In this way it is possible to determine the *type* of terminals provided by the different fiber systems after an autoradiographic exposure time of only one month. However, in order to approximate the *number* of terminals provided by the different fiber systems a much longer exposure time was required since an exposure time of at least 4 months was required to lable 80% of the large terminals in the AV thalamic nucleus after tritiated leucine injections in the mammillary bodies. Obviously this very long exposure time is one of the major drawbacks of the EM autoradiography technique. Yet it should be kept in mind that labeling of 80% of one of terminal is only possible with this technique and cannot be achieved by means of the degeneration technique probably because of the rapid and asynchronous nature of the degeneration due to which only a limited number of degenerating terminals can be recognized at any given time.

On the basis of these findings the labeled amino acid transport technique was concluded to be much more efficient

than the degeneration technique in labeling a great many terminals of different fiber systems. In addition, the labeled amino acid transport technique combines this capacity with the striking advantage that it leaves the cytoarchitecture of the labeled terminals and of the surrounding tissue unchanged which makes it possible to determine the structural characteristics of the terminal in the same way as in normal EM material. In addition, the labeled amino acid transport technique makes it possible to study terminals of fiber systems originating from cell groups which are embedded in other fiber bundles without the masking effect of the additional labeling of the terminals of passing fibers which is virtually unavoidable when employing the lesion and degeneration technique.

SUMMARY

In this study an attempt was made to determine whether the labeled amino acid axonal transport technique combined with electron microscopy (EM) autoradiography can be used to identify reliably the terminals and synapses of different afferents to a cell group in the brain, and whether this technique is as good or even better than the EM lesion and degeneration technique in identifying such terminals and synapses. For this purpose the antero-ventral (AV) thalamic nucleus of the rat was used as a model and the type of terminals and synapses of the afferent fibers from the cingulate cortex, hippocampus and mammillary bodies were investigated.

First, the structure of the AV thalamic nucleus has been investigated in normal light microscopy (LM) and EM material (see chapter II). Nissl stained sections showed that the rat's AV thalamic nucleus contained mainly relatively light stained neurons which in Golgi material were found to possess tufted dendrites. In EM material three types of synaptic terminals were found which showed a topical distribution over the neuronal surface. A few medium-sized terminals with flattened vesicles establish symmetrical synaptic junctions with cell somas and stem dendrites. Large terminals with spherical vesicles establish asymmetrical synaptic junctions with proximal dendrites while many small terminals with spherical vesicles establish asymmetrical synaptic junctions with distal dendrites.

Subsequently the type of terminals and synapses of the 3 different afferent sources to the AV thalamic nucleus was determined with both the EM autoradiographic (see chapter III) and the EM degeneration (see chapter IV) techniques. For this purpose in one group of rat's ^{3}H-leucine was injected in

cingulate cortex, hippocampus and mammillary bodies, respectively, and in another group lesions were made in these structures. With one month autoradiographic exposure time it could be demonstrated that after cortical and hippocampal injections the radioactivity was transported to small asymmetrical synaptic terminals on distal dendrites, while after mammillary body injections it was transported to large asymmetrical synaptic terminals on proximal dendrites. After 4 months exposure altogether 6% of the small terminals and 80% of the large ones had been labeled.

In agreement with the EM autoradiography studies the small terminals were found to degenerate after both cingulate cortex and hippocampal lesions and the large terminals after lesions of the mammillary bodies. However, the number of degenerating small terminals and of degenerating large terminals were only half of the number of autoradiographically labeled small terminals and only one tenth of that of the autoradiographically labeled large terminals, respectively after 4 month exposure. It was therefore concluded that the labeled amino acid axonal transport technique combined with EM autoradiography can be used to identify reliably the type of terminals and synapses of different afferent fibers to a cell group in the brain and that in this respect this technique appears to be much more efficient than the EM degeneration technique.

SAMENVATTING

In deze studie is getracht na te gaan of de techniek gebaseerd op het transport van met tritium-gelabelde aminozuren gecombineerd met electron microscopische (EM) autoradiografie gebruikt kon worden om de uiteindigingen en synapsen van verschillende afferente zenuw vezels naar een celgroep in de hersenen te identificeren, en of deze techniek even goed of misschien beter was dan de EM degeneratie techniek voor de identificatie van zulke uiteindigingen en synapsen. Voor dit doel werd de nucleus antero-ventralis (AV) van de thalamus bij de rat gebruikt als model en werden de uiteindigingen en synapsen van de afferente zenuw vezels van de cortex cingulum, hippocampus en corpora mammillaria geidentificeerd.

Allereerst werd de structuur van de AV nucleus bestudeerd in normaal licht microscopisch (LM) en EM materiaal (zie hoofdstuk II). Coupes volgens Nissl gekleurd toonden dat de AV kern van de thalamus bij de rat voornamelijk is opgebouwd uit relatief licht gekleurde neuronen die in Golgi materiaal 'tufted' dendrites bezitten. In EM materiaal werden 3 typen synaptische uiteindigingen gevonden die een topografische verdeling over het neuronale oppervlak vertoonden. Een paar middel grote uiteindigingen met platte blaasjes maken symmetrisch synaptische contacten met cellichamen en stamdendrieten. Grote uiteindigingen met ronde blaasjes maken asymmetrisch synaptische contacten met proximale dendrieten terwijl vele kleine uiteindigingen met ronde blaasjes asymmetrisch synaptische contacten maken met distale dendrieten.

Vervolgens werden de uiteindigingen en synapsen van de 3 verschillende afferent bronnen naar de AV thalamus geidentificeerd met zowel de EM autoradiografie (zie hoofdstuk III) als met de EM degeneratie (zie hoofdstuk IV) techniek. Voor dit

doel werd in een groep ratten ^{3}H-leucine geinjiceerd in de cortex cingulum, hippocampus en corpora mammillaria, respectievelijk, en in een ander groep werden lesies gemaakt in deze structuren. Na een maand autoradiografische belichting kon aangetoond worden dat na corticale en na hippocampale injecties de radioactiviteit getransporteerd was naar kleine asymmetrisch synaptische uiteindigingen op distale dendrieten, terwijl na injectie van de corpora mamillaria het getransporteerd was naar grote asymmetrisch synaptische uiteindigingen op proximale dendrieten. Na 4 maanden autoradiografische belichting waren in totaal 6% van de kleine en 80% van de grote uiteindigingen gelabeld.

In overeenstemming met de EM autoradiografische studies werd gevonden dat de kleine uiteindigingen degenereren na lesies van de cortex cingulum en hippocampus en dat de grote uiteindigingen degenereren na lesies van de corpora mammillaria. Echter, het aantal gedegenereerde kleine en grote uiteindigingen, respectievelijk, bedroeg slechts de helft van het aantal autoradiografisch gelabelde kleine en slechts een tiende van de autoradiografisch gelabelde grote uiteindigingen, respectievelijk, na 4 maanden belichting.

Geconcludeerd kon daarom worden dat de techniek gebaseerd op het axonaal transport van met tritium-gelabelde aminozuren en gecombineerd met EM autoradiografie gebruikt kan worden om de uiteindigingen en synapsen van verschillende afferente zenuw vezels naar een celgroep in de hersenen te identificeren en dat deze techniek in dit respect veel efficienter blijkt te zijn dan de EM degeneratie techniek.

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