BRAINSTEM PROJECTIONS TO MOTONEURONS IN THE LUMBAR SPINAL CORD

An Ultrastructural Study in Rat

Verbindingen van de hersenstam met motoneuronen in het lumbale ruggemerg

Een electronen microscopisch onderzoek bij de rat

PROEFSCHRIFT

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In de wetenschap gelijken wij op kinderen,
die aan de oever der kennis
hier en daar een steentje oprapen,
terwijl de wijde oceaan van het onbekende
zich voor onze ogen uitstrekt.

Newton

Aan mijn ouders
Aan Edith

Dit proefschrift is gebaseerd op de volgende publicaties:

Holstege, J.C. and Kuypers, H.G.J.M. (1987) Brainstem projections to lumbar motoneurons in rat. I. An ultrastructural study using autoradiography and the combination of autoradiography and horseradish peroxidase histochemistry. Neurosci. 21, 345-367.

Holstege, J.C. (1987) Brainstem projections to lumbar motoneurons in rat. II. An ultrastructural study by means of the anterograde transport of wheat-germ agglutinin coupled to horseradish peroxidase and using the tetramethyl benzidine reaction. Neurosci. 21, 368-376.

Holstege. J.C. and Kuypers, H.G.J.M. (1987) Brainstem projections to spinal motoneurons: an update. Neurosci. 23, 809-821.

Holstege, J.C. and Vrensen, G.F.J.M. (1988) Anterograde tracing in the brain using autoradiography and HRP-histochemistry. A comparion at the ultrastructural level. J. Microsc. 150, 233-243.

Overige publicaties:

Holstege, J.C. and Dekker, J.J. (1979) Electron microscopic identification of mammillary body terminals in the rat's AV thalamic nucleus by means of anterograde transport of HRP. A quantitative comparison with the EM degeneration and EM autoradiographic techniques. Neurosci. Lett. 11, 129-135.

Holstege, J.C. and Kuypers, H.G.J.M. (1982) Brain stem projections to spinal motoneuronal cell groups in rat studied by means of electron microscopy autoradiography. In Descending Pathways to the Spinal Cord, Progress in Brain Research (eds. Kuypers H.G.J.M. and Martin G.F), Vol. 57, pp. 177-183. Elsevier Biomedical Press, Amsterdam.

De Zeeuw, C.I., Holstege, J.C., Calkoen, F., Ruigrok, T.J.H. and Voogd, J. (1988). A new combination of WGA-HRP anterograde tracing and GABA-immunocytochemistry applied to afferents of the cat inferior olive at the ultrastructural level. Brain Res. 447, 369-375.

De Zeeuw, C.I., Holstege, J.C., Ruigrok, T.J.H. and Voogd, J. (1989) The GABA-ergic, cerebellar and mesodiencephalic innervation of the glomeruli in the cat inferior olive. A comparison at the ultrastructural level. Exp. Brain Res.17, 111-117.

De Zeeuw, C.I., Holstege, J.C., Ruigrok, T.J.H. and Voogd, J. (1989) An ultrastructural study of the GABA-ergic, the cerebellar and the mesodiencephalic innervation of the cat medial accessory olive combining anterograde tracing with immunocytochemistry. J. Comp. Neurol. 284, 12-35.

CHAPTER I

GENERAL INTRODUCTION

I.1.

INTRODUCTION

The tracing of fiber systems in the brain has always been one of the chief aims in neuro-anatomy. Although anatomists described and named several fiber bundles in the brain during the past centuries, major advances in neuro-anatomy only occurred around the turn of the century because of two important developments. In the first place new staining techniques were developed (for a review see Voogd and Feirabend, 1981), most notably the Weigert and the Nissl stains and the Golgi method. The Nissl stain (Nissl, 1894) mainly stains the cell body of neurons and glial cells, but not their processes. Nissl staining makes it possible to identify cytoarchitectonic subdivisions in light microscopic sections of brain tissue. The Golgi method (Golgi, 1873), on the other hand, stains the whole cell, including its processes. The cells, which become impregnated by a silver deposit, clearly stand out against a clear background of unstained cells, because -for some unknown reason(s)- only a fraction of all cells in a section react. Therefore the Golgi technique is especially suited for studying the details of individual neurons and glial cells. The Weigert stain (Weigert, 1884) was developed from an already existing staining technique (the carmine technique) and is specific for myelin. The Weigert stain made it easier to identify myelinated fiber tracts within the brain. Silver impregnation techniques were introduced by Bielschowsky (1902) and Cajal (1903). These techniques are based on the staining of neurofibrils within neurons and their processes.

With the development of the various new staining techniques around the turn of the century, the discussion on two opposing theories on the organisation of the brain came to a climax. One theory stated that all neurons are part of a continuous network. This "reticular theory" was strongly defended by Golgi. The other theory, known as the "neuron theory", held that all nerve cells were individual entities, which were not in protoplasmic continuity. According to Cajal, in his Nobel Prize Lecture in 1906 (Cajal, 1906), the individual nerve cells were connected through "a granular cement or special conducting substance, which serves to keep the neuronal surfaces in very intimite contact". The word "synapse" for such functional contact between nerve cells had already been introduced by Sherrington (Sherrington, 1897) almost ten years earlier. Cajal strongly defended the neuron theory. He put forward many arguments in favour of it, mainly based on his own detailed descriptions of Golgi impregnated single cells from all areas of the brain. By the time Golgi and Cajal shared the Nobel prize for Medicine in 1906 the discussion on the reticular and neuron theory had been decided in favour of the neuron theory. The proof for the validity of the neuron theory was finally completed by the early electron microscopical studies, in which the synaptic structure could actually be visualized (see e.g. Palade and Palay, 1954; Palay, 1956). The neuron theory was of crucial importance for the further development of neuroantomy, because it formed the basis for the interpretation of findings obtained with the different tracing techniques.

At the end of the nineteenth century only few methods were available for the identification of connections between specific nuclei in the brain. Myelinization and demyelinization of long pathways could be studied with the Weigert stain, but these types of studies did not give information on the location of the cell bodies from which these pathways originated. On the other hand, cells which had degenerated after their axon had been cut could sometimes be identified in Nissl stained sections by the displacement of the nucleus and the Nissl bodies towards the periphery of the cell. This retrograde degeneration technique for identifying connections in the brain was given a further impetus by techniques which specifically stained degenerating fibers, like the Marchi technique (Marchi and Algeri, 1885), which stains the myelin of degenerating fibers. The Marchi technique is less well suited for tracing the terminal arborizations of nerve fibers, since these are generally not myelinated. For the purpose of identifying degenerating fibers and terminals distal to a lesion

(anterograde degeneration) the silver impregnation techniques of Bielschowsky or Cajal can be used. However, these techniques also stain normal fibers which made it often complicated to obtain reliable data. Only much later the silver impregnation techniques were modified to make them specific for degenerating fibers and terminals (Glees, 1946; Nauta and Gygax, 1954; Fink and Heimer, 1967).

The application of the Nauta technique for visualizing degenerating axons and the introduction of electron microscopy for studying the nervous system (for a review see Peters et al., 1976) resulted in a rapid expansion of our knowledge of the brain with its many different fiber systems. Up to 1970 the axonal degeneration technique remained the most important tool for tracing connections in light and electron microscopical studies of the brain. Around 1970 two new techniques for tracing nervous connections became available. They did not rely on the pathological changes in damaged nerve cells as the degeneration technique, but were based on normal physiological uptake and transport mechanisms of the neuron. In the next paragraphs these techniques will be described in more detail.

I.2.

A DESCRIPTON OF SOME RECENT NEURO-ANATOMICAL TECHNIQUES

In the past decades many new techniques have been developed for studying the anatomy of the brain. The basic principles of some of these techniques will be described below. They include the axonal transport techniques, the histochemical and immuno-histochemical techniques.

I.2.A. AXONAL TRANSPORT

In 1948 Weiss and Hiscoe were the first to clearly demonstrate the existence of an active anterograde transport system within the axon of a neuron. They described the accumulation of material proximal to a ligature of a nerve and, after the constriction had been removed, the anterograde movement of the material with a velocity of 1-2 mm/day. Application of tritium labelled amino acids around neurons and the subsequent movement of radioactive proteins within the axon confirmed this transport rate (Droz and Leblond, 1962). Other quantitative studies on transport velocities in axons (see e.g. Grafstein, 1967) showed that much faster transport rates (upto 400 mm/day) also existed. Apart from the slow and fast transport rates there may also exist one or more intermediate transport rates (Grafstein and Forman, 1980).

The material that is transported by the fast anterograde flow consists mainly of membranous structures like smooth endoplasmatic reticulum, plasma membranes, synaptic and other types of vesicles and some mitochondria. These membrane structures generally consist of proteins, glycoproteins and lipids. However, small molecules like amino-acids, which may be contained as neurotransmitters within synaptic vesicles, are also part of the fast flow as well as sugars and nucleosides. The slow anterograde transport generally consists of cytoplasmic materials, mainly proteins which are part of the structural elements of the axon (neurofilaments, microtubules and some mitochondria). Other proteins like actin, cadmodulin and various metabolic enzymes are also part of this slow flow (for details and references see Grafstein and Forman, 1980).

Apart from the anterograde transport system of the axon there also exists a retrograde transport system. This is best shown by the application of certain substances around axon terminals, while monitoring the uptake of these substances and subsequent retrograde transport (Kristensson, 1971; Lavail and Lavail, 1972). The velocity of retrograde transport is estimated at rates between 2 and 300 mm/day. Fast and slow transport rates have been distinguished, although only one protein has been identified to be associated with the slow retrograde flow (Fink and Gainer, 1980). Furthermore it was found that on the average the retrograde transport velocities are slower than the anterograde ones. Several substances were found to be transported retrogradely within neurons, including proteins, glycoproteins, phospholipids and small molecules like GABA, glycine and serotonin. Since the fast retrograde and the anterograde transport have many substances in common, a large part of the material in the retrograde transport flow may be derived from a reversal of the anterograde flow. Several proteins and small molecules can be taken up from the extracellular space and subsequently enter the retrograde flow (for details and references see Grafstein and Forman, 1980). In this respect it is of interest to note that some extra-cellularly applied substances (e.g. nerve growth factor, tetanus toxin, cholera toxin and wheat germ agglutinin) are easily taken up by an axon terminal or a cell soma even at low extracellular concentrations (Dumas et al., 1979). Other substances, like horseradish peroxidase (HRP) are taken up in detectable amounts only if a high extra cellular concentration is achieved. This difference in uptake efficiency

is probably due to the presence of receptors in the neuronal membrane, which facilitate the uptake of only a few specific substances like the ones mentioned above. Specific receptors for horseradish peroxidase probably do not exist (Stoeckel and Thoenen, 1975), which would explain the relatively high extra-cellular concentration which is needed to obtain sufficient uptake of HRP by the cell soma or the axon terminals (see also Ch. I.2.B.).

Both anterograde and retrograde axonal transport are essential for maintaining the integrity of the neuron: newly synthesized enzymes, proteins and membrane constituents are transported to the part of the axon or terminal where they are needed. Vica versa redundant materials from the axon or terminal or substances which have been taken up from the extracellular space are transported back to the soma, where they are recycled or degradated in lysosomes. Apart from the anterograde and retrograde transport in axons there is also evidence for such transport in dendrites (Lux et al., 1970).

The basic mechanisms which underlie the axonal transport systems are still unclear, although several hypotheses have been proposed (for details see Grafstein and Forman, 1980). At any rate the transport mechanism critically depends on energy which is provided locally within the axon. Even after the axon has been disconnected from the cell soma, transport will continue for a few hours (Ochs and Ranish, 1969). Furthermore it has been shown (Ochs and Ranish, 1969; see also Grafstein and Forman, 1980) that transport in an axon is independent of its electrical activity. Thus the transport velocity in non-active nerve fibers (e.g. in an anaesthetized animal) is the same as in an electrically active nerve. Anoxia and blockers of oxidative metabolism put a stop to the transport and agents which disrupt the microtubules (like colchicine, vincristine and others) have a similar effect (for a review see Samson, 1976). The microtubules themselves do not seem actively involved in the transport of substances. They may serve as a structural support for certain proteins, which are capable of producing force along the surface of microtubules, provided that ATP is present. One of these proteins (kinesin) is involved in anterogade transport (Schnapp and Reese, 1986), while another protein (dynein) is especially associated with retrograde transport (Vallee et al., 1989). The exact relation between these proteins and the transported organelle is at present unclear.

I.2.B. AXONAL TRANSPORT TECHNIQUES

The techniques which use axonal transport for tracing connections in the brain are based on the neuronal uptake of certain substances from the extracellular space and their active transport in an anterograde or retrograde direction. Most of these substances are taken up by the soma and/or the terminals. In addition there is evidence for the uptake and subsequent transport of substances (in particular HRP) by axons. Probably this uptake will only occur when the axons are damaged (Wakefield and Shonnard, 1979; Brodal et al., 1983). Although the active transport of extra-cellularly applied substances has been clearly established, it cannot be excluded that a fraction of these substances actually diffuse within the axon. The recent finding that in post-mortem or even in fixed material substances like HRP (Beach and McGeer, 1987) and certain carbocyanide dyes (Godement et al., 1987) "passively" move through axons at a very slow rate, may point in this direction.

The substances which are used for tracing connections in the brain are generally referred to as tracers. There are several tracers which can be used and their characteristics will be shortly described below.

Tritiated amino acids and other radioactive substances

The use of radioactive tracers is based on the fact that their presence can be detected in the tissue by virtue of their radiation. Generally radioactive tracers are produced by using a non-radioactive tracer, which is then radioactively labelled by substituting one or more hydrogen atoms for tritium. Other isotopes like carbon-14, phosphorus-32, sulphur-35 or iodine-125 can also be used but are less often applied, because in comparison to tritium, their halflife, mean particle energy or commercial availability is often less suitable for tracing purposes (for details see Williams, 1977). Tritium emits ß radiation (with a mean particle energy of 0.018 MeV), which penetrates in tissue for a maximum distance of 3 µm. It has a half life of approximately 12.2 years. In tissue the radiation of e.g. tritium can be detected and localized by the applying to a tissue section an emulsion layer containing silver-bromide crystals, or by liquid scintillation counting of small samples. When a silver-

bromide crystal in the emulsion layer is "hit" by a ß-particle, it is sensitized and will be converted into a grain of metallic silver during photographic development. Non sensitized crystals will be dissolved during fixation, leaving behind the silvergrains (for details and references: see Rogers, 1979).

Lasek et al. (1968) were one of the first to use a tritiated amino acid as a tracer at the light microscopical level. They injected ^{3}H-leucine in a dorsal ganglion of cats and toads. After a survival time (ranging from 1 to 7 days) the animals were perfused and their spinal cord and brainstem were cut in transverse sections. Slides carrying the sections were covered with a photographic emulsion, exposed for several weeks and then developed and fixed. After counterstaining, the sections were coverslipped and viewed in the light microscope. They found silvergrains overlying the dorsal and ventral horns and the dorsal column nuclei in the lower brainstem, indicating the presence of a radioactive source in those areas. They concluded that the injected ^{3}H-leucine had been taken up by the ganglion cells and was subsequently transported to their terminals in the spinal cord and brainstem. The distribution of the silvergrains was identical to the distribution of degenerated elements observed in earlier degeneration experiments (Sprague, 1958).

In 1972 Cowan et al. performed a similar experiment in various central neuronal systems. They made detailed observations on the autoradiographic technique and summarized its advantages over the degeneration technique. They pointed out that 1) ^{3}H-leucine was taken up by cell somata and not by terminals and passing axons 2) only anterograde transport was involved 3) a fast flow, which mainly labelled the terminals and a slow flow, which labelled the entire axon including its terminals could be distinguished 4) in some cases additional projections could be visualized which had not been observed with the degeneration technique and 5) the autoradiographic tracing technique made use of the physiological transport system of the cell and did not depend on pathological changes.

Apart from the advantages, the autoradiographic technique also has certain drawbacks. In the first place it is often difficult to determine the size of the injection site. It has been shown (Swanson, 1981) that after survival times longer than one week, the size of the injection site tends to become increasingly smaller. Furthermore the exposure time also influences the size of the injection site. In the second place, the autoradiographic technique is an indirect technique: the silvergrains give an indication that the underlying structures are radioactively labelled. Sometimes it is difficult to determine from the pattern of the silvergrains whether these structures are axons or terminals or both (e.g. terminals en passage). These problems can be overcome by using other anterograde tracers, which give more structural detail (like phaseolus vulgaris leucoagglutinin or WGA-HRP, see later) or by using retrograde tracers to determine the exact location of the cells of origin of a pathway. A third drawback associated with the autoradiographic technique is the background activity in autoradiograms, i.e. the silvergrains produced by factors other than the radioactive tracer. Background grains may be produced by other (naturally occurring) radioactive sources in the tissue, by cosmic radiation, by heat or mechanically (when applying the emulsion, handling the slides etc.). Therefore the technique must be carried out in such a way that low background levels are obtained, otherwise "light projections" cannot be distinguished from the background activity. However, when these drawbacks have been taken into account, the autoradiographic tracing technique has proven to be a valuable tool for the identification of pathways in the brain.

In the past 15 years the autoradiographic tracing technique has become standard in many laboratories. In due course it was found that, in addition to ^{3}H-leucine, several other tritiated substances could be used for tracing nervous connections. Anterograde transport was obtained with tritiated amino acids like proline, alanine, glycine, lysine, serine and valine and with fucose, a monosaccharide (Repérant et al., 1985). Two of these radioactive tracers (^{3}H-proline and ^{3}H-fucose) have been shown to be transported transneuronally (Grafstein and Laureno, 1973; Repérant et al., 1985), i.e. from the nerve terminal into the postsynaptic neuron. Transneuronal transport may be recognized in a particular experiment since the postsynaptic neuron which has taken up the transneuronally transported radioactive tracer will become radioactive itself. In such a case it must be excluded that retrograde transport from the injection site to this neuron has occurred. In fact retrograde transport of tritiated amino acids has been reported (for a review see Cuénod et al., 1982). These include aspartate, glutamate, glycine, gamma-amino-butyric acid (GABA) and proline. Since all these amino acids are considered as putative neurotransmitters, it has been assumed that

their uptake depends on the presence of high affinity uptake systems, which are located within the terminal membrane of certain neurons and which are specific for a particular transmitter. Indeed other transmitters like dopamine, (nor-) adrenalin, serotonin, choline (a precursor of acetyl-choline) are also selectively taken up by those terminals which secrete them as a transmitter and are then transported back to the soma. The existence of high affinity uptake systems has been demonstrated for biogenic amines, amino acids and choline (see e.g. Hökfelt and Ljungdahl, 1975; Descarries and Beaudet, 1983) and retrograde transport of labelled neurotransmitters has been used to identify transmitter-specific connections.

The techniques for transmitter-specific retrograde transport are essentially based on the presence of a high affinity uptake systems for specific transmitters. Thus when an injection is made with e.g. ^{3}H-glycine in area A and autoradiographically labelled cells are seen in area B, this finding would imply not only that there exists a connection between A and B, but also that glycine is present as a transmitter in this connection. On the other hand, the absence of retrogradely labelled cells does not exclude the presence of a glycinergic connection, but merely signifies that there is no high affinity uptake system for this particular transmitter. After a ^{3}H-GABA injection in the spinal cord no labelling of nearby interneurons (known to be GABA-ergic) was found (Rustioni and Cuénod, 1982), indicating that the terminals from these neurons do not have a high affinity uptake system for GABA. Similar findings were obtained in the GABA-ergic projection from the cerebellar cortex to the vestibular nuclei (Wiklund et al., 1983). Apart from these "false negative" results there may also be false positive results since high affinity uptake systems for different biogenic amines show some "cross reactivity". In addition it has been shown that some of the transmitter-like substances are taken up aselectively, especially when high extracellular concentrations are produced (for references and a discussion on these problems see Cuénod and Streit, 1983). Taken together the various data indicate that the results obtained with the neurotransmitter-specific transport techniques (including the negative results) should be interpreted with care. Nevertheless it represents a promising and simple tool for tracing chemically identified axonal pathways. At this point it should also be mentioned that a high affinity uptake system for ^{3}H-leucine does not seem to exist, since retrograde transport of ^{3}H-leucine has never been demonstrated. Thus for general purposes ^{3}H-leucine is a good and reliable anterograde tracer, without retrograde or transneuronal transport and without uptake by passing axons.

Horseradish peroxidase

The horseradish peroxidase (HRP) tracing technique was introduced in the early seventies (Kristensson and Olsson, 1971; Lavail and Lavail, 1972; for a review see Mesulam, 1982). Usually the HRP is applied to an appropriate region, where it is taken up by neuronal somata and terminals in the injected area and transported by way of the anterograde and/or retrograde axonal flow. HRP may also be applied to intact, cut or crushed nerves or to muscle tissue. In the central nervous system the HRP is passively taken up by the soma or terminal through micro-pinocytosis. Therefore the amount of HRP which will have entered the neuron may be largely dependant on the extracellular concentration of the HRP (Sawchenko and Gerfen, 1985). Axons (of passage) may also take up HRP, but only when they are injured either by the injection or intentionally by a knife cut (Wakefield and Shonnard, 1979; Brodal et al., 1983). After its uptake HRP is transported both in anterograde and retrograde directions. After 2 to 5 days survival time, the animal is perfused and the tissue is cut in frozen sections or in slabs using a Vibratome slicer. The sections or slabs are incubated with a chromogen and hydrogen peroxide. At the site of the HRP, the chromogen is oxidized and precipitates, marking the location of the HRP. Originally diamino benzidine (DAB) was used as a chromogen (Graham and Karnovsky, 1966), but since that time several other substances have been introduced (De Olmos and Heimer, 1977; Hanker et al., 1977). The application of tetramethyl benzidine (TMB) as a chromogen (Mesulam, 1978) has substantially increased the sensitivity of the technique and made it possible to detect connections which could not be demonstrated with DAB (see e.g. Mesulam and Brushart, 1979; Chapter 2). However, the TMB reaction product is rather unstable, and may disappear when the incubated sections have to be further treated e.g. for immunohistochemistry or electron microscopy. Therefore several methods have been proposed to stabilize the TMB reaction product (Adams, 1980; Rye et al., 1984). A further increase in

sensitivity was obtained by coupling HRP to wheat germ agglutinin (WGA) (Gonatas et al., 1979). This increase in sensitivity is probably due to the fact that WGA will specifically bind to compounds like N-acetyl-glucosamide and sialic acid, which are generally found in neuronal membranes (see e.g. Sawchenko and Gerfen, 1985). These membrane constituents may act as a receptor, enhancing the uptake of WGA-HRP. Both retrograde (Trojanowski et al., 1982) and anterograde (Trojanowsky et al., 1981) transport were found to be enhanced when using WGA-HRP instead of free HRP. It was also found that, similar to free WGA (Ruda and Coulter, 1982), the WGA-HRP conjugate was transported transneuronally, both retrogradely (Harrison et al., 1984; Wiesendanger and Wiesendanger, 1985) and anterogradely (Itaya and Van Hoesen, 1982). Transneuronal transport of free HRP has never been shown, but transganglionic transport, which is a transport within one cell, does occur (Mesulam and Brushart, 1979). Transneuronal transfer of WGA-HRP seems to be enhanced towards those second order neurons which have been most active during the survival period (Jankowska, 1985; Alstermark et al., 1987). Thus WGA-HRP combined with TMB as a chromogen is a very sensitive technique for tracing axonal connections. However the possibility of transneuronal transfer always should be taken into account when using WGA-HRP, whereas with free HRP this phenomenon does not seem to occur.

Fluorescent tracers

One of the first retrograde tracers to be used in neuro-anatomy was the fluorescent tracer Evans Blue, combined with bovine albumin (Kristensson, 1970). In the late seventies Kuypers and collaborators demonstrated that Evans Blue by itself is also transported. They discovered also several other fluorescent compounds which were transported retrogradely from the terminals to the cell soma (Kuypers et al., 1977) and some of them were transported anterogradely as well (Rosina, 1982). These fluorescent tracers include diamidinophenyl-indole (DAPI), primulin, granular blue, true blue, fast blue, nuclear yellow and diamidino yellow (Bentivoglio et al., 1979, 1980; Kuypers et al. 1977; 1979; 1980; Keizer et al., 1983; for a review see Kuypers and Huisman, 1984). More recently other fluorescent tracers were found, most notably fluorogold (Schmued and Fallon, 1986) and rhodamine labelled latex beads (Katz et al., 1984). One of the great advantages of the fluorescent tracers is the possibility to combine them in double labelling experiments. The combination of two fluorescent tracers e.g. DAPI/primulin with Evans Blue (Van der Kooy and Kuypers, 1979) or either fast or true blue with diamidino yellow (Keizer et al., 1983) and even three (de Olmos and Heimer, 1980; Bentivoglio and Molinari, 1982) fluorescent tracers makes it possible to identify axon collaterals. Thus one tracer is applied to the terminals of the stem axon and the other tracer to the terminals of the collateral axon. After retrograde transport to the parent cell body the tracers can be identified and distinguished in the cell, using fluorescence microscopy. Double labelling of neurons for the purpose of identifying axon collaterals can also be achieved by combining the retrograde transport of (WGA-)HRP with tritiated enzymatically inactive (WGA-)HRP (Hayes and Rustioni, 1981). Fluorescent tracers have also been used in combination with histofluorescent (Björklund and Skagerberg, 1979) and immuno-fluorescent (Skirboll et al., 1984) techniques for tracing chemically identified pathways. Some fluorescent tracers, which accumulate in the cell body can be used as cell markers during development (O'Leary et al., 1981; Innocenti, 1984). Many of the fluorescent tracers have one or more disadvantages in comparison with (WGA-)HRP. There may be transport over short distances only, leakage out of the labelled cells and fading of the fluorescence in the sections may occur. In addition fluorescent tracers cannot be visualized in the electron microscope, although recently a method for photoconversion of some fluorescent markers to a diamino benzidine product (which can be visualized in the electron microscope) has been described (Sandell and Masland, 1988). Fluorescent tracers are mainly used for double labelling experiments or other purposes (see above) for which they are especially suitable. For single tracing experiments the use of (WGA)-HRP is still to be preferred.

Lectins, bacterial toxins and viruses

Lectins are proteins which can be obtained from plant extracts (for review see Sawchenko and Gerfen, 1985). Best known for tracing purposes are tritium vulgaris agglutinin (from wheat germ) (WGA) and phaseolus vulgaris

leucoagglutinin (from the red kidney bean) (PHA-L). Lectins are known to bind to receptors (glycoproteins and glycolipids) in the neural membrane, which enhance their uptake of lectins. Both WGA and PHA-L can be used for tracing fiber connections, but their characteristics are rather different.

WGA is transported anterogradely and retrogradely (Lechan et al., 1981) and may also be transported transneuronally (Ruda and Coulter, 1982). There is evidence that the uptake and transport of WGA is system- and species-dependant, because some neurons lack the specific receptors for WGA (Schnyder and Künzle, 1983; see also Sawchenko and Gerfen, 1985). WGA can be detected by autoradiography of ^{3}H-WGA (Schwab et al., 1978) or by immuno-cytochemical methods (Ruda and Coulter, 1982) and it can be labelled with gold particles (Menétrey, 1985). The most common method is to use WGA coupled to HRP, which can be detected histochemically (see earlier).

The lectin phaseolus vulgaris leucoagglutinin (PHA-L) was found to possess some unique properties as a tracer (Gerfen and Sawchenko, 1984). When PHA-L is delivered iontophoretically (pressure injection does not yield satisfactory results) it will be taken up by a limited number of cells. These cells become completely stained, including the axon and its terminals, giving a Golgi-like appearance. Thus detailed observations can be made, not only on the terminal arborizations of the labelled axons in the target areas, but also on the presence of axon collaterals may also be identified (Van der Want et al., 1989). Phaseolus vulgaris, which appears to work especially well in rats, remains in the neuron for a long time. Therefore long survival times can be used, which may be needed to trace connections over relatively long distances. Initially it was found that PHA-L is transported only in an anterograde direction, but more recent findings have shown that retrograde transport may also occur (Lee et al., 1988). Uptake by passing fibers and transneuronal transfer did not seem to be present, although in a recent report (Van der Want et al., 1989) it was found that these phenomena did occur to a limited extent. Since PHA-L is detected immuno-histochemically with the PAP method (Ch. I.2.C.), it can also be identified at the ultrastructural level, either by directly visualizing the PAP reaction product (Wouterlood and Groenewegen, 1985) or by substituting the PAP reaction products by a silver deposit (Van der Want, personal communication; Van den Pol et al., 1985).

Bacterial toxins may also be used for anterograde and retrograde tracing. Two toxins (or non-toxic fragments of them) have been succesfully applied: cholera toxin and tetanus toxin. In analogy to lectins, the two toxins bind to specific substances (gangliosides) in the neuronal membrane, enhancing their uptake (see e.g. Stoeckel et al., 1977). Tetanus toxin and cholera toxin are powerful retrograde tracers, which can be identified immuno-histochemically (see e.g. Horikawa and Powell, 1986; Luppi et al., 1987; Fishman and Carrigan, 1987) or autoradiographically, when labelled with 125-I (Schwab et al., 1977, Stoeckel et al., 1977). The retrograde transport of cholera toxin has also been used in combination with transmitter immunocytochemistry of the retrogradely labelled cells (Luppi et al., 1987; 1988). The conjugate of cholera toxin with HRP is also a valuable tracer, both for retrograde and anterograde tracing (Trojanowski et al., 1981; 1982; for review see Trojanowski, 1983). Tetanus toxin injected in a muscle, is transported transneuronally to the terminals contacting the motoneurons (Schwab and Thoenen, 1976), but does not seem to be further transported to the cell bodies of the second order neurons (Fishman and Carrigan, 1987). Until recently cholera and tetanus toxin or their conjugates with HRP were not extensively used for tracing connections in the brain. Therefore further reports have to be awaited in order to precisely determine the characteristics of these tracers.

Finally the use of viruses should be mentioned. So far only a few studies have used viruses for tracing purposes (Kristensson et al., 1982; Ugolini et al., 1987; Ugolini et al., 1989). The main advantage of using a virus (like herpes simplex or rabies) is the fact that it is transported transneuronally and is replicated in the recipient neurons. The amount of virus in the second order neuron will therefore increase during the survival time. This will enhance the possibility of detection by immunocytochemistry, leading to a larger number and a more complete labelling of the second order neurons. This efficacy of the transneuronal labelling is an advantage of viral transport over transneuronal labelling with WGA-HRP, since in the latter case the labelling in the second order neurons is always much weaker.

I.2.C. HISTOCHEMICAL AND IMMUNO-HISTOCHEMICAL TECHNIQUES

The introduction of the histofluorescence Falck-Hillarp technique for demonstrating biogenic monoamines in neuronal cell bodies and terminals (Falck et al., 1962), represented an essential step in the development of "chemical neuroanatomy". This technique, which was based on aldehyde induced fluorescence of different monoamines, enabled the localisation of several catecholamines such as adrenaline, noradrenaline and dopamine as well as indolamines such as serotonin (for review see Björklund, 1983). In their classical studies Dahlström and Fuxe (1964) used this technique for mapping the different monoamine cell bodies and terminals in the brain and some of their projections (Dahlström and Fuxe, 1965). The histofluorescence technique gradually became replaced by the more sensitive and more specific immuno-histochemical technique (Coons, 1958). This technique, was introduced in neuroanatomy in 1969 (Geffen et al., 1969). It is based on the identification of specific compounds (antigens) by means of labelled antibodies. Any compound in the tissue may act as an antigens be it neurotransmitter or its synthesizing enzyme, a specific proteins or a lipid. Foreign substances such as tracers may also act as antigens and can also be identified by appropriate antibodies (Ch. I.2.B.). Antibodies can be obtained in two ways (a) by using "polyclonal antisera" from animals injected with the purified antigen and (b) by using a "clone" derived from one individual lymphoid cell which produces only one type of antibody, i.e. a monoclonal antibody (Köhler and Milstein, 1975). In contrast to the monoclonal antibodies, polyclonal antisera usually contain several different antibodies, directed against different parts of the antigen. In order to locate an antigen, indirect immuno-histochemical labelling techniques are now commonly used, involving the application of a second antibody directed against the primary one (for review see Cuello, 1983; Larsson, 1983 and Polak and Van Noorden, 1986). The second antibody can be labelled with various markers: fluorescent compounds, enzymes (e.g. HRP, visualized with DAB as a chromogen) and ferritin or gold particles of different sizes. The labelling with HRP ferritin or gold particles carries the advantage that the labels can also be visualized in the electron microscope (for review see Polak and Varndell, 1984). A further modification of the above techniques was provided by the peroxidase-anti-peroxidase (PAP) method (Sternberger et al., 1970). When the PAP complex, which contains three HRP molecules is applied to the section it is firmly bound by the second antibody which thereby becomes strongly labelled (for review see Sternberger, 1979). In light microscopy the identification of transmitters in neurons by means of the PAP method has been succesfully combined with retrograde HRP labelling of the same neurons (Bowker et al., 1983). A more recently developed indirect immuno-histochemical method is the avidin-biotin technique, which is based on the high affinity of biotin (a vitamin) for avidin (an egg-white protein) or streptavidin (a bacterial avidin). The technique usually involves the application of a second antibody coupled to biotin. In the next step avidin coupled to a specific marker (HRP, a fluorescent substance or gold particles) is applied. This specifically binds to the biotin, thereby labelling the second antibody. Instead of avidin coupled to a marker, a (strept)avidin-biotin complex, which includes several HRP molecules, may also be used (the ABC method) (Hsu et al., 1981). The avidin-biotin and PAP methods are so far, the most sensitive ones and yield very little background staining. They can also be used for electron microscopic immuno-cytochemistry (Bonnard et al., 1982; Priestley and Cuello, 1983). A completely different technique for demonstrating monoamines and other putative transmitters is based on high affinity uptake of tritiated transmitters or their precursors, which can subsequently be visualized by light and electron microscopical autoradiography (for review see Descarries and Beaudet, 1983). However, this method can only be used with transmitters for which there exists a high affinity uptake system; peptides therefore can not be identified with this method.

I.3.

ANTEROGRADE TRACING AT THE ULTRASTRUCTURAL LEVEL

I.3.A. INTRODUCTION

It is the aim of anterograde tracing studies to unravel the intricate afferent connections (input) of the various neuronal cell groups in the brain. However, for understanding the functional importance of the different fiber system it is not sufficient to determine from which area(s) of the brain afferent input is conveyed to a particular neuronal cell group. It is of equal importance to determine which transmitters are used in a particular system and whether their postsynaptic action is excitatory or inhibitory. For this purpose electro-physiological techniques are indespensable. However they generally measure the overall effect of a specific input to a neuron or neuronal cell group, but cannot always decide whether the effects are monosynaptic or relayed through interneurons. Anterograde tracing techiques at the ultrastructural level are meant to fill this gap by studying the local characteristics of the afferent systems i.e. the strategic location of their terminals on the neuronal membrane. It can be determined whether synaptic contacts are established with a cell soma (axo-somatic), with a dendrite (axo-dendritic) or with another terminal (axo-axonic). The size and shape of the terminals and their synaptic vesicles as well as the proximity to other synapses may be important parameters all of which determine the functional properties of a particular afferent connection. This chapter only deals with three anterograde tracing techniques which are presently most widely used in electron microscopy: the degeneration technique, the autoradiographic technique and the horseradish peroxidase (HRP) tracing technique.

I.3.B. THE ANTEROGRADE DEGENERATION TECHNIQUE

The anterograde degeneration technique is based on the fact that a lesion of the soma or the axon of a particular neuron will result in pathological changes (degeneration) in that part of the axon (including its terminals), located distally to a lesion. A degenerating axon or terminal should be identified in the electron microscope before it has been engulfed by glia or has disappeared by phagocytosis. Thus the phenomenon of anterograde degeneration can be used for anterograde tracing of fibers and terminals. Three types of early degenerative changes in terminals have been described: the electron-dense type, showing darkening of mitochondria and axoplasm (Colonnier, 1964), the hypertrophic type, showing hypertrophy of the terminal filaments (Gray and Hamlyn, 1962) and the electron-lucent type, characterized by swollen terminals containing only aggregations of vesicles close to the synaptic junction (Gentchev and Sotelo, 1973). In all three types of early degeneration the type and shape of the synaptic vesicles and that of the synaptic junction may still be identified, allowing for morphological characterization of the terminal.

The anterograde degeneration technique has some major drawbacks. In the first place it is often difficult to lesion a particular cell group or fiber bundle without interrupting fibers passing through the area. These passing fibers may also terminate in the area to be studied, which may lead to confusion or misinterpretation of the origin of the degenerated terminals. Secondly, the process of degeneration is asynchronous. This makes

it impossible to identify, at one particular moment, all the degenerating terminals of a lesioned system as degenerating. Thus after a certain survival time some terminals show no signs of degeneration while others are already in an advanced stage of degeneration and cannot be characterized anymore or have already disappeared. Therefore only a limited number of degenerating terminals can be identified as such. Thus the survival time must be carefully chosen in order to obtain the maximum number of degenerating terminals. As a result it is often difficult to obtain a sufficient number of degenerating terminals in which certain features of their "normal" morphology can still be recognized (see e.g. Conradi, 1969d). The finding that some terminals may show spontaneous degeneration (Sotelo and Palay, 1971; Rustioni and Sotelo, 1974) is an additional complicating factor in this technique.

I.3.C. The Autoradiographic Tracing Technique

For tracing axonal connections in the central nervous system the electron microscopic autoradiography technique was introduced by Hendrickson (Hendrickson, 1969). In most studies ^{3}H-leucine is used as a tracer (see e.g. Dekker and Kuypers, 1975; 1976) sometimes in combination with ^{3}H-proline. The tracer is injected into the appropriate brain area using a syringe or a glass micropipette. Usually a survival time of 1 to 7 days is used. Longer survival times have also been used especially for tracing over long distances (Holstege, G., 1982). After fixation, the tissue is routinely processed for electron microscopy. Ultrathin sections are cut from the plastic blocks and placed on formvar or collodion coated slides and contrasted with uranyl acetate and lead citate. A carbon layer is evaporated on the slides, which are then dipped in a liquid photographic emulsion (Vrensen, 1970). Other mehods for emulsion application have been used (e.g. the loop method). Detailed descriptions of the various techniques can be found elsewhere (Rogers, 1979). The sections are exposed in the dark at 4 °C for 2 months up to more than a year, depending on the intensity of the labelling. After photographic developing and fixation, the autoradiograms can be examined in the electron microscope.

I.3.D. The Analysis of EM Autoradiograms of Brain Tissue

The purpose of anterograde tracing at the EM level is the characterization of specific synaptic terminals. Morphological characterization of terminals is generally based on one or more of the following criteria (see also Ch. I.4.C.): the size and shape of the terminals, the number and type of their synaptic vesicles, the size and shape of their synaptic junctions, the kind of postsynaptic structure: a soma, a dendrite (distal or proximal, spinous or non-spinous) or a terminal. The relation to other terminals or glial elements may be an additional criterion. At the electron microscopic level the neuropil is composed of various profiles of neuronal cell bodies, myelinated and unmyelinated axons, terminals, dendrites, glial elements and bloodvessels. Several of these profiles can be easily recognized: e.g. terminals on account of their vesicle content or axons on account of their myelin etc. However many other profiles, especially if they are small, contain only few structural markers. Therefore even experienced neurocytologists can denominate only a small percentage of these profiles (cf. Peters et al., 1976). This denomination problem and the phenomenon of cross fire (see below) seriously complicate a proper analysis of EM-autoradiograms of brain tissue. Additional complicating factors may arise from the presence of background grains (silvergrains which are produced by factors other than the radioactive tracer), from the simultaneous labelling of axons and terminals of the afferent systems and (depending on the tracer) from occassional transsynaptic labelling. The problems associated with autoradiographic cross fire and the denomination problem will be described next.

Autoradiographic cross fire

The interpretation of EM autoradiograms poses several problems, which may be explained as follows. It is a fundamental aspect of radioactivity that radiation is emitted in every direction with equal probability. Even for the low energetic radioisotopes used in EM autoradiography, the range of the emitted radiation appreciably exceeds the dimensions of cellular or subcellular structures. This means that there is a fair chance that silver grains in the EM autoradiograms are located

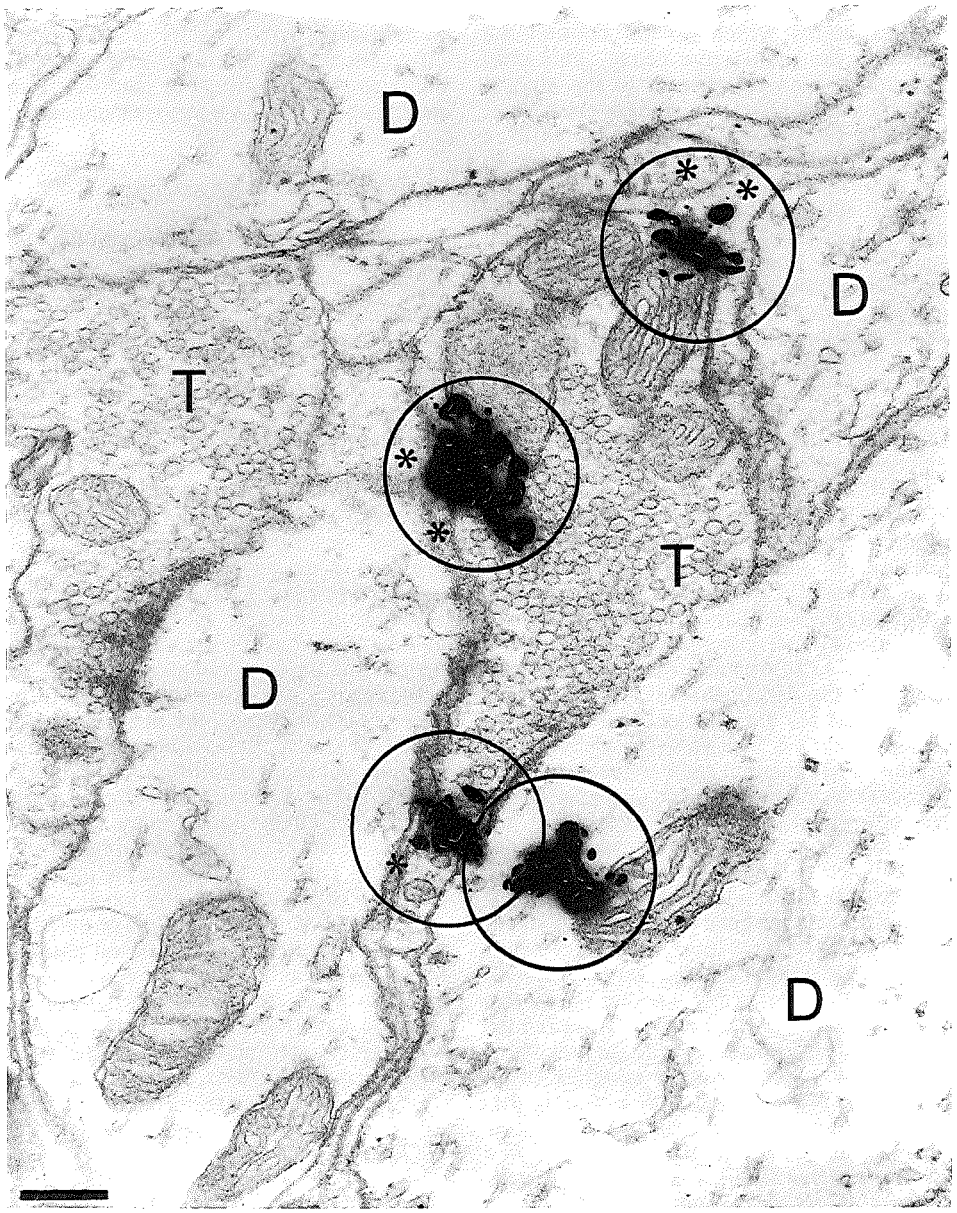


Fig. 1. Electron microscopical autoradiograph of rat lumbar motoneuronal cell groups after a ^{3}H-leucine injection in the raphe pallidus obscurus. Four silver grains are present over the neuropil. Terminal (T) and dendritic (D) profiles can be easily recognized. The circle drawn around each of the silver grains represents a 50% probability circle. All profiles located within the circle are potential radioactive sources. The profiles which cannot be reliably denominated are indicated by an asteriks. For details see text. 5 months exposure time; bar = 0.2 μm.

over structures which do not contain the source of the radioactive decay. This phenomenon, which is inherent to the autoradiography technique, is called cross fire. Williams (1969) was the first to offer a solution for this fundamental problem and in later years other approaches have been described by Blackett and Parry (1973) and Downs and Williams (1978). More recently Friedman et al., (1986), Miller et al., (1985) and Markov (1986) have extended these methods of analysis. Basic in all these approaches is their use of a probabilistic estimate of cross fire. This estimate indicates the distance away from the source, within which e.g. 50% of the silvergrains are formed in the emulsion. This 50% probability distance (half distance: HD) has been empirically estimated by Salpeter et al., (1969) using hot lines. HD values between 80-145 nm have been determined for tritium, a commonly used isotope. With these experimental data it could be calculated that in an actual EM autoradiograph there is a probability of 50% that the radioactive source giving rise to a silvergrain is located within a distance of 135-245 nm around the center of the grain: the 50% probability circle. This implies that all the (sub)cellular structures located within the 50% probability circle must be viewed as potential radioactive sources. It follows from the probabilistic character of this approach that an individual silvergrain has only a limited meaning, since it can not always be attributed to one individual structure. In order to obtain reliable information from the autoradiograms we have to carry out a statistical analysis using a large number of silver grains (the real grain distribution). In addition it has to be demonstrated whether or not this real grain distribution is random. Therefore the real grain distribution over the various items i.e. individual, compound or complex structures has to be compared with a random distribution of hypothetical grains over the same items (for details see Williams, 1977). In fact this random distribution is to some extent comparable to the volume density of the various items within the tissue or cell. The currently used methods of analysis differ mainly in the way they compare the real grain distribution with the random (or hypothetical) grain distribution. Thus the physical process of radioactive decay makes it necessary to use a statistical approach in order to obtain reliable information from the EM autoradiograms. As a consequence the outcome of an EM autoradiographic study can only be presented in terms of probability. It is therefore impossible to determine with certainty whether one specific cellular or subcellular structure actually contains radioactivity. For neuro-anatomical tracing studies this is a serious handicap.

The problem of denomination

Whatever the differences between the various methods of analysis, they have one obvious element in common: the potential sources of radioactivity must be denominated i.e. they must be cellular or subcellular components that can be distinguished on account of their fine structural characteristics. For autoradiography studies investigating the subcellular distribution of radioactivity that is seldom a serious problem: endoplasmic reticulum, ribosomes, Golgi-fields, etc. are distinct and clearly identifiable structures. However, the denomination of fine structural objects raises a serious problem in the study of the neuropil. When considering e.g. the silver grains overlying the neuropil in Fig. 1, the problem becomes evident. Some of the grains can be attributed unequivocally to a synaptic terminal which subsequently can be characterized. In many other cases, however, the probable origin of the silver grains can be denominated only as a profile without additional characteristics regarding its axonal, dendritic or terminal origin. This means that many silver grains cannot be ascribed to a meaningful cellular structure. Thus in addition to the intrinsic uncertainty of autoradiography localization, the analysis of EM-autoradiograms of the neuropil is handicapped by the lack or paucity of meaningful characteristics in several structures. In other words autoradiograms of the neuropil have two superimposing sources of uncertainty: autoradiographic cross fire and denomination problems.

The cluster analysis

One way out of this problem of superimposed uncertainties is to reduce one of them to a neglectable level. As illustrated in Fig. 2, the problems due to autoradiographic cross fire can be drastically reduced by taking into account only those structures which carry a large number of silvergrains (e.g. a cluster of at least four silvergrains). The probability that the structure under consideration (in this case a terminal) is the source of radioactivity may be estimated as at least 90% (the larger the number of silvergrains in the cluster, the higher the

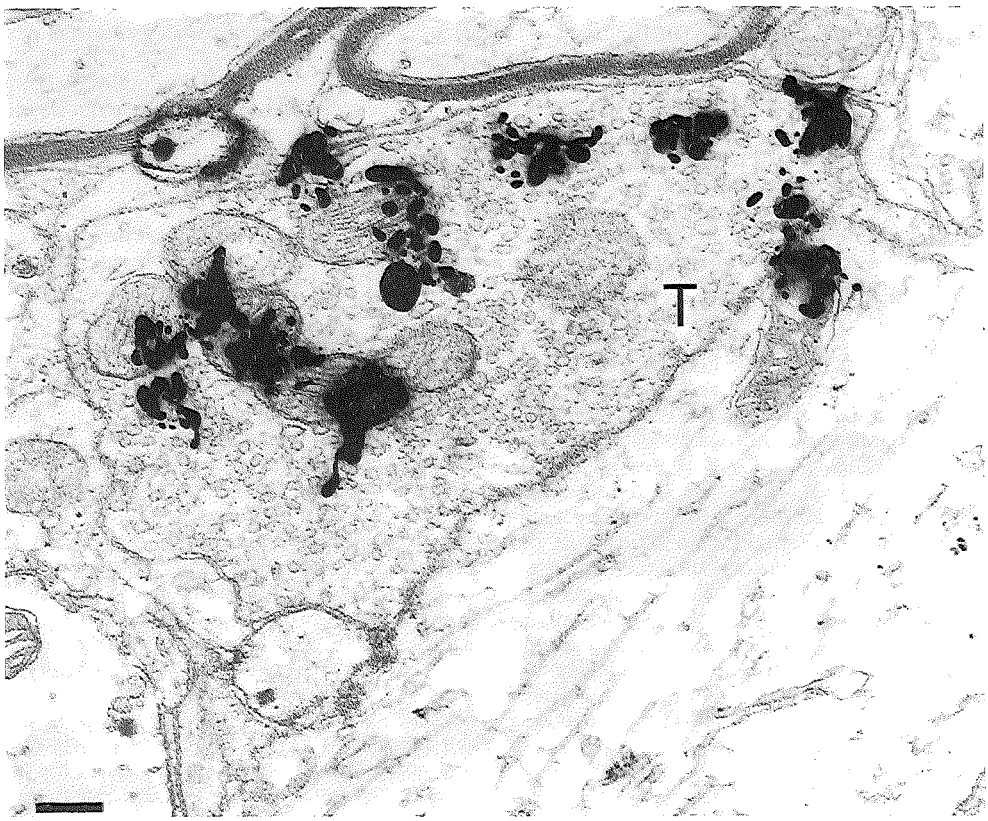


Fig. 2. Electron microscopical autoradiograph of rat lumbar motoneuronal cell groups (after a ^{3}H-leucine injection in the medial reticular formation of the lower brainstem), showing a cluster consisting of at least eleven silver grains which is centered on a terminal (T). There is a very high probability that this cluster-labelled terminal actually contains radioactivity. In such cases an analysis using a 50% probability circle may be omitted. For details see text, 6 months exposure time; bar = 0,2 μm.

probability). In such a case the cluster-labelled structure may be considered as the actual source of radioactivity. The morphoogical details of the cluster-labelled terminals can be carefully analysed according to the criteria outlined in the introduction (I.3.A). In this way the problem of autoradiographic cross fire and the associated statistical analysis can be largely circumvented. Thus we can meet with the purpose of anterograde labelling of terminals, as described before. The "cluster approach" relies on the presence of heavily labelled structures. In order to achieve this aim longer exposure times are generally needed. In addition a large amount of the radioactive tracer may be injected and a somewhat longer survival time can be used. The cluster method has some disadvantages: structures which do not contain a relatively large amount of radioactivity may not carry a cluster of silver grains and will not be detected. In addition the numerous silvergrains in a cluster may mask important fine structural details. Another point of concern when using the cluster analysis may arise from the occurrence of α-tracks originating from uranyl acetate staining. However, as outlined by Williams (1977), α-tracks are infrequent in electron microscopic autoradiographs. In addition they characteristically appear as straight tracks of silver grains, outrising the diameter of most profiles in the neuropil. Therefore α-tracks are easily distinguished from grain clusters.

I.3.E. The Horseradish Peroxidase Tracing Technique

Since the beginning of the seventies, HRP has been used as an anterograde tracer, both at the light (Lynch et al., 1972; 1973) and the electron (Winfield et al., 1975) microscopical level (for a review see Mesulam, 1982). The introduction of WGA-HRP and the use of tetramethyl benzidine (TMB) as a chromogen significantly increased the use of (WGA-)HRP for anterograde tracing. For electron microscopical studies DAB was routinely used as a chromogen, because the DAB reaction product is very stable under various conditions. However, the efficacy of DAB is low in comparison with that of ^{3}H-leucine in combination with the EM autoradiographic tracing technique and about equal to the efficacy of the anterograde degeneration technique (Holstege, J.C. and Dekker, 1979). The introduction of the sensitive TMB as a chromogen (Mesulam, 1978) greatly increased the efficacy of the anterograde HRP technique. Basically the technique used in light microscopy for detecting HRP with the TMB chromogen can also be applied for electron microscopy (Carson and Mesulam, 1982; see also Ch II.2). However, the amount of TMB reaction product that could still be identified in the electron microscope was found to be very low or even absent. It appeared that most of the TMB reaction product (the oxidized TMB) dissolved when the tissue was routinely processed for electron microscopy, especially during the dehydration and osmification steps. Several solutions to this problem have been proposed: speeding up the dehydration step (Stürmer et al., 1981), using chemical dehydration with dimethoxypropane (see also Ch. II.2.), changing the osmification procedure by using a lower pH, a higher temperature and a shorter time (Sakumoto et al., 1980; Carson and Mesulam, 1982) or stabilizing the TMB reaction product with DAB-cobalt (Lemann et al., 1985). These precautions result in a better preservation of the TMB reaction product during processing for electron microscopy, but some loss has to be accepted. In the electron microscope the TMB reaction product has an electron-dense crystalline appearance, which can be easily recognized. The crystals are usually confined within one cellular strucure, but occasionally part of the crystal may pierce through the membrane. TMB crystals are generally large and may even completely obscure the labelled structure. Therefore this technique cannot be used for labelling subcellular structures. On the other hand the TMB crystals are almost certainly located in the cellular elements containing the (WGA-)HRP and not in neighbouring structures. Thus the problem of cross fire encountered in the autoradiography technique does not apply to the HRP technique. Moreover "background" crystals are virtually absent. This implies that the analysis of the labelling is relatively easy and complicated statistical procedures are not needed (for details see Ch. II.2.). For tracing connections in the brain, the use of WGA-HRP instead of free HRP has the advantage of a greater sensitivity, while processing for EM is identical for both tracers. However WGA-HRP may be transported transneuronally, whereas free HRP is not (for details see Ch. I.2.B). This should be taken into account when analysing the results.

I.4.

THE ULTRASTRUCTURE OF THE SPINAL MOTONEURON POOL

I.4.A. INTRODUCTION

The vertebrate spinal cord is generally subdivided in segments, i.e the parts of the spinal cord where the fibers from one dorsal root enter the cord. Each segment receives sensory information mainly by way of the dorsal roots, while axons from motoneurons and autonomic preganglionic neurons leave the spinal cord by way of the ventral roots. The motoneuronal axons innervate the skeletal muscles of the body, with the exception of the muscles of the face and part of the neck. The neurons of the spinal cord are located in the grey matter, which occupies a central position and is surrounded by white matter, containing ascending and descending axons. The spinal white matter can be subdivided in different funiculi, named after their location in the spinal cord, i.e. the dorsal, lateral and ventral funiculi. The spinal grey matter has the shape of an 'H' with the central canal in the center. It can be subdivided in the dorsal and the ventral horns. Groups of motoneurons occupy the ventro-medial and the lateral parts of the ventral horn; they are referred to as the medial and lateral motoneuronal cell groups respectively. The area between the central nucleus of the dorsal horn (the nucleus proprius) and the area of the motoneuronal cell groups is known as the intermediate zone. Based on the cytoarchitecture of the spinal grey of the cat a more detailed subdivision of the spinal grey in different laminae was proposed by Rexed (1952; 1954). In terms of Rexed's laminae the different parts of the dorsal horn correspond to laminae I to VI. Laminae V and VI can be subdivided in medial and lateral parts, especially in the cervical and lumbar segments (Rexed, 1954). In these areas the medial parts of these laminae are often considered as part of the dorsal horn, while the lateral parts are considered part of the intermediate zone (c.f. Kuypers, 1981), together with laminae VII and VIII. Lamina IX comprises the motoneuronal cell groups and lamina X corresponds to the area surrounding the central canal. The same laminar division can be applied to the spinal cord of other mammals like the rat (McClung and Castro, 1978; Molander et al. 1984), the monkey (Noback and Harting, 1971) and the human (Truex and Taylor, 1968; Schoenen, 1973). The dorsal horn mainly acts as a processing center and relay station for sensory information, which enters the cord by way of the dorsal root fibers. The intermediate zone contains a heterogeneous mixture of cells with various sizes and shapes which makes it difficult to distinguish the various laminae and nuclei. The large majority of the neurons located in the intermediate zone are interneurons which distribute their fibers within the spinal cord. Their axons ascend or descend within the white matter of the funiculi close to the spinal grey which they re-enter in order to terminate on the target neurons in the intermediate zone and in the motoneuronal cell groups (Molenaar and Kuypers, 1978). Some interneurons also project to supraspinal levels, especially the lower brainstem, the thalamus and the cerebellum (see e.g. Verburgh and Kuypers., 1987). The input of interneurons is derived from dorsal root fibers, descending supraspinal fibers and from other interneurons.

Lamina IX contains the motoneurons, which can be easily recognized by their large size. They constitute the final common pathway of the motor system, since they innervate the skeletal muscles of body and limbs. In the following paragraphs a detailed description will be given of the ultrastructural morphology of motoneurons, including the relevant data from light microscopical studies. Subsequently the different types of terminals, which are present in the spinal motoneuron pool will be described, including data on the location of their parent cell bodies and their putative transmitters.

I.4.B. THE MOTONEURONS IN THE SPINAL CORD

Since the appearance of the electron microscope in the field of neuro-anatomy (Hess and Young, 1952; Palade and Palay, 1954; Palay, 1956; Wyckoff and Young, 1955; Luse, 1956) the ultrastructural morphology of motoneurons and the surrounding neuropil in brain stem and spinal cord have been a focus of attention for electron microscopists (Palay, 1956; Rosenbluth, 1962; Charlton and Gray, 1966; Uchizono, 1966; Bodian, 1966; Ralston, 1967; Wuerker and Palay, 1969; Conradi, 1969a,b,c,d; Poritsky, 1969; McLaughlin, 1972a; Bernstein and Bernstein, 1976; Goshgarian and Rafols, 1984; Atsumi and Ohsato, 1984; Pullen, 1988b). From these studies a detailed picture of the ultrastructural morphology of the motoneuronal cell groups has emerged. A description of these data becomes meaningful if combined with data from light microscopical studies. In this respect studies using intracellular injections of motoneurons are most relevant since they yield very detailed morphological data. Until the introduction of the intracellular staining technique (Stretton, 1968), the various modifications of the Golgi impregnation technique (see Ch I.1) had been the main tool for studying the morphology of motoneurons (see e.g. Aitken and Bridger, 1961; Gelfan et al., 1970). The intracellular staining technique has the advantage that it reveals many morphological details of individual motoneurons and at the same time it offers the possibility to study the electrophysiology of the cell before it is injected. This makes it possible to correlate the function of a neuron with its anatomy. For the intracellular injection of motoneurons several substances were used such as Procion Yellow (Barrett and Crill, 1974), ^{3}H-glycine (Lux et al., 1970) and HRP (Cullheim and Kellerth, 1976; Jankowska et al., 1976; Snow et al., 1976). Of these intracellular markers, HRP proved most successful mainly because it allowed for a more detailed study of the dendritic tree. Therefore, in all of the more recent studies of the morphology of individual motoneurons, HRP has been used for intracellular injection. HRP has the additional advantage that it can be visualized in the electron microscope. This makes it possible to correlate the morphology of a labelled structure at the light microscopical level with its morphology at the electron microscopical level. This is especially relevant since detailed information on the general morphology of a specific motoneuron including its dendritic tree is difficult to obtain with the electron microscope, unless elaborate serial section analysis is performed.

Motoneurons can be subdivided in two basic types: α–motoneurons, innervating extrafusal muscle fibers and γ–motoneurons innervating intrafusal fibers (i.e striated fibers located within the muscle spindles). The γ–motoneurons are relatively small and play an essential role in the regulation of muscle tone and musle length (for a review see Henneman and Mendell, 1981). They lie intermingled with the α–motoneurons in the motoneuronal cell groups of the ventral horn (Strick et al., 1976). In addition there are a limited number of ß-motoneurons which innervate both intra- and extrafusal fibers of some muscles (Emonet-Denard et al., 1975). The motoneurons are organized in two large aggregates located ventro-medially and laterally in the ventral horn (Sprague, 1948); they are commonly referred to as the medial and lateral motoneuronal cellgroups respectively. Motoneurons in the medial motoneuronal cell groups innervate axial muscles through the dorsal ramus of the spinal nerves (Sprague, 1948; Brink et al., 1979; Richmond et al., 1978), whereas motoneurons in the lateral motoneuronal cell groups innervate the skeletal muscles of the body wall and of the extremities through the ventral ramus of the spinal nerves as shown in the rat (Goering, 1928), cat (Romanes, 1951; Sterling and Kuypers, 1967) and monkey (Sprague, 1948). In the thoracic cord the medial and lateral motoneuronal cellgroups fuse into a single group in the ventral horn. The motoneurons are somatotopically organized in longitudinal columns which innervate particular muscles. This columnar arrangement is especially clear in the lateral motoneuronal cell groups of the cervical and lumbar enlargements. The length of these columns ranges from one to three segments (Romanes, 1951; Nicolopoulos-Stournaras and Iles, 1983) and their organization appeared to be such that motoneurons which supply distal muscles are located dorsolaterally to motoneurons supplying more proximally located muscles.

The motoneuron soma

The findings obtained in earlier Golgi studies in respect to the soma size of α–motoneurons were basicly similar to the more recent

findings obtained in intra-cellular studies. α–Motoneurons range in size from about 40 to 75 μm, with an average of approximately 55 μm (Strick et al., 1976; Burke et al., 1977; Ulfake and Cullheim, 1981). The soma size of a motoneuron is closely related to the type of muscle it innervates, such that motoneurons innervating slow twitch muscle fibers tend to be smaller than those innervating fast twitch muscle fibers (Kernell and Zwaagstra, 1981; Burke et al., 1982; Ulfake and Kellerth, 1982). Smaller motoneurons are generally more easily recruited than the larger ones, a phenomenon known as the size principle (Henneman et al., 1965; for details see Henneman and Mendell, 1981). These differences cannot be attributed to size alone, since the electrical membrane properties may also differ for motoneurons of different size (Kernell, 1986). α–Motoneurons are much smaller with a size range of 20 to 45 μm and an average of approximately 30 μm (Burke et al., 1977; Strick et al., 1976; Ulfake and Cullheim, 1981). Therefore the size of motoneurons is often used to discriminate between α– and γ–motoneurons.

The somata of motoneurons are characterized by the presence in the cytoplasm of several large stacks of flattened cysternae made up of granular endoplasmatic reticulum. These structures were already observed in Nissl stained material, hence their name: Nissl bodies. For the rest the motoneuron cytoplasm contains much the same organelles as other neurons in the central nervous system such as a large nucleus often with a nucleolus, a Golgi apparatus, smooth endoplasmatic reticulum, lysosomes, clusters of ribosomes, mitochondria, microtubules and neurofilaments. The last four organelles can also be found in dendrites and, with the exception of ribosomes, in axons (Wuerker and Palay, 1969; Peters et al., 1976).

The axon

The axons of α–motoneurons were extensively studied by Cullheim and Kellerth (1978) after intracellular injections of HRP. They found that axons usually originate from the cell soma, although in a few cases they arise from a stem dendrite. The axons radiate away from the soma and travel through the gray matter before they enter the white matter to reach the ventral root. The area of the axon between its origin at the soma (or stem dendrite) and the beginning of the myelin sheath is subdivided in two parts. The proximal part is called the axon hillock and the relatively smaller distal part is called the initial segment. The axon hillock has a broad base and becomes smaller distally. It contains many neurofilaments and tubuli, while lacking granular endoplasmatic reticulum. It is covered with terminals and glial elements. The initial segment, on the other hand, is relatively straight and narrow (3-4μm) with a mean length of 26 μm (Cullheim and Kellerth, 1978). It contains many tubuli and is not contacted by terminals at all. The cell membrane of the initial segment characteristically shows an undercoating of electron-dense material. The initial segment is the area with the lowest threshold for initiating an axon potential: the trigger zone of the motoneuron (Coombs et al., 1957). Just distally to the beginning of the myelin sheath the diameter slowly widens to its normal size (± 6μm). Most (but not all) axons give off one or more collaterals within the gray matter and occasionally in the white matter as well. The collaterals emerge from the axon at a node of Ranvier, the area between the termination of one segment of the myelin sheath and the beginning of the next one. Motor-axon collaterals are usually myelinated and ramify into several branches. Most of these branches terminate on Renshaw cells in the intermediate zone, but approximately 20% of them (Burke, 1981) terminate in the same motoneuronal cell groups or even the on same motoneuron from which they originate (Cullheim et al., 1977).

The dendritic tree

It was already observed in Golgi studies (Aitken and Bridger, 1961; Romanes, 1964; Sprague, 1964; Scheibel and Scheibel, 1966) and confirmed in intracellular studies (Cullheim and Kellerth, 1976; Brown, 1981; Rose, 1981; Ulfake and Kellerth, 1981; Kernell and Zwaagstra, 1981; Zwaagstra and Kernell, 1981; Burke et al., 1982; Egger and Egger, 1982; Ulfake, 1984; Vanner and Rose, 1984; Rose et al., 1985; Cullheim et al., 1987) that the dendritic trees of motoneurons were not confined to the area of the motoneuronal cell groups in lamina IX and extended widely over the ventral horn; in cat up to 1,5 mm from the soma. Some motoneurons distributed dendrites even to lamina VI and V, while other motoneurons distributed their dendrites into the white matter surrounding the ventral horn (see also Romanes, 1964). Some of these white matter dendrites even reached the outer

edge of the spinal cord. This situation is more common in lower vertebrates like reptiles (Ruigrok et al., 1985) and amphibia (van Mier et al., 1985). Motoneurons located ventromedially in the ventral horn may distribute their dendrites into the contralateral white and grey matter, through the anterior commissure (Light and Metz, 1978; Abrahams and Keane, 1984; c.f. Cajal, 1909). In a study of motoneurons innervating neck muscles it was established that the (ipsilateral) white matter dendrites were contacted by terminals in areas which also contained myelinated and unmyelinated axons, thus resembling grey matter neuropil (Rose and Richmond, 1981; personal observations). It is still unclear whether these white matter dendrites receive a specific synaptic input.

The dendrites of a motoneuron radiate in all directions from the soma (Cullheim et al., 1987). There may be a predominant distribution of the dendritic tree of a motoneuron in a particular part of the spinal cord. It was noted from Golgi studies (Scheibel and Scheibel, 1966; Sterling and Kuypers, 1967) that in the brachial cord the orientation of the dendritic trees of motoneurons was predominantly longitudinal, although in more recent intracellular studies (Light and Metz, 1978; Rose, 1981) motoneurons were observed with dendritic trees that had a predominant orientation in other planes. As a whole it seems likely that the orientation of the dendritic tree of a motoneuron is closely related to the location of its soma in the ventral horn and similar to the orientation of the dendrites of neighbouring motoneurons, innervating the same or functionally similar muscles (Rose, 1981). With respect to the number of dendrites which take their origin from the motoneuron soma (stem dendrites), their number varies from 8 to 22 stem dendrites (Barrett and Crill, 1974). On the average most intracellular studies (for references: see above) report an average of 10-12 stem dendrites per motoneuron. These studies also showed that the dendrites of motoneurons are smooth, with only an occasional spine. These observations are in line with electron microscopical observations that the occurrence of spines, both on the soma and on dendrites, is rare (personal observations). Additional quantitative data on the dendritic tree of motoneurons are given in table 1.

TABLE 1

SOME CHARACTERISTICS OF α-MOTONEURONAL DENDRITES

Number of dendrites	-	11 (average)
Spines	-	occasionally
Radius of dendritic tree	-	1400 μm
Last order branches (total)	-	120
Total dendritic length	-	80.000-120.000 μm
Total dendritic surface area	-	400.000-600.000 μm^2
Total surface area of soma	-	2 - 4% of total dendritictic surface area.

I.4.C. THE TERMINALS ON SPINAL MOTONEURONS

In the early studies (see above) on spinal motoneurons with the aid of the electron microscope it was noted that various terminal swellings contacted somata and dendrites in the motoneuronal cell groups. In the classical studies of Conradi (1969a,b,c,d) an extensive description was given of the motoneuronal cell groups in the cat.spinal cord. In one of these studies (Conradi, 1969a) a subdivision of the terminals in the neuropil was proposed, which was largely based on an earlier study of Bodian in the monkey (Bodian, 1966). The different types of terminals were distinguished on the following criteria: the size of the terminal, the morphology of the synaptic vesicles within the terminal (see later), the morphology of the synaptic complex (see later) and the postsynaptic element. The morphology of the vesicles has become a most important criterion for distinguishing different types of terminals. In aldehyde- and osmium-fixed material, which is routinely processed for electron microscopy there are two main types of vesicles which can be distinguished: granular vesicles, containing an electron-dense core, and agranular vesicles, the content of which appears clear in the electron microscope. Terminals containing many granular vesicles are relatively rare in the motoneuronal cell groups. The large majority is formed by terminals containing agranular vesicles, although in these terminals an occasional granular vesicle may be present. Furthermore it was observed that the shape of the agranular vesicles could be spherical or flattened. Bodian (1966) has shown that this difference in shape of the agranular vesicles is largely due to the osmo-

larity of the fixation and rinsing fluid prior to postfixation with osmium tetroxide. Since it seems likely that in vivo all vesicles are spherical, the difference of vesicle shape should be regarded as an artefact. However this artefact is very reproducable in all parts of the brain and may very well be due to a structural difference between the two types of synaptic vesicles. In any case it has become a widely used criterion for the distinction of different types of terminals. In addition the occurence of terminals with either many flattened or merely spherical vesicles may also be important from a functional point of view. Uchizono (1965) has shown that in the cerebellum terminals presumed to be excitatory contain mainly spherical vesicles, while terminals presumed to be inhibitory contained many flattened vesicles. In other parts of the brain (including the motoneuronal cell groups, see later) a similar relation was found between the morphology of the vesicles within a terminal and its presumed function. Reliable evidence as to whether a terminal is excitatory, inhibitory, modulatory or otherwise in function can only be obtained by identification of the transmitter(s) present within the terminal and the receptors on the postsynaptic neurons, combined with physiological and pharmacological studies on the system to which the terminals belong.

Another important criterion for distinguishing different types of terminals is the morphology of the synaptic complex i.e. the pre- and postsynaptic membranes and the synaptic cleft. Gray (1959) noticed that in the cerebral cortex, fixed with osmium tetroxide (OsO_4) and stained with phospho-tungstic acid the synaptic complexes of terminals contacting dendrites was different from the synaptic complexes of terminals contacting cell somata. On this basis he distinguished two types of synaptic complexes: a type I complex, characterized by a thick postsynaptic density and a broad (30 nm) synaptic cleft with an intermediate dense line, and a type II complex, characterized by a thinner postsynaptic density and a relatively narrow (20 nm) synaptic cleft without an intermediate dense line. Collonier (1968) reexamined the synaptic structure of the cat visual cortex, which was aldehyde-fixed, postfixed with OsO_4 and counterstained with uranyl acetate and lead citrate. He found that in his aldehyde-fixed material a synaptic complex could not always be easily classified following the criteria of Gray, especially with respect to the width of the synaptic cleft and the dense line associated with it. Therefore Colonnier suggested to distinguish synaptic complexes merely on the basis of the thickness of the postsynaptic membrane. He referred to the synaptic complexes with a thick postsynaptic density as asymmetric and to those with a thin postsynaptic density (almost equal to the presynaptic membrane) as symmetric. This classification is basically similar to that of Gray (type I is asymmetric, type II is symmetric), but is much less rigorous, and therefore more easy to apply. In the present study the terminology of Colonnier will be used. At this point it seems of importance to note that asymmetric synaptic complexes have been associated with an excitatory function in the cerebellar cortex (Landis and Reese, 1974) and the olfactory bulb (Landis et al., 1974). Furthermore, neurochemical evidence (Carlin et al., 1980) has shown that postsynaptic densities of asymmetric synaptosomes (isolated synaptic complexes) contain different proteins than the postsynaptic densities of symmetric synaptosomes. Proteins present in the postsynaptic densities of asymmetric synaptosomes could be associated with mediating excitatory effects and visa versa (see also Cohen et al., 1982). These findings and the fact that terminals with spherical vesicles often show an asymmetric synaptic complex further strengthens the hypothesis that terminals with merely spherical vesicles and asymmetric synaptic complexes are excitatory, while terminals with many flattened vesicles, which often show a symmetric synaptic complex, are inhibitory in function. This hypothesis does not hold true in every case, e.g. terminals from Golgi cells in the cerebellum are known to be glycinergic and/or GABA-ergic, but the vesicles in these terminals were not clearly flattened and their synapses were not clearly symmetric (Otterson et al., 1987). It may be concluded that there is a high probability that terminals with spherical vesicles and asymmetric synapses are excitatory, while terminals with flattened vesicles and symmetric synapses are inhibitory in function. The ultrastructural morphology of a specific terminal may therefore give an indication of its functional properties. This may be helpful, especially when no other data are available.

In the motoneuronal cell groups 6 different types of terminals were distinguished, using the various criteria of Conradi and Bodian mentioned earlier. These terminals include S-type terminals (containing mainly spherical vesicles), F-type terminals (containing many flattened vesicles), G-type terminals (containing granular vesicles), C-type terminals (with a subsynaptic cistern), M-type terminals

(containing multiple synapses), P-type terminals (presynaptic to other terminals) and T-type terminals (with subsynaptic dense bodies, also known as Taxi bodies).

S(pherical)-type terminals contain merely spherical agranular vesicles, although an occasional granular vesicle may be present. S-type terminals usually established asymmetrical synaptic contacts. F(lattened)-type terminals, on the other hand, contain a large number of flattened vesicles, besides a few, mostly small, spherical vesicles and an occasional dense core vesicle. They usually establish symmetrical synaptic contacts. Both S- and F-type terminals contact cell somata, proximal and distal dendrites. However in comparison with the S-type terminals, a larger number of F-type terminals is present on the cell soma and the primary dendrites, while on the medium-sized and distal dendrites the situation is the reverse. For the purpose of identifying the origin of the type(s) of terminal(s) belonging to propriospinal or descending supraspinal fibers, degeneration studies were performed after spinal cord transection in cat (McLaughlin, 1972b) and monkey (Bodian, 1975). It was found that some terminals of the S- and F-types degenerated in segments below a spinal cord transection. After unilateral motor cortex ablation in monkey (Bodian, 1975) only S-type terminals were found to degenerate. Injections of ^{3}H-leucine in the somato-sensory cortex (Ralston and Ralston, 1985) also resulted in radioactive labelling of S-type terminals in the motoneuronal cell groups, but in this case a few F-type terminals were also labelled. S- and F-type terminals are also derived from the caudal raphe nuclei, the adjoining ventro-lateral part of the medial reticular formation and the area of the locus coeruleus and subcoeruleus. These projections are extensively described in Ch. II. In monkey, injections of WGA-HRP in the red nucleus resulted in labelling of S-type terminals in the lateral motoneuronal cell groups (Ralston et al., 1988). Terminals from recurrent axon collaterals of HRP-injected motoneurons, which contacted other α-motoneurons, all contained a large number of spherical vesicles (Lagerbäck et al., 1981). Therefore it seems likely that some of the S-type terminals on motoneuronal dendrites belong to motor-axon collaterals from other motoneurons in the same area and that they mediate direct synaptic interaction between spinal α-motoneurons.

Taken together, the various data indicate that the S- and F-type terminals in the motoneuronal cell groups constitute a mixed population originating from the spinal cord and from supraspinal levels. This population would include terminals from the motor cortex (in the monkey), several brainstem nuclei, propriospinal neurons, dorsal root afferents (see later) and recurrent axon collaterals from α-motoneurons.

With respect to the transmitters in the terminals derived from the various systems mentioned above, no direct ultrastructural evidence is available at present. From physiological and pharmacological studies (for review see Krnjevic, 1981) it seems likely that the following transmitters are present in the S- and F-type terminals of the various systems. The S-type terminals derived from the motor cortex probably use glutamate or perhaps aspartate as a transmitter. The transmitters in the F- and S-type terminals derived from the brainstem are still unknown (but see Ch. III). S- and F-type terminals derived from interneurons may contain either glutamate and/or aspartate or glycine and/or GABA respectively. S- (or M-) type terminals derived from dorsal root afferents probably contain glutamate, while S-type terminals belonging to motor axon collaterals almost certainly contain acetyl-choline. Furthermore, immuno-cytochemical (McLaughlin et al., 1975; Van den Pol and Gorcs, 1988) and transmitter uptake studies (Matus and Dennison, 1971; Ljungdahl and Hökfelt, 1973) at the ultrastructural level have shown the presence of GABA and glycine mainly in F-type terminals. In view of these data the hypothesis that terminals with mainly spherical vesicles and asymmetric synaptic complexes (i.e. S-type terminals) are excitatory and that terminals with many flattened vesicles and symmetric synaptic complexes (i.e. F-type terminals) are inhibitory also seems to hold for the terminals in the motoneuronal cell groups.

G(ranular)-type terminals are characterized by their content of a large number of dense cored (granular) vesicles. In addition they contain clear vesicles of various forms and sizes. They represent only a minority of the total number of terminals in the motoneuronal cell groups (approximately 1% according to Bernstein and Bernstein, 1976 and Conradi, 1969a) and establish asymmetrical synaptic contacts mostly with proximal and distal dendrites and occasionally with a cell soma. These terminals are in all likelihood derived from the lower brainstem (for details see Ch.

II). Several transmitters have been shown to be present in G-type terminals. These include serotonin, substance P, thyrotropin releasing hormone and GABA (for details see Ch.III).

C(istern)-type terminals contain flattened and/or spherical vesicles and are characterized by the presence of a subsynaptic cistern in the postsynaptic structure which is either a proximal dendrite or a cell soma. In the monkey, C-type terminals were designated as L bulbs (Bodian, 1966). A pre- or postsynaptic density is not clearly visible in osmium-fixed material, which makes it hard to define the location of the synapse. However, studies using staining with ethanolic phosphotungstic acid (a substance which specifically stains synaptic membrane specializations) revealed the existence of presynaptic dense projections and a thin postsynaptic density along the entire length of the apposition (Schröder, 1979; Pullen, 1988a). This indicates that C-type terminals establish conventional synapses which, however, exhibit a unique ultrastuctural morphology. With respect to the origin of the C-type terminals, it was found that C-type terminals never showed any signs of degeneration after spinal cord transection in cat (McLaughlin, 1972b) and monkey (Bodian, 1975). It was therefore concluded that the C-type terminal belonged to short inter- or intrasegmental propriospinal fibers. In a degeneration study in the cat cervical spinal cord (Matsushita and Ikeda, 1973) a few degenerating C-type terminals were observed in segments immediately below and above a transection. These findings may indicate that C-type terminals originate from (short) propriospinal neurons and from the lower brainstem (see Ch.II). Furthermore it has been shown in the cat (Pullen and Sears, 1978; 1983) that in the area between two hemisections of the cord there is an absolute increase in the number of C-type terminals. Under these circumstances the size of the C-type terminals also increased. This increase involved the postsynaptic cistern and the postsynaptic Nissl body, which showed a marked hypertrophy. These phenomena may indicate a specific trophic function of the C-type terminal. From a functional point of view, it may be interesting to note that C-type terminals are absent on γ-motoneurons (Lagerback, 1985), for which, so far there has been no explanation. Finally it should be mentioned that up to now there is no clue which transmitter(s) may be present in C-type terminals.

M(ultiple synapse)-type terminals are large terminals with a large number of spherical vesicles and multiple asymmetric synaptic junctions, characteristically asssociated with subsynaptic dense bodies (Taxi bodies) in the postsynaptic structure. M-type terminals are often postsynaptic to a P(resynaptic)-type terminal: a small terminal containing several slightly flattened vesicles. In the monkey the M-type terminal was designated as R bulb (Bodian, 1966), whereas in rat motoneuronal cell groups the M- and P-type terminals were not observed (Bernstein and Bernstein, 1976), although the P-type does exist in the rat (McLaughlin et al., 1975; personal observations). The M-type terminal is considered to be of dorsal root origin since transection of the dorsal root resulted in a reduction in the number of M-type terminals, mainly on primary dendrites (Conradi, 1969d; McLaughlin, 1972c). However, in these degeneration studies degenerated terminals could not be observed. In a similar study in monkey (Bodian, 1975) a few large degenerated terminals of the S-type were noticed. They were postsynaptic to a P-type terminal. Similarly, after injection of ^{3}H-leucine in the lumbar and sacral dorsal ganglia of the monkey (Ralston and Ralston, 1979) the radioactivity was also located in large S-type terminals some of which were also postsynaptic to small unlabelled terminals (P bulbs). However in this latter case the labelled S-type terminals contacted small to medium sized dendrites, which is in contrast with the degeneration results of Conradi (1969d) and McLaughlin (1972c). The terminals of I-a afferent fibers on dendrites in the motoneuronal cell groups were also identified after intra-axonal injection of HRP in physiologically identified I-a afferent fibers (Conradi, 1983). With this technique it was also found that large S-type terminals were labelled and that several of them were postsynaptic to small P-type terminals. Thus in the autoradiographic and the intra-axonal HRP studies of dorsal root afferents, the M-type of terminal was never found to be labelled. Therefore it seems likely that the terminals on motoneurons, which are of dorsal root origin are represented by large S-type terminals (which themselves are postsynaptic to small P-type terminals) and not by the M-type terminal. Whether the M-type terminal should be regarded as a seperate type or as a large S-type terminal with subsynaptic densities and whether it represents a specific type of dorsal root afferent (e.g. type II) is at present unclear. If the M- and large S-type terminals are indeed of dorsal root origin, it seems most likely that they contain glutamate as a trans-

mitter, also because glutamate has recently been shown to be present in dorsal root fibers in the dorsal horn (De Biasi and Rustioni, 1988). The P-type terminal is the only axo-axonic terminal in the motoneuronal cell groups. It almost certainly contains GABA as a transmitter as indicated by physiological, pharmacological and immuno-cytochemical studies (Eccles, 1954; McLaughlin et al., 1975; Holstege, J.C.et al., 1987).

T(axi)-type terminals are characterized by the presence of subsynaptic dense bodies, also known as Taxi bodies (Taxi, 1961), located directly underneath the postsynaptic membrane. T-type terminals contain spherical vesicles and establish asymmetric synaptic contacts. They were labelled after intracellular injections of α-motoneurons, indicating that they originate from motor-axon collaterals (Lagerbäck et al., 1981). Since the same collateral fiber also gave rise to S-type terminals, which in fact are morphologically similar to the T-type terminal (except for the Taxi bodies), it seems likely T-type terminals are a "modification" of the S-type terminal and do not necessarily originate from other sources than the S-type terminals.

A summary of the origin of the different types of terminals and their (presumed) transmitter(s) is given in table 2.

TABLE 2.

ORIGIN AND TRANSMITTERS OF THE VARIOUS TYPES OF TERMINALS IN THE LATERAL SPINAL MOTONEURONAL CELL GROUPS

ORIGIN	TYPE OF TERMINAL	TRANSMITTER(S)
- (motor)cortex	S-type	glutamate and/or aspartate
	F-type	GABA??
- red nucleus	S-type	?
- vestibular nuclei		
MVST	F-type ??	GABA ?? glycine ??
LVST	S-type??	aspartate ?? acetyl-choline ??
- (sub)coeruleus	E-type	nor-adrenalin ??
	S-type	?
- raphe pallidus, obscurus and adjoining ventro-medial reticular formation	G-type	serotonin, substance P, TRH
	F-type	GABA ; glycine ??
	S-type	acetyl-choline ??
	C-type	?
- propriospinal	S-type ?	aspartate ?? acetyl-choline ??
	F-type ?	GABA ? glycine ?
	P-type ?	GABA
	C-type ?	?
- dorsal root	large S-type	glutamate ? aspartate ??
- motor axon collateral	S-type	acetyl-choline

I.5.

A SHORT SURVEY OF THE DESCENDING PROJECTIONS FROM THE BRAINSTEM TO THE SPINAL CORD

I.5.A. INTRODUCTION

According to Kuypers (1982; 1985) the pathways descending to the spinal cord may be divided into three groups. The first group comprises the cortico-spinal fibers which terminate in the dorsal horn (Cheema et al., 1984), the intermediate zone and -especially in higher primates- also in the somatic motoneuronal cell groups (Armand et al., 1985; for review see Kuypers, 1987). The second group comprises the descending pathways to the spinal cord which originate in the brainstem. These fibers, which largely parallel the corticospinal fibers in their projections to the intermediate zone, may be subdivided into a medial and a lateral system (Kuypers, 1981); a division, which is based on their terminal distribution in the intermediate zone and which also corresponds to their localization in the spinal white matter. The third group comprises the descending brainstem pathways which originate from neurons in the area of the nucleus coeruleus and subcoeruleus and from those in the medullary raphe nuclei and adjoining ventro-medial reticular formation. These pathways project in a diffuse non-focussed manner to all laminae of the spinal cord, including the motoneuronal cell groups. In the short survey below the various descending brain stem pathways will be briefly described, except for those belonging to the third group (i.e. the descending projections from the raphe nuclei and the adjoining ventro-lateral part of the medial reticular formation and those from the locus coeruleus and subcoeruleus), since they will be extensively dealt with in the following chapters.

I.5.B. THE MEDIAL SYSTEM OF THE DESCENDING BRAINSTEM PATHWAYS

The medial system of descending brainstem pathways is derived from several areas in the brainstem: the medial reticular formation of the mesencephalon, pons and medulla, the superior colliculus, the nucleus interstitialis of Cajal, the vestibular complex and the cerebellar nuclei (for details see below). A few of the fibers from the above areas terminate directly in medial motoneuronal cell groups. However, the bulk of the fibers terminate in the medial part of the spinal intermediate zone, where many long propriospinal neurons are located. These neurons in turn distribute their fibers throughout the length of the spinal cord to terminate in the medial part of the intermediate zone and in the medial motoneuronal cell groups (Molenaar, 1978; Molenaar and Kuypers, 1978; Matsushita et al., 1979).

The mesencephalic reticular formation

The medial part of the mesencephalic reticular formation, including the lateral part of the central gray, sends fibers to the spinal cord as shown in degeneration and axonal transport studies (Waldron and Gwyn, 1969; Kuypers and Maisky, 1975; Castiglioni et al., 1978; Crutcher et al., 1978; Martin et al., 1979). Along their trajectory some of these fibers are distributed to several nuclei in the lower brain stem (Kuypers, 1981). In the spinal cord the fibers are located in the ventral funiculus and terminate bilaterally in lamina VII and VIII

of the spinal cord, mainly at cervical levels; a few fibers however may reach lumbar levels as well (Kuypers, 1981). In the area of the nucleus tegmenti pedunculo-pontinus in the mesencephalic tegmentum the mesencephalic locomotor center is situated (Shik et al., 1966; Garcia-Rill, 1986). This center is defined not so much anatomically but rather functionally by its capacity to induce locomotion upon electrical stimulation. Recent studies (Rye et al., 1988; Goldsmith and Van der Kooy, 1988; Spann and Grofova, 1989) have shown projections from the area of the nucleus tegmenti pedunculo pontinus to the ventro-medial medulla in the lower brainstem and to the spinal cord, in addition to its ascending projections (Saper and Loewy, 1982; Woolf and Butcher, 1986; Rye et al., 1987). It appeared that the two descending projections originated from separate neuronal populations, since the projections to the medulla carry acetyl-choline as a transmitter (Garcia-Rill and Skinner, 1987), while those to the spinal cord do not (Goldsmith and Van der Kooy, 1988). The termination site in the ventro-lateral medulla appeared especially important, since application of acetyl-choline to neurons in the ventro-lateral medulla resulted in locomotor effects which were similar to those obtained from the mesencephalic locomotor center, whereas application of acetyl-choline antagonists blocked the locomotor effects, obtained after electrical stimulation in the mesencephalic locomotor center (Garcia-Rill and Skinner, 1987). No such data are available regarding the function of the fibers descending from the nucleus tegmenti pedunculo pontinus to the spinal cord.

The superior colliculus

Neurons in the intermediate and deep layers of the superior colliculus give rise to the crossed tectospinal tract, which does not descend beyond the cervical spinal segments (Huerta and Harting, 1982). The tectospinal tract terminates contralaterally mainly in lamina VI and VII with some endings in lamina VIII (Petras, 1967; Martin, 1969). There is also evidence for a few direct projections to motoneurons innervating neck muscles (Peterson et al, 1979; Huerta and Harting, 1982). These fibers are involved mainly in movements of the head and neck especially in relation to eye movements.

The nucleus interstitialis of Cajal.

This nucleus was given its name because of its location in between the fibers of the medial longitudinal fasciculus (MLF) in the rostral part of the mesencephalon. The fibers from this nucleus descend through the brainstem by way of the ipsilateral MLF. In the spinal cord the fibers are located in the ventral funiculus and are distributed to lamina VII and VIII throughout the spinal cord (Nyberg-Hansen, 1966b; Carpenter et al., 1970; Kuypers and Maisky, 1975). Electrophysiological data (Fukushima et al., 1979) showed that at upper cervical levels the interstitio-spinal fibers also terminate on the medially located motoneurons innervating neck muscles.

The vestibular nuclei.

The vestibular complex is located dorsolaterally in the brainstem. It consists of four subnuclei: the superior, the medial, the lateral and the descending vestibular nucleus (Brodal and Pompeiano, 1957). In addition, several small nuclei have been identified. Except for the superior vestibular nucleus, all vestibular subnuclei give rise to descending projections to the spinal cord (Kuypers and Maisky 1975, Crutcher et al., 1978, Zemlan et al., 1979). The lateral vestibular nucleus of Deiters gives rise to the lateral vestibulo-spinal tract (LVST). It descends entirely ipsilaterally in the periphery of the ventral funiculus. From lower cervical levels downward, the LVST gradually shifts to the medial part of the ventral funiculus. During their course in the funiculus most axons give off collaterals, which leave the stem-axon in the funiculus at right angles (Shinoda et al., 1986). It has also been demonstrated electro-physiologically (Abzug et al., 1974) and anatomically (Huisman et al., 1984) that in the lateral vestibular nucleus at least 50% of the neurons distribute axon collaterals to both the cervical and lumbosacral segments. The LVST terminates ipsilaterally in lamina VIII, in the ventro-medial part of lamina VII and possibly in the lateral motoneuronal cell groups (Shinoda et al., 1986). The actions of the LVST in the spinal cord appeared to be excitatory (Lund and Pompeiano, 1968).

The fibers, which form the medial vestibulo-spinal tract (MVST) originate from neurons in the medial vestibular nucleus and,,

to a lesser extent, in the descending and lateral vestibular nuclei (for review see Wilson and Peterson, 1981). The MVST fibers run in the contralateral as well as the ipsilateral MLF. The fibers terminate medially in lamina VIII and part of lamina VII, mostly at cervical levels. There are also monosynaptic connections with medially located motoneurons, innervating neck and back muscles. These connections are mostly inhibitory in action (Wilson et al., 1970).

The pontine and medullary medial reticular formation

Neurons in the medial part of the pontine tegmentum send their fibers ipsilaterally and to a limited extent also contralaterally to the spinal cord. These fibers run in the ventral funiculi and terminate mainly in the medial and central parts of the intermediate zone (lamina VII and especially lamina VIII) throughout the spinal cord (Nyberg-Hansen, 1965; Petras, 1967; Holstege, G. and Kuypers, 1982; Jones and Yang, 1985). A similar projection is obtained from the dorsal part of the medial tegmental field in the medulla (Basbaum et al., 1978; Holstege, G. and Kuypers, 1982). However, the bulk of the spinal projections from the medial reticular formation in the medulla is derived from its ventral part and from the midline raphe nuclei. Neurons in these areas send a massive projection to all laminae of the spinal cord. These projections are described in more detail in Ch. II and III.

The cerebellar nuclei

The fastigial nucleus and the adjoining interpositus nucleus send a projection to the spinal cord (Matsushita and Hosoya, 1978; for a review see Bentivoglio, 1982). Many of these spinal projecting fibers send collaterals to the brainstem and the thalamus (Bharos et al., 1981; Bentivoglio and Kuypers, 1982). The majority of the fibers from the cerebellar nuclei project to the cervical cord, but some fibers may reach lumbar levels (Achenbach and Goodman, 1968; Ware and Mufson, 1979; Kuypers, 1981). They terminate mainly in the intermediate zone (Ware and Mufson, 1979). In monkey some of the cerebello-spinal fibers terminate on spinal motoneurons (Batton et al., 1977).

I.5.C. The Lateral System of the Descending Brainstem Pathways

According to Kuypers (1981) the descending brain stem pathways which terminate mainly in the dorso-lateral part of the intermediate zone, are considered as part of the lateral system of descending brain stem pathways. The majority of the brainstem fibers which terminate in this area, are derived from the contralateral red nucleus (Kuypers, 1964; Nyberg-Hansen and Brodal, 1964; Petras, 1967; Kuypers and Maisky, 1975; 1977). In addition, some of the fibers terminating in this area originate in the mesencephalic reticular formation, adjacent to the contralateral red nucleus, and in the ventro-lateral part of the pontine tegmentum on the contralateral side (Basbaum and Fields, 1979; Holstege, G. and Kuypers, 1982). The bulk of the fibers from the red nucleus are derived from its caudal magnocellular part (see e.g. Kneisly et al., 1978). The rubrospinal tract is located contralaterally in the dorso-lateral funiculus and distributes its fibers to the lateral part of lamina V and VI and also to the dorsal part of lamina VII (Kuypers, 1964; Nyberg-Hansen and Brodal, 1964; Petras, 1967; for a review see Kuypers, 1981). In this area many of the "interneurons" are located with short propriospinal connections to nearby interneurons and motoneurons. In cat and monkey, a few of the rubro-spinal fibers terminate in motoneuronal cell groups innervating distal extremity muscles (Holstege, G. and Tan, 1988; Holstege, G. et al., 1988; Shapovalov et al., 1971). The rubro-spinal projections display a somatotopic organization, such that the medial and dorsal parts of the red nucleus project mainly to the cervical cord, while the ventral and ventro-lateral parts project mainly to the lumbar cord. This relatively focussed organization is further examplified by the finding that very few rubro-spinal neurons send collaterals to both the cervical and the thoracic and/or lumbar spinal cord (Huisman et al., 1982), although they do exhibit collaterals within a limited number of segments (Shinoda, et al., 1977). The fibers from the ventro-lateral pontine tegmentum and the mesencephalon generally show a similar course and termination pattern as those from the nucleus ruber, except for the fact that these fibers are also distributed to the superficial layer of

the dorsal horn (Holstege, G. and Kuypers, 1982) and that they distribute collaterals to a relatively more extensive portion of the spinal cord than the rubrospinal fibers (Huisman et al., 1982).

I.5.D. Functional Aspects

Important clues regarding the function of a particular system may be obtained from its anatomical organization. Thus the fact that many fibers of the medial system send axon collaterals to the cervical, thoracic and/or lumbar spinal cord (Wilson and Peterson, 1981; Huisman et al., 1984) indicates that the medial system (in contrast to the lateral system) is organized in a relatively diffuse non-focussed manner. This organization seems especially well suited for the coordination of whole body movements and synergistic limb movements (for details see Kuypers, 1981). Observations in freely moving monkeys with pyramidal lesions and a superimposed lesion of the medial system confirmed this idea (Lawrence and Kuypers, 1968). These animals showed, besides their pyramidal symptoms, great difficulties with steering axial and proximal limb movements, whereas their capacity to execute independant distal extremity movements was hardly impaired. On the other hand, lesions of the lateral system in monkeys with pyramidal lesions caused little impairment of whole body movements like walking and climbing, but greatly affected the execution of independant distal extremity movements such as taking morsels of food by means of independant finger movements (Lawrence and Kuypers, 1968). This is in line with anatomical findings (see above) that the descending pathways of the lateral system display a somatotopic organization and give off collaterals only to a restricted number of spinal segments.

The various propriospinal neurons, which receive input from the medial and lateral descending brainstem pathways respectively, are also contacted by corticospinal fibers. These fibers were found to originate from different areas in the precentral and adjoining frontal areas and terminated in different parts of the spinal cord and in different parts of the intermediate zone (for a review see Kuypers, 1987). Thus the area of the motor cortex representing the hand projects contralaterally to the dorsal and lateral parts of the intermediate zone at cervical and high thoracic segments, while the foot representation area projects to similar parts of the intermediate zone at low thoracic and lumbar segments and the first sacral segment (Armand et al., 1985). This projection largely coincides with the projections of the lateral system of descending brainstem pathways, notably the rubro-spinal tract. Other parts of the precentral gyrus project bilaterally to the medial part of the intermediate zone throughout the spinal cord (see Kuypers, 1987). This projection largely coincides with that of the medial system of the descending brainstem pathways. Thus the corticospinal system appears to be superimposed on the brainstem spinal projections. This arrangement and the fact that cortical fibers also contact several of the brainstem neurons which give rise to descending projections (Kuypers, 1981), provides the cortex with the possibility to control and adjust the influence exerted by the brainstem on the spinal cord. Furthermore, in monkey there also exist direct cortico-spinal projections to motoneurons innervating muscles of the hand and foot (Kuypers, 1964). In higher primates like chimpanzee and man, direct connections are also established with motoneurons innervating axial and proximal extremity muscles (Kuypers, 1964; Schoen, 1964). These direct connections with motoneurons provide the cortex with an extra means by which it can exert direct control over the execution of specific movements, including highly fractionated movements (for review see Kuypers, 1981).

CHAPTER II.

BRAINSTEM PROJECTIONS TO LUMBAR MOTONEURONS IN RAT

II.1

AN ULTRASTRUCTURAL STUDY USING AUTORADIOGRAPHY AND THE COMBINATION OF AUTORADIOGRAPHY AND HORSERADISH PEROXIDASE HISTOCHEMISTRY.

II.1.A. INTRODUCTION

Anterograde degeneration studies, conducted since the first two decades of this century (Rasdolsky, 1923; Szentagothai-Shimert, 1941; Kuypers et al. 1962; Nyberg-Hansen, 1965; Petras, 1967; Martin et al. 1975), failed to demonstrate the existence of brainstem projections to spinal motoneurons. Such projections were first demonstrated by means of histofluorescent techniques (Dahlström and Fuxe, 1965; Nygren and Olson, 1977) and by means of electrophysiological techniques (Grillner and Lund, 1968; Lund and Pompeiano, 1968; Shapovalov, 1975; Peterson et al., 1979). More recent light microscopy studies (LM) showed these projections also in rat (Jones and Yang, 1985; Holstege, J.C. and Kuypers, 1980; Martin et al., 1985), opossum (Martin et al., 1979) and cat (Holstege G. et al., 1979; Holstege, G. and Kuypers, 1982) by means of the anterograde transport of radioactivity after injection of labelled amino acids (Lasek et al., 1968; Cowan et al., 1972). After injections of ^{3}H-leucine in several brainstem areas, the transported radioactivity was present in the motoneuronal cell groups of the ventral horn throughout the spinal cord. In an earlier electron microscopical (EM) autoradiographic study (Holstege, J.C. and Kuypers, 1982) it was shown that in rat a major part of the radioactivity in the motoneuronal cell groups of the ventral horn was located in terminals making synaptic contacts with neurons in this area. In the present electron microscopical autoradiographic study the distribution of the radioactivity in the lumbar motoneuronal cell groups after ^{3}H-leucine injections in the various brainstem areas was studied in more detail. The types of labelled terminals and their postsynaptic structures were determined. In addition an attempt was made to ascertain at the ultrastructural level that the neurons in the ventral horn which were contacted by radioactively labelled terminals after ^{3}H-leucine injections in the brainstem actually represented motoneurons. For this purpose in one and the same animal terminals of the brainstem fibers were labelled anterogradely by means of ^{3}H-leucine and lumbar motoneurons were labelled retrogradely with horseradish peroxidase (HRP).

II.1.B. EXPERIMENTAL PROCEDURES

Autoradiography

Thirteen adult rats were used. Eleven rats received brainstem injections of L-(4-5) ^{3}H-leucine (S.A. ±100 Ci/mmol) in distilled water. In two rats the brainstem injections were combined with HRP injections in the ipsilateral hindleg muscles. All brainstem injections were made with a glass micropipette and the muscle injections were made with a 10 µL Hamilton microsyringe and a 26 G needle. All rats were operated under pentobarbital anaesthesia. The ^{3}H-leucine injections in the eleven rats were made in three different brainstem areas. Three rats each received two injections of ±20 µCi ^{3}H-leucine in 0.2 µL in the ventro-lateral medial reticular formation (vlMRF) 1.2 mm off the midline between the facial and hypoglossal nuclei. Four other rats received two injections of ±20 µCi ^{3}H-leucine in 0,2 µL distilled water in the raphe nuclei and the adjoining MRF between the facial and hypoglossal nuclei. The remaining four rats each received four injections of ±15 µCi ^{3}H-leucine in 0.15 µL distilled water in the area of the nucleus coeruleus and subcoeruleus. The animals were perfused 12 days after the injections. They were deeply anaesthetized with pentobarbital and perfused transcardially with 0.9% NaCl in cacodylate buffer (pH 7.3) at 37°C, followed by a cacodylate buffered fixation solution (3%

glutaraldehyde and 2,5% paraformaldehyde, pH 7,3) at room temperature.

The spinal cords of the animals were kept in the fixation fluid overnight. The L5 and L6 segments were embedded in plastic as described earlier (Holstege, J.C. and Kuypers, 1982). Semithin sections (2 μm) which were cut from the plastic blocks and processed for light microscopical (LM) autoradiography (Holstege, J.C. and Kuypers, 1982) together with the brainstem injection sites using a 1-month exposure time. Those blocks, the semithin sections of which showed the largest number of silvergrains over the motoneuronal cell groups, were selected for EM autoradiography. The blocks were trimmed to pyramids containing the entire L5 or L6 lateral motoneuronal cell groups. From these pyramids both semithin and pale gold ultrathin sections were cut. The semithin sections were processed for LM autoradiography (Holstege, J.C. and Kuypers, 1982). The ultrathin sections were placed on formvar or collodion-coated slides and stained with aqueous uranyl acetate followed by lead citrate. A thin layer of carbon was evaporated on these slides which were then coated with Ilford L4 emulsion by dipping using a mechanical device (Kopriwa, 1967). For optimal results a slightly overlapping monolayer of silver bromide crystals should be used, which is characterized by a purple-blue interference colour. Test slides were dipped, their interference colour was determined and the thickness of the emulsion layer was checked directly in the electron microscope. If necessary, the dilution of the emulsion was adjusted. The slides carrying the ultrathin sections were kept in the dark at 4 °C for 4 to 9,5 months (see results) and were then developed with freshly made D19 and fixed with 28% sodium thiosulphate. The formvar or collodion films carrying the sections were floated off the slides on distilled water and 200-mesh grids were placed on the sections. The grids carrying the ultrathin EM autoradiographs were recovered and then viewed with a Philips 300 electron microscope.

In the ultrathin autoradiographs the lateral motoneuronal cell groups were identified after first studying the semithin autoradiographs of the same pyramid by LM. A series of drawings of the grids carrying the ultrathin autoradiographs were made and the outlines of the ventral horn and the lateral motoneuronal cell groups were indicated on these drawings (see also Holstege, J.C. and Kuypers, 1982).

In the various EM autoradiographic sections the background activity was estimated in the four grid holes which contained a corner of the section. This was done by counting the silver grains which were present on the resin free of tissue or the collodion or formvar film next to the four corners of the section. An average background activity of 13 grains per 10.000 μm^2, with a maximum of 21 grains per 10.000 μm^2 was found, which is comparable to findings in other studies (Schonbach et al. 1971; Ruda and Gobel, 1980; Ralston and Ralston, 1985).

Autoradiography in combination with HRP histochemistry

Two rats each received 2 injections of ±20 μCi ^{3}H-leucine in 0,2 μL distilled water in the vlMRF, 1.2 mm off the midline between the levels of the facial and hypoglossal nuclei. Nine days later 200 μL of 20% HRP (Miles) in 2% dimethylsulphoxide (DMSO) (Keefer, 1978) was injected ipsilaterally in the gastrocnemius and soleus muscles. After three days the animals were deeply anaesthetized with pentobarbital and perfused transcardially with 0.9% NaCl in cacodylate buffer (pH 7.3) at 37°C, followed by a cacodylate buffered fixation fluid (3.5% glutaraldehyde and 1% paraformaldehyde, pH 7.3) at room temperature. After perfusion, the brainstem injection sites were prepared for LM autoradiography (Holstege, J.C. and Kuypers, 1982). Immediately after dissection the L4-L6 spinal segments were cut transversely in slabs (70 μm) on a vibratome. The slabs were treated for HRP histochemistry using 3,3',5,5'-tetramethyl benzidine (TMB) as a chromogen (Mesulam, 1978) but plain acetate buffer (0.01 M, pH 3,3) was used as a stabilizer. Those slabs, which showed the largest number of retrogradely labelled motoneurons were trimmed down to the ventral horn. They were kept in phosphate buffer (pH 7.2, 20°C) and then transferred to phosphate buffer (pH 7.3, 20°C) containing 8% glucose (±650 mosmol). The slabs were postfixed with 2% osmium tetroxide in phosphate buffer (pH 6.0, 45°C) (Carson and Mesulam, 1982; Sakumoto et al., 1980) for 45 minutes and then thoroughly rinsed in distilled water. They were dehydrated by passing them through two changes (8 minutes each) of acidified di-methoxypropane (Muller and Jacks, 1975) and embedded in Araldite. Semithin sections (2-3 μm) were cut from the blocks and processed for LM autoradiography (Holstege, J.C. and Kuypers, 1982). Those blocks, which

showed both intense HRP labelling of motoneurons and a large number of silver grains, were trimmed to pyramids. From these pyramids ultrathin sections were cut and processed for EM autoradiography.

II.1.C. Collection and Analysis of the Data

EM autoradiography

The three rats with an injection in the ventrolateral part of the medial reticular formation were studied first. In each rat 2 blocks were taken from the L5 and L6 segments and two ultrathin sections were used from each block, i.e. 12 sections in total. In these sections an attempt was made to determine to which tissue compartment the radioactivity had been transported. A standardized band of tissue (10 µm in width) adjacent to the grid bar at the right side of each grid hole within the motoneuronal cell groups was completely photographed on a 35 mm film which was reversal-developed. This film was projected and the analysis was performed on the projected image. In pale gold ultrathin sections covered with a slightly overlapping monolayer of Ilford L4 emulsion and developed with D19, the source of the radioactivity (i.e. the tritium isotope), which gives rise to a silver grain in the emulsion layer, can be located with 50% confidence within a 480 nm circle centered on the silvergrain (Salpeter et al., 1969; Salpeter and Szabo, 1972). Therefore a circle, drawn on a transparent sheet and corresponding to a diameter of 480 nm, was centered on each silver grain (including those located in a cluster, see later) in the projected electron micrographs. The tissue compartment present within the circle was registered. When two or three tissue compartments were present, they were all registered because they all had to be considered with equal probability to represent the source of the radioactivity. This method of analysing will be referred to as 'the circle method' (Williams, 1969; 1977). The single tissue compartment or the combination of tissue compartments found within the circle will be referred to as an item; some items were grouped together. In the motoneuronal cell groups the following tissue compartments were encountered: axon (Ax), dendrite (D), terminal (T) and cell soma (CS). The remaining tissue compartments (R) comprised glial element, blood-vessel and unidentified structure. In presenting the data the following items were distinguished (see also Fig. 1):

(1) Terminal: T.
(2) Terminal in combination with axon: T/Ax.
(3) Terminal in combination with axon and cell soma (T/Ax/CS), terminal in combi nation with axon and dendrite (T/Ax/D), terminal in combination with axon and R (T/Ax/R).
These three items were grouped together as: T/Ax/CS-D-R
(4) Terminal in combination with dendrite (T/D), terminal in combination with den drite and R (T/D/R).
These two items were grouped together as: T/D-R.
(5) Terminal in combination with cell soma (T/CS), terminal in combination with cell soma and dendrite (T/CS/D), terminal in combination with cell soma and with R (T/CS/R).
These three items were grouped together as: T/CS/D-R.
(6) Terminal in combination with R: T/R.

The remaining items are the same as above but without T:
(7) Axon: Ax.
(8) Axon in combination with cell soma or with dendrite or R.
These three items were grouped together as: Ax/CS-D-R.
(9) Dendrite, or dendrite in combination with R.
These two items were grouped together as: D-R.
(10) Cell soma or cell soma in combination with dendrite or with R.
These three items were grouped together as: CS/D-R.
(11) R(emaining): R.

For each section the frequency with which an item or group of items was found in the photographed band was expressed as a percentage of the total number of items found in that band (Fig. 1). When a terminal profile was present within the circle, either alone or in combination with other tissue compartments, the type of the terminal (Table 1) was registered. For each section the frequency of the silver grains overlying items with a given type of terminal was expressed as a percentage of the total number of silver grains overlying items

Table 1 The different types of terminals distinguished in the rat motoneuronal cell groups.

S-type	Terminals containing spherical vesicles (40-50 nm. in diameter) and an occasional large dense core vesicle (80-120 nm.). They usually showed asymmetrical synaptic junctions.
F-type	Terminals containing flattened synaptic vesicles (25-35 x 50-60 nm. in diameter) or a combination of flattened and spherical vesicles (40-50 nm. in diameter). They usually showed symmetrical synaptic junctions.
E-type	Terminals containing small spherical vesicles (microvesicles) (15-25 nm. in diameter). Small elongated vesicles (15-25 x 40-60 nm.) were usually present and canaliculi-like structures could occasionally be observed. Dense-cored vesicles (60-80 nm.) and relatively large agranular vesicles (50-60 nm. in diameter) were rarely observed. The terminals usually showed asymmetrical synaptic junctions.
G-type	Terminals, characteristically containing large granular vesicles (70-120 nm.), which were sometimes elongated (up to 180 nm.). In addition many clear vesicles were present, which were spherical or flattened and ranged in size from 15 to 50 nm. One or two large clear vesicles were often present. The terminals usually showed asymmetrical synaptic junctions.
C-type	Terminals containing spherical or flattened vesicles. The synaptic junctions lacked the pre- and postsynaptic membrane thickenings. The postsynaptic element was either a cell soma or a proximal dendrite. A postsynaptic subsurface cistern was present along the entire length of the synaptic apposition, with a Nissl body located beneath the cistern.
T-type	Terminals containing spherical vesicles (40-50 nm. in diameter) and characteristically showing a postsynaptic dense body (Taxi-body).

In the 12 sections studied the percentages showed some variations. In order to determine the contributions of the three different rats and the six different blocks to these variations, a variance analysis was performed (Snedecor and Cochran, 1980). In the vast majority of cases the differences between the percentages were statistically not significant. Only in a very few cases the differences were either significant or not significant but relatively large ($F > 2$). However these differences did not show a consistent pattern. From this it was inferred that the variations in the percentages could not be attributed either to differences between the animals or to differences between the blocks. It was therefore concluded that they could only be attributed to differences between the sections, resulting from random variation and errors of observation. This made it possible to calculate the 95% confidence limits of the mean of the percentages, which were obtained in each of the 12 sections. The study of the EM autoradiographs by means of the circle method (see above) can only lead to conclusions if it can be shown that the silver grains are not distributed randomly. Therefore the distribution of the "real grains" was compared with that of randomly distributed "hypothetical grains", (effective area measurement, Williams, 1969; 1977). The electron micrographs of the standardized band of tissue were projected on a white cardboard carrying 100 horizontal and 100 vertical lines, which produce 10.000 intersection points. For every electron micrograph two to four intersection points were chosen randomly with the aid of a table of random numbers and the center of a circle with a diameter corresponding to 480 nm was placed on these points. The items located within the circle were then registered. The number of hypothetical grains produced in a section was matched with that of the real grains found in the same section. The content of a total of 6592 random circles was thus analysed. The results were expressed as percentages per item or group of items as was done for the real grains. On these percentages the same type of variance analysis was performed as on the real grain percentages, which led to the same conclusions. The mean percentages per item or group of items in the hypothetical grain analysis (effective area measurement) were compared with those in the real grain analysis (Fig. 1). Obviously the effective area measurement is not related to the type and site of injection. In addition it was shown by the variance analysis that the results of the effective area measurements in the

individual rats were not statistically different. Therefore these measurements could also be used in the rats with injections in other brainstem areas (see below).

The four rats with injections in the raphe nuclei and the adjoining MRF were studied as follows. Two blocks were taken from the L5 and L6 segments and from each block two sections were used, i.e. 16 sections in toto. Since in these four rats no effective area measurements had to be made, only the silver grains in the standardized band of tissue were photographed. The tissue compartments carrying these silvergrains were analysed by means of the circle method (see above). The variance analysis performed on the different percentages led to the same conclusions as in the rats injected in the vlMRF.

In the remaining four rats with injections in the area of the nucleus coeruleus and subcoeruleus, 16 sections were obtained which were analysed as described for the four rats injected in the raphe nuclei. The variance analysis led to the same conclusions as in the rats injected in the vlMRF. In the EM autoradiographs many clusters of silvergrains were present over the motoneuronal cell groups in addition to more diffusely distributed silver grains. A group of silvergrains was considered to form a cluster if six or more silvergrains were present within a circle with a diameter of 3.5 μm. It was assumed that each of these clusters resulted from radioactivity in the tissue compartment located under the center of the cluster. The sections analysed with the circle method were also scrutinized for clusters. This search for clusters was not limited to the standardized band of tissue but covered the entire gridhole (i.e. approximately nine times the square surface of the standardized band). The cluster-labelled structures were photographed on reversal film, projected and analysed. This analysis will be referred to as the cluster method. Every tissue compartment on which a cluster was centered was registered and the frequency was expressed for each section as a percentage of the total number of clusters found in that section. The cluster-labelled terminals were characterized as in the circle method. A variance analysis performed on the different percentages thus obtained gave the same results as described in the real grain analysis by means of the circle method (Fig 3, Table 4,5 and 6).

EM autoradiography in combination with HRP histochemistry

Two rats were studied. Three blocks from each of the two rats were used and from each block three sections were examined, i.e. 18 sections in total. In these sections only clusters of six or more silvergrains were taken into account and only terminal profiles carrying such clusters were studied. Every section was scrutinized for clusters. When a terminal profile carrying a cluster was encountered, its type was registered. Occasionally a terminal profile was disregarded because it could not be reliably classified either due to the fact that the profile was obscured by the abundance of silvergrains overlying it, or due to fixation artefacts, or both. If a labelled terminal profile established a synaptic contact, the postsynaptic structure was identified and registered. In addition it was determined whether the postsynaptic structure contained HRP reaction product. A total of 684 cluster-labelled terminal profiles were examined (Table 7).

II.1.D. Results

Light microscopy autoradiography.

The injection sites were studied in frozen sections treated for LM autoradiography with a 1-month exposure time. In the three rats with vlMRF injections, a dense accumulation of silvergrains was present mainly over the ventral part of the MRF at the levels between the rostral part of the facial nucleus and the caudal part of the inferior olive. The accumulation also covered the lateral part of the inferior olive (Fig. 4A). In the four rats injected in the raphe nucleus and the adjoining MRF a dense accumulation of silvergrains was present bilaterally over the nuclei raphe magnus, raphe obscurus and raphe pallidus and over the ventral part of the adjoining MRF as well as over the inferior olive (Fig. 5A). The injection site extended from the caudal part of the facial nucleus to the caudal end of the inferior olive. In the four rats with injections in the nucleus coeruleus and subcoeruleus a dense accumulation of silvergrains

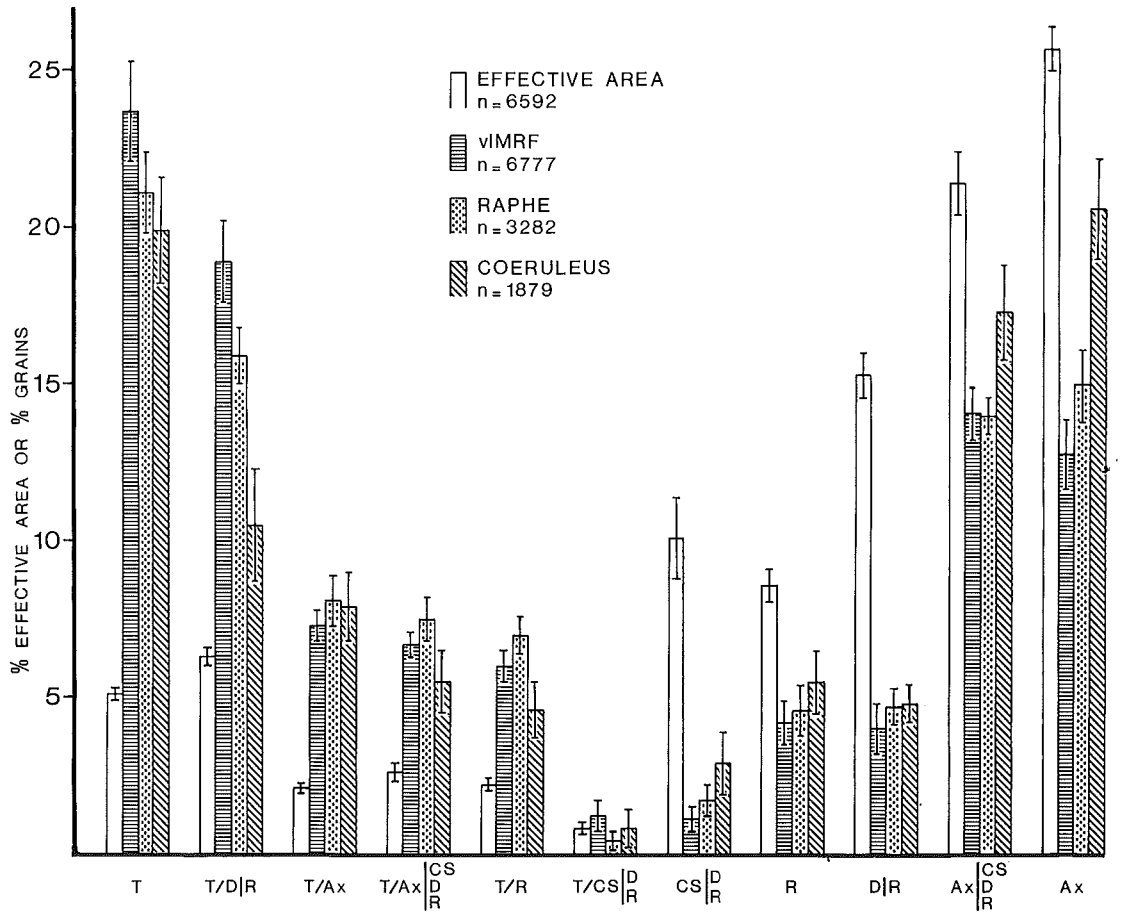
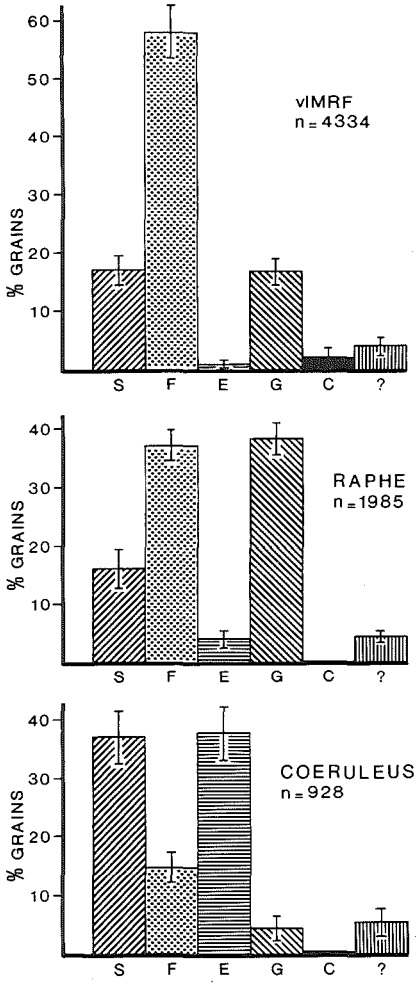


Fig. 1. Distribution of the silvergrains and the hypothetical grains over the various items and groups of items. Values indicated are mean percentages (±95% confidence limits) and total numbers (n). For details and abbrevations see Collection and Analysis of the Data.

Fig. 2 Frequency of the silvergrains overlying items with s given type of terminal.



Values indicated are mean percentages (±95% confidence limits) and total numbers (n). See also Collection and Analysis of the Data.

Table 2 Frequency of the silvergrains overlying items with synaptic terminals of the different types of terminals.

	MRF (%)	Raphe (%)	Coeruleus (%)
Synaptic S-type	51 ± 4 (n = 381)	51 ± 7 (n = 143)	39 ± 10 (n = 124)
Synaptic F-type	52 ± 3 (n = 1304)	50 ± 5 (n = 379)	51 ± 8 (n = 67)
Synaptic E-type	— (n = 0)	12 ± 10 (n = 15)	7 ± 5 (n = 24)
Synaptic G-type	9 ± 6 (n = 71)	8 ± 4 (n = 62)	— (n = 0)

Values given are mean percentages (±95% confidence limits) and total numbers (n). See also Collection and Analysis of the Data.

Table 3 Frequency of the silvergrains overlying items with synaptic terminals of the different types of terminals, contacting cell soma, proximal dendrite or distal dendrite.

	MRF (%)	Raphe (%)	Coeruleus (%)
S→CS	5 ± 7	2 ± 3	4 ± 5
S→pD	79 ± 12	78 ± 10	72 ± 15
S→dD	16 ± 10 (n = 381)	20 ± 9 (n = 143)	24 ± 12 (n = 124)
F→CS	7 ± 4	4 ± 3	6 ± 6
F→pD	84 ± 6	77 ± 8	65 ± 18
F→dD	9 ± 4 (n = 1304)	19 ± 8 (n = 379)	29 ± 19 (n = 67)
E→CS	—	0	0
E→pD	—	60 ± —	0
E→dD	— (n = 0)	40 ± — (n = 15)	100 (n = 24)
G→CS	0	0	—
G→pD	100	62 ± 20	—
G→dD	0 (n = 71)	38 ± 20 (n = 62)	— (n = 0)
C→CS	52 ± 29	—	—
C→pD	48 ± 29 (n = 95)	— (n = 2)	— (n = 4)

Values given are mean percentages (±95% confidence limits) and total numbers (n). CS, cell soma; pD, proximal dendrite; dD, distal dendrite.See also Collection and Analysis of the Data.

was present over these nuclei. The injection site also involved the mesencephalic nucleus of the trigeminus, the brachium conjunctivum, the parabrachial nuclei, the lateral part of the central grey, part of the superior vestibular nucleus and the dorsal part of the rostral half of the trigeminal motor nucleus. The injection site extended into the caudal part of the mesencephalic reticular formation (Fig 6A). In the two rats with ^{3}H-leucine injections in the vlMRF as well as HRP injections in the ipsi-lateral hindleg muscles a dense accumulation of silvergrains was present (Fig. 7A) over the same brainstem area as described in the first three rats. In the semithin autoradiographs of the L5 and L6 segments of the rats injected in the vlMRF and in the raphe nuclei respectively, a large number of silvergrains were

present bilaterally over the ventral horn, including the lateral and medial motoneuronal cell groups, where the silvergrains were evenly distributed. In the autoradiographs of L5 and L6 from the rats injected in the area of the nucleus coeruleus and subcoeruleus, the silvergrains were much less numerous but were also evenly distributed over the motoneuronal cell groups. Concentrations of silver grains over cell bodies were never observed.

Electron microscopy autoradiography

In the EM autoradiographs many diffusely distributed silvergrains were present as well as clusters of silvergrains. In the 11 rats with various brainstem injections the source of the radioactivity producing the silvergrains was determined by means of the circle method and by means of the cluster method (see Collection and Analysis of the Data).

The circle method

In the 12 EM autoradiographs of the three rats injected in the vlMRF (5,5 months exposure time) a total of 6777 silvergrains was found in the standardized bands of tissue (calculated average ±300 silvergrains per gridsquare of 7225 μm^2). In the 16 sections of the four rats with injections in the raphe nuclei and the adjoining MRF (4 months exposure time) 3282 silvergrains were found (calculated average of ±130 silvergrains per gridsquare). In the 16 sections of the 4 rats with injections in the area of the nucleus coeruleus and subcoeruleus (9,5 months exposure time) 1879 silvergrains were found (calculated average of ±80 silvergrains per gridsquare). For the effective area measurement 6592 hypothetical silvergrains were analysed. Fig. 1 shows the real grain percentages and the effective area percentages in the three groups of experiments. Each item or group of items was considered to contain radioactivity only if its real grain percentage was larger than its effective area percentage and if the 95% limits of confidence of these percentages did not overlap, indicating a statistical significant difference. From these data (Fig.1) it was concluded that only the items and groups of items containing a terminal profile (T) could with confidence be regarded to contain radioactivity. These items and groups of items were regarded to derive their radioactivity from this terminal profile since the other tissue compartments in these items could not with confidence be considered to contain radioactivity (Fig. 1). However, the group of items T/CS/D-R, despite the presence of a terminal profile (T), could not with confidence be considered to contain radioactivity, because the 95% limits of confidence of its real grain percentages overlapped with that of its effective area percentage. Little weight has been attached to this exception since this group carried only 1% of all silvergrains. In the three groups of experiments a difference in the distribution of the silvergrains over various types of terminals was observed (Fig. 2). After injections in the ventro-lateral medial reticular formation 4334 silvergrains were overlying items containing a terminal profile (Fig. 4). A large percentage of these silvergrains (58%) was located over items containing F-type terminal profiles, while 17% was located over items containing S-type and 17% over those containing G-types. Only 1 and 2% of the silvergrains were overlying items containing E- and C-type respectively. After the injections in the raphe and the adjoining medial reticular formation 1985 silvergrains were overlying items containing a terminal profile (Fig. 5). These silver grains displayed a slightly different distribution than those after vlMRF injections. A relatively large percentage (37%) was found over items containing F-type terminals but also over those containing G-type (38%). A further 16% was found over items containing S-type and 4% over those containing E-type terminal profiles. After injections involving the area of the nucleus coeruleus and subcoeruleus 928 silvergrains were overlying items containing a terminal profile (Fig. 6), but these silvergrains showed an entirely different distribution than in the preceeding cases. The largest percentages were located over items containing E-and S-type terminal profiles (38 and 37% respectively) while much fewer were found over items containing F-type (15%) and very few over those containing G-type (4%). T-type terminals were never seen to carry silvergrains. In regard to the synaptic terminals (Table 2), in the three groups of experiments between 39 and 52% of the grains overlying items containing an F-type terminal or an S-type terminal were located over synaptic terminals, while only a small percentage (ranging from 7 to 12%) of the silver grains overlying items containing an E- or G-type terminal were found over synaptic terminals. Silvergrains overlying C-type terminal profiles were always found above synaptic terminals.

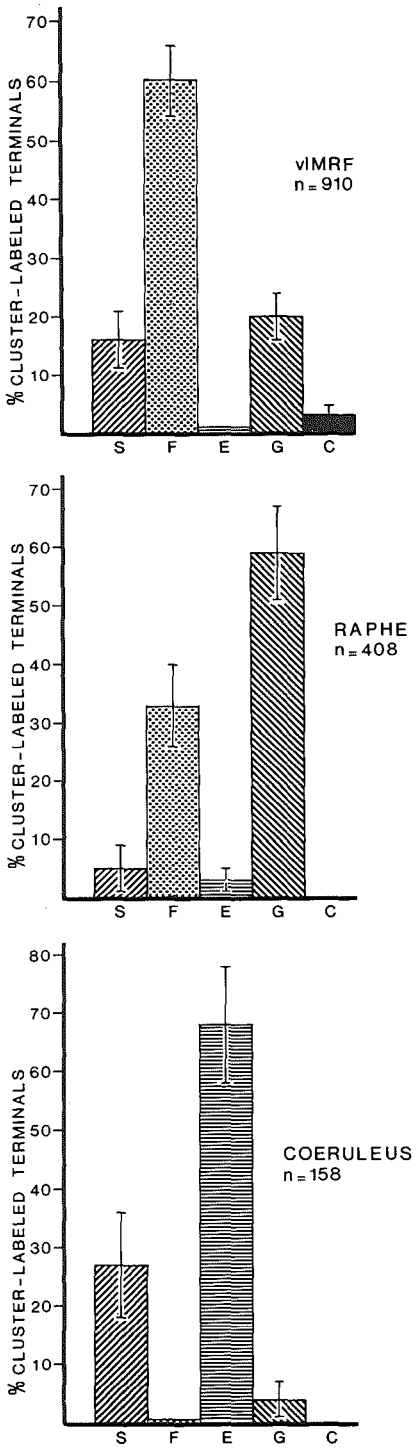


Fig. 3. Frequency of the silvergrains overlying items with a given type of terminal. Values indicated are mean percentages (±95% confidence limits) and total numbers (n). See also Collection and Analysis of the Data.

Table 4 Distribution of the clusters over the various tissue compartments.

	MRF (%)	Raphe (%)	Coeruleus (%)
Terminal	70 ± 3	82 ± 4	66 ± 4
Axon	18 ± 3	10 ± 4	23 ± 6
Unclear	12 ± 1	8 ± 3	11 ± 4
	(*n* = 1293)	(*n* = 501)	(*n* = 239)

Values given are mean percentages (±95% confidence limits) and total numbers (n). See also Collection and Analysis of the Data.

Table 5 Frequency of synaptic terminals of the different cluster-labelled types of terminals.

	MRF (%)	Raphe (%)	Coeruleus (%)
Synaptic S-type	41 ± 5 (*n* = 56)	34 ± — (*n* = 6)	52 ± 18 (*n* = 25)
Synaptic F-type	51 ± 2 (*n* = 284)	55 ± 11 (*n* = 84)	0 (*n* = 0)
Synaptic E-type	0 (*n* = 0)	8 ± — (*n* = 1)	11 ± 7 (*n* = 13)
Synaptic G-type	9 ± 4 (*n* = 19)	14 ± 6 (*n* = 34)	0 (*n* = 0)

Values given are mean percentages (±95% confidence limits) and total numbers (n). See also Collection and Analysis of the Data.

In the various experiments (Table 3),the silvergrains overlying F- and S-types of synaptic terminals were overlying terminals which mostly contacted proximal dendrites, containing ribosomes (percentages ranging from 65 to 84%). Only few silvergrains were overlying terminals contacting cell somata (2-7%) or distal dendrites (9-29%). Similar results were obtained in respect to the E- and G-type terminals (Table 3). Silvergrains overlying synaptic terminals making axo-axonic contacts were not observed.

The cluster method

A total of 2033 clusters containing six or more silvergrains were studied (Table 4). After injections in the vlMRF, in the raphe nuclei plus the adjoining MRF and in the area of the nucleus coeruleus and subcoeruleus respectively 18%, 10% and 23% of the clusters were centered over myelinated and unmyelinated axons. In all these experiments approximately 10% of the clusters were centered either over structures which could not be identified or over terminal profiles which could not be classified because of the abundance of silver grains overlying them. The remaining clusters 70, 82 and 66% respectively were centered over terminal profiles (cluster-labelled terminals) which could be classified (Figs. 4, 5 and 6). After injections in the ventrolateral medial reticular formation the majority of the cluster-labelled terminals (60%) was of the F-type, 20% of the G-type and 16% of the S-type. On a few occasions cluster-labelled C-type terminals were also identified (3%, Fig. 3). After injections in the raphe nuclei and the adjoining MRF the large majority of the cluster-labelled terminal profiles were of the G-type (59%) while 33 % were F-type. Only a few S- (5%) and E- (3%) types were cluster-labelled (Fig. 3). C-type terminal profiles were not cluster-labelled. After injection in the area

Table 6 Frequency of the different types of cluster-labelled synaptic terminals contacting cell soma, proximal dendrite or distal dendrite.

	MRF (%)	Raphe (%)	Coeruleus (%)
S→CS	5 ± 6	0	0
S→pD	80 ± 9	100	84 ± 14
S→dD	15 ± 8	0	16 ± 14
	($n = 56$)	($n = 6$)	($n = 25$)
F→CS	8 ± 4	4 ± 4	—
F→pD	76 ± 5	71 ± 14	—
F→dD	16 ± 4	25 ± 14	—
	($n = 284$)	($n = 84$)	($n = 0$)
E→CS	—	—	0
E→pD	—	—	61 ± —
E→dD	—	—	39 ± —
	($n = 0$)	($n = 1$)	($n = 13$)
G→CS	0	4 ± 5	—
G→pD	82 ± 14	66 ± 16	—
G→dD	18 ± 14	30 ± 16	—
	($n = 19$)	($n = 34$)	($n = 0$)
C→CS	62 ± 10	—	—
C→pD	38 ± 10	—	—
	($n = 26$)	($n = 0$)	($n = 0$)

Values given are mean percentages (±95% confidence limits) and total numbers (n). CS, cell soma; pD, proximal dendrite; dD, distal dendrite. See also Collection and Analysis of the Data.

Table 7
Results obtained after combining EM autoradiography with HRP histochemistry.

1 Type of terminal	2 Synaptic terminal	3 Postsynaptic structures	4 HRP-labelled	5 HRP-labelled structures (total)
S-type		CS $n = 6$ (7%)	$n = 6$ (100%)	
$n = 144$ (21%)	$n = 80$ (56%)	pD $n = 55$ (69%)	$n = 31$ (56%)	$n = 41$ (51%)
		dD $n = 19$ (24%)	$n = 4$ (21%)	
F-type		CS $n = 22$ (9%)	$n = 21$ (95%)	
$n = 409$ (60%)	$n = 246$ (60%)	pD $n = 165$ (67%)	$n = 96$ (58%)	$n = 138$ (56%)
		dD $n = 59$ (24%)	$n = 21$ (36%)	
E-type				
$n = 0$ (0%)				
G-type		CS $n = 0$ (0%)		
123 (18%)	$n = 12$ (10%)	pD $n = 6$ (50%)	$n = 3$ (50%)	$n = 6$ (50%)
		dD $n = 6$ (50%)	$n = 3$ (50%)	
C-type		CS $n = 3$ (38%)	$n = 2$ (67%)	
$n = 8$ (1%)	$n = 8$ (100%)	pD $n = 5$ (62%)	$n = 4$ (80%)	$n = 6$ (75%)

Values indicate: frequency (in actual numbers and in percentages) of the different cluster-labelled types of terminals (1), of their synaptic terminals (2), of the postsynaptic structures contacted (3) and frequency of HRP labelling of these variouspostsynaptic structures (4). The values in the last column (5) indicate frequency of HRP labelling of all postsynaptic structures contacted by the different-cluster labelled types of terminals (2).

of the nucleus coeruleus and subcoeruleus a completely different distribution was observed. The large majority of the cluster-labelled terminal profiles were of the E-type (68%), a smaller proportion were of the S-type (27%) and few of the G-type (4%). Only one cluster-labelled F-type profile was observed (<1%) (Fig. 3). C-type terminals were not cluster-labelled. In all three groups of experiments (Table 5) the percentages of the cluster labelled F- and S-type terminals that exhibited a synaptic contact varied between 34% and 55%. However, only a limited percentage (varying between 0 and 14%) of the cluster-labelled E- and G-type terminal profiles exhibited a synaptic contact, while the C-type always exhibited a synaptic contact. The cluster-labelled F-, S-, G- and E-type terminals (Table 6) contacted mainly proximal dendrites (containing ribosomes), and to a much lesser extent distal dendrites. In only a few instances a synaptic contact with a cell soma was observed. C-type terminals behaved differently and contacted cell somata more frequently than proximal dendrites. The cluster-labelled terminals were never seen to establish axo-axonic synaptic contacts.

EM autoradiography combined with HRP histochemistry

The L4-L6 spinal segments from the two rats which received ^{3}H-leucine injections in the vlMRF combined with HRP injections in the ipsilateral hindleg muscles, were first treated for HRP histochemistry and subsequently processed for EM autoradiography using 6 months exposure time. In the EM autoradiographs attention was paid only to cluster-labelled terminal profiles that could be reliably classified plus their postsynaptic structures. Furthermore it was determined whether the different postsynaptic structures contained TMB crystals. Table 7 summarizes the different findings and shows that nearly all postsynaptic cell somata were found to contain TMB crystals and that also many postsynaptic proximal dendrites contained such crystals (percentages ranging from 50 to 80%). Distal dendrites which were contacted by cluster-labelled terminals, were less frequently observed to contain TMB crystals (percentages ranging from 21 to 50%, fig 7B-E).

II.1.E. DISCUSSION

Axonal transport studies (Kuypers and Maisky, 1975; Leichnitz et al., 1978; Holstege G.et al., 1979; Martin et al., 1979; Holstege J.C. and Kuypers, 1980; Holstege G. and Kuypers, 1982; Martin et al., 1985; Jones and Yang, 1985) have shown that the brainstem neurons which project to spinal motoneurons are concentrated in the raphe nuclei, the ventral part of the MRF and in the area of the nucleus coeruleus and subcoeruleus, as demonstrated also by histofluorescent and histochemical techniques (Dahlström and Fuxe, 1965; Nygren and Olson, 1977; Satoh et al., 1977; Commissiong et al., 1978; Bowker et al., 1982; Björklund and Skagerberg, 1982; Westlund et al., 1983; Skagerberg and Björklund; 1985). The original studies of Dahlström and Fuxe (1965) in addition showed that many projections from the raphe nuclei and the adjoining MRF were serotonergic while many of those from the nucleus coeruleus and subcoeruleus were noradrenergic.

The present EM findings demonstrated that after ^{3}H-leucine injections in the above brainstem areas the radioactivity transported to the lumbar motoneuronal cell groups was mainly located in terminals. Moreover, the findings in the last group of experiments demonstrated that in the motoneuronal cell groups many of the terminals labelled from the vlMRF contacted motoneurons. It has been assumed that the labelled terminals in the motoneuronal cell groups received their radioactivity directly from their parent cell bodies in the brainstem. However these terminals might have received their radioactivity transneuronally i.e. through spinal neurons located e.g. in the intermediate zone. This is most unlikely because: a) ^{3}H-leucine is transported transneuronally only to a minor extent (Grafstein and Laureno, 1973); b) in the semithin autoradiographs of the three groups of experiments no indication of labelling of neurons in the intermediate zone was found, despite the large amount of radioactivity present in the motoneuronal cell groups as indicated by the many clusters of silvergrains in the EM autoradiographs; and c) labelling of terminals in the motoneuronal cell groups was only observed after injections in the vlMRF and in the raphe nuclei and not following injections in the

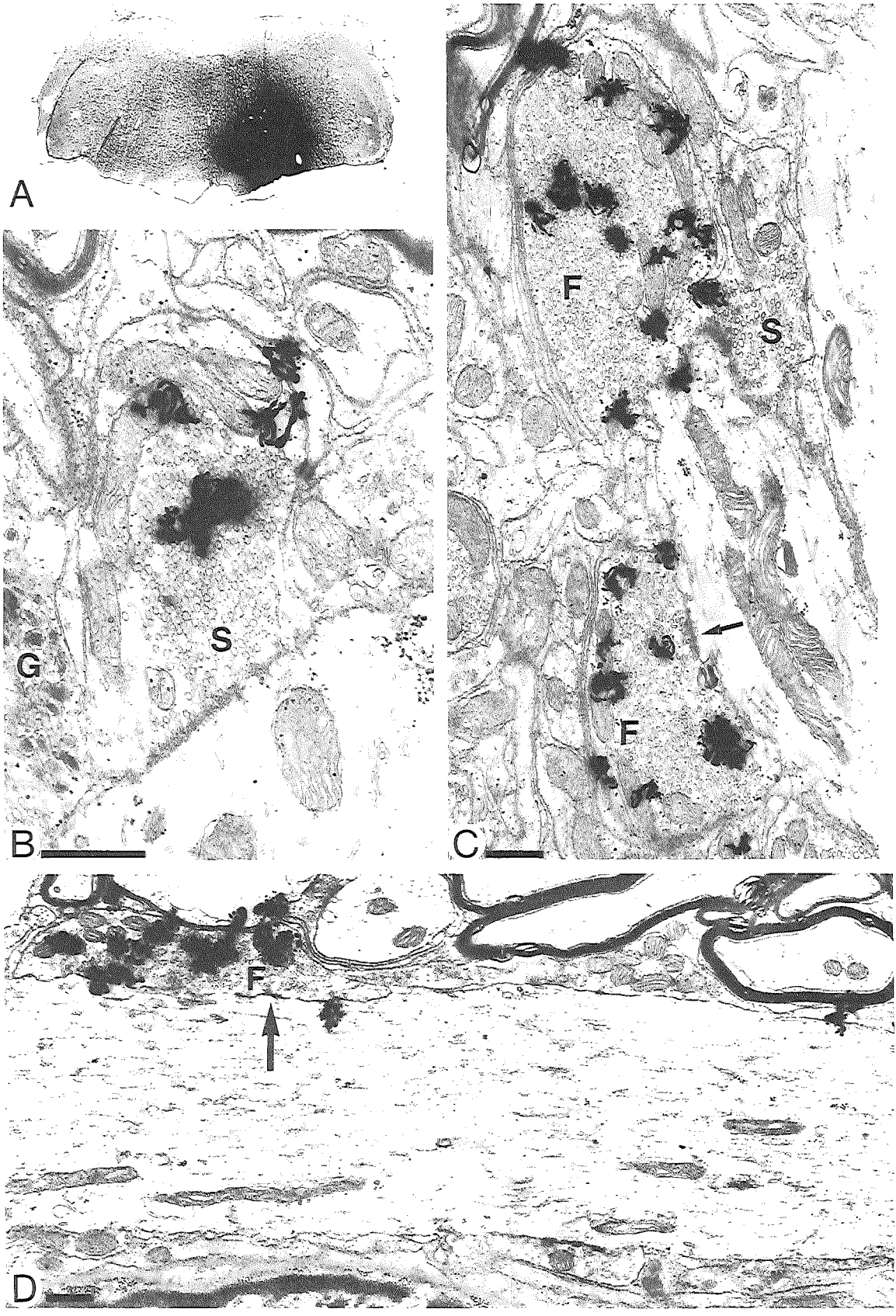
Fig. 4 (A). Light microscopical autoradiograph of the ^{3}H-leucine injection area in the vlMRF at the level of the most rostral part of the inferior olive (1-month exposure time). (B)-(J) Electron micrographs of autoradiographically labelled terminal profiles in the L5 and L6 lateral motoneuronal cell groups after ^{3}H-leucine injections in the vlMRF and using 5.5 months exposure time. Bar = 0,5 μm. (B) A labelled S-type terminal synapsing with a proximal dendrite. (C) Two cluster-labelled F-type terminals; the lower one establishing a synapse (arrow) on a proximal dendrite. (D) Photomontage showing three cluster-labelled F-type terminals synapsing (arrows) with a proximal dendrite. (E)-(G) A serial sectioned, cluster-labelled F-type terminal synapsing (arrows) with a proximal dendrite. Depicted are three profiles out of a row of six. (H) A cluster-labelled C-type terminal synapsing with a cell soma. Note the subsynaptic cistern and the Nissl body associated with it. (I). An S-type terminal carrying one single silver grain. (J) A cluster-labelled G-type terminal exhibiting an asymmetrical synaptic contact (arrow) with a proximal dendrite.

Fig. 5. (A) Light microscopical autoradiograph of the ^{3}H-leucine injection area in the raphe nuclei and the adjoining MRF at the level of the inferior olive (1-month exposure time). (B)-(K) Electron micrographs of autoradiographically labelled terminal profiles in the L5 and L6 motoneuronal cell groups after ^{3}H-leucine injections in the raphe and the adjoining MRF and using 4 months exposure time. Bars = 0.5 μm. (B) Two labelled F-type terminals; the upper one is carrying 5 silver grains and establishes a synaptic contact (arrows) with a proximal dendrite while the lower terminal profile is labelled by a cluster of six silver grains and does not exhibit a synapse. (C)-(D) Cluster-labelled S-type terminals exhibiting an asymmetrical synaptic contact with a distal dendrite (C) or a proximal dendrite (D). (E)-(F) Labelled G-type terminals synapsing (arrows) with a proximal dendrite (E) or a distal dendrite (F). (G). A cluster-labelled G-type terminal. A synaptic specialization is not visible in this section. (H) A cluster-labelled G-type terminal establishing an asymmetrical synapse with a proximal dendrite (ribosomes not visible). (I)-(K) A serial sectioned, labelled profile of the G-type. Note that the two G-type terminal profiles [(I), (arrows)] become interconnected by a short axonal segment [(J), (arrow), and (K)]. Depicted are three profiles from a row of five.

Fig. 6. (A) Light microscopical autoradiograph showing the extent of the ^{3}H-leucine injection area. It includes the nucleus coeruleus and subcoeruleus as well as other structures (for details see text). (B)-(F) Electron micrographs of autoradiographically labelled terminal profiles in the L5 and L6 lateral motoneuronal cell groups after ^{3}H-leucine injections in the area containing the nucleus coeruleus and subcoeruleus and using 9.5 months exposure time. Bars = 0,5 μm. (B)-(C) Cluster-labelled S-type terminals synapsing with proximal dendrites containing ribosomes (not visible on (B)). (D). A cluster-labelled E-type terminal containing many very small vesicles and some elongated vesicles (arrow). A synaptic specialization is not visible. Note the difference in vesicle size and shape as compared with the neighbouring unlabelled S-type terminal. (E) A labelled preterminal axon passing into a cluster-labelled E-type terminal. (F) A labelled E-type terminal without a visible synaptic specialization. Note the presence of many microvesicles and small elongated vesicles (arrows), whereas the size of the vesicles present in the neighbouring S- and F-types terminals is much larger.

Fig. 7 (A) Light microscopical autoradiograph of the ^{3}H-leucine injection area in the vlMRF at the level of the inferior olive (1-month exposure time). (B)-(F) Electron micrographs of autoradiographically labelled terminal profiles and HRP-labelled motoneurons located in the L4 and L5 motoneuronal cell groups from rats which received both a ^{3}H-leucine injection in the MRF and an HRP injection in the hindleg muscles. The tissue was reacted with TMB and processed for electron microscopical autoradiography using 6 months exposure time. Bars = 0,5 μm. (B) An F-type terminal, autoradiographically labelled with a cluster of silver grains, establishing a synaptic contact (open arrows) with a motoneuronal dendrite containing TMB crystals (arrows). (C) A cluster-labelled F-type terminal synapsing (open arrows) with a motoneuronal proximal dendrite containing TMB crystals (arrows). (D) Two cluster-labelled terminals are present: on the right side an F-type terminal establishing a synaptic contact (open arrows) with a motoneuronal distal dendrite containing TMB crystals (arrows). In the middle an S-type terminal synapsing (open arrow) with the soma of a motoneuron containing TMB crystals (arrow on the left). (E)-(F) Cluster-labelled F-type terminals establishing a synaptic contact (open arrows) with motoneuronal proximal dendrites containing TMB crystals (arrows).

Figure 4



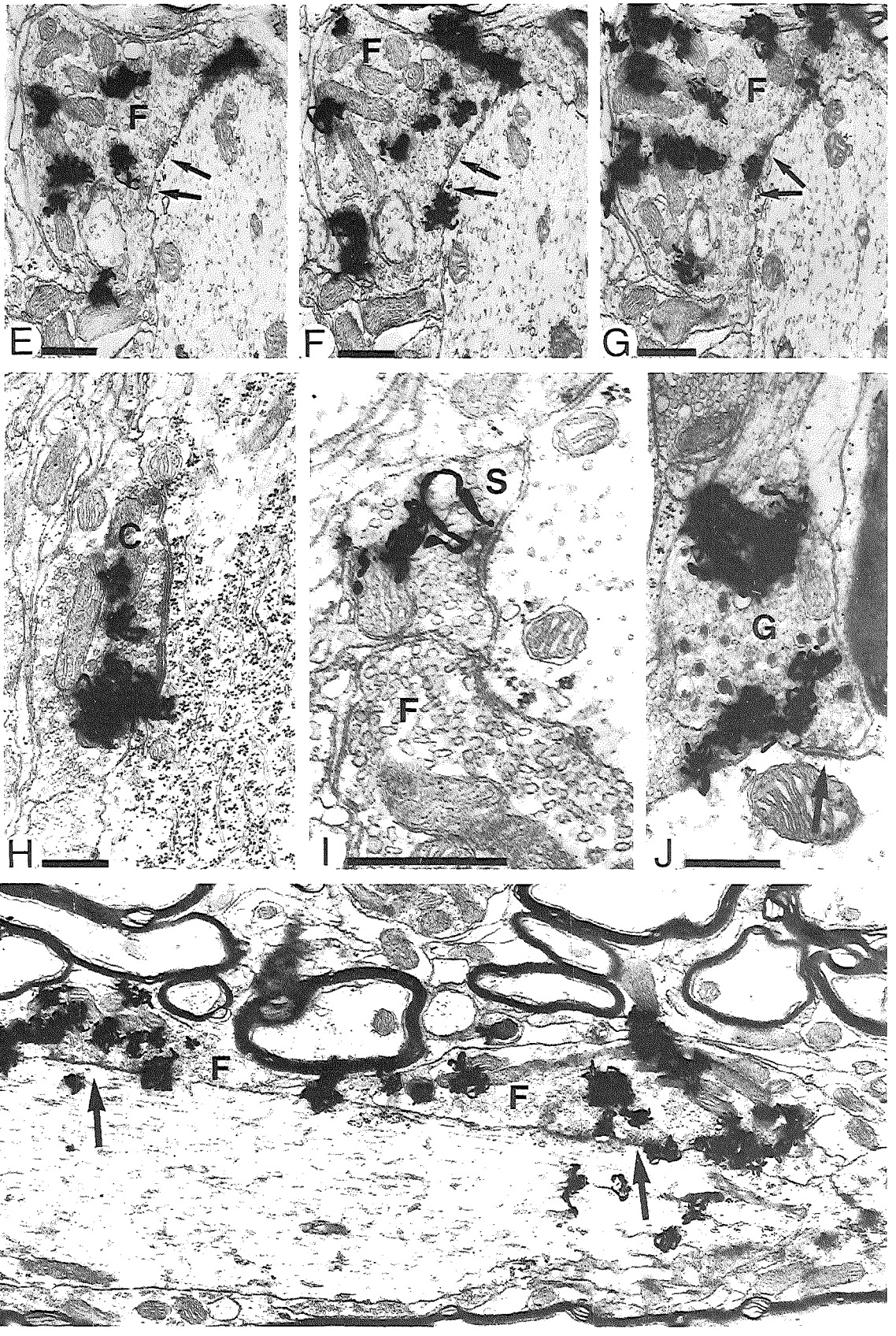

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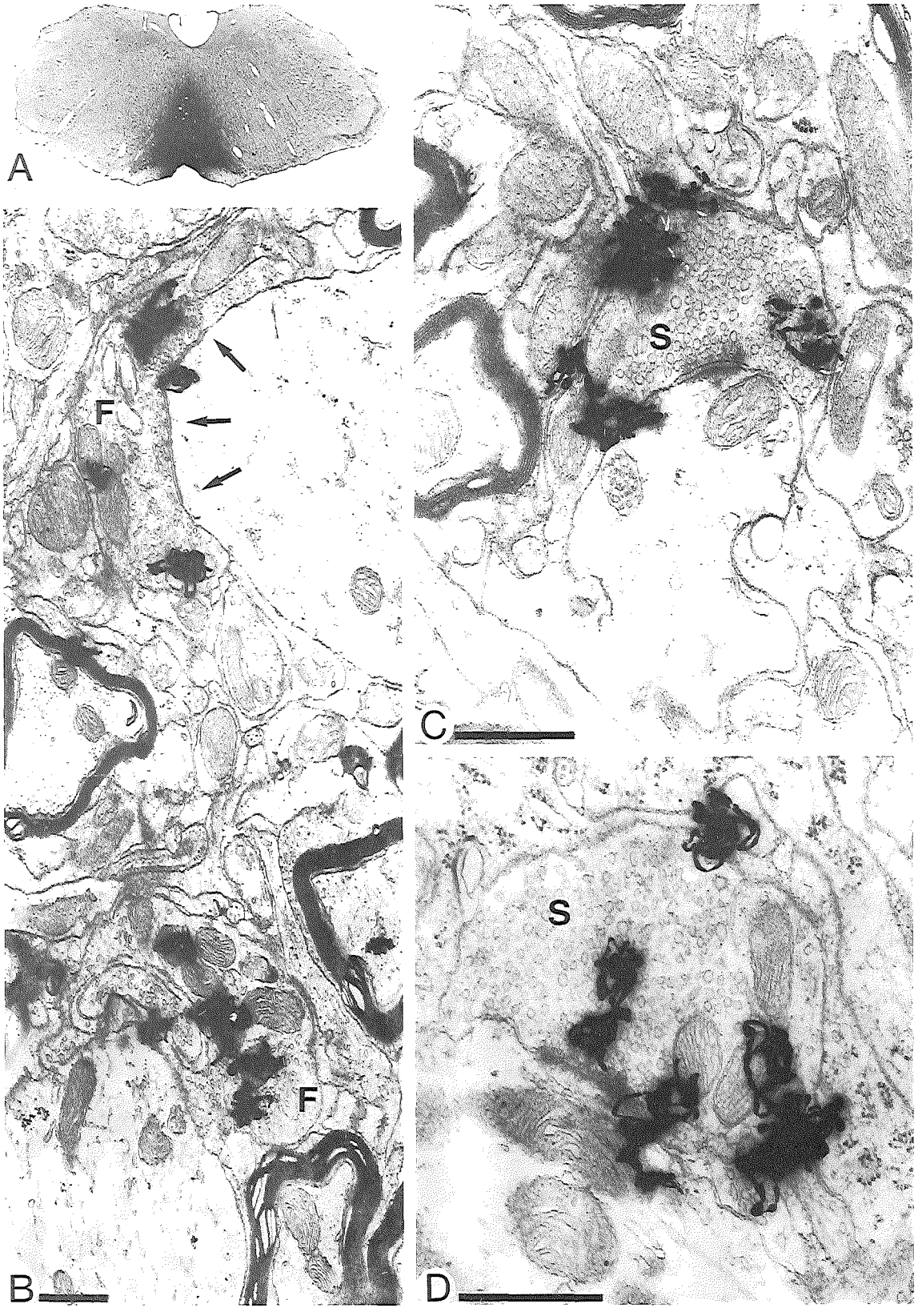
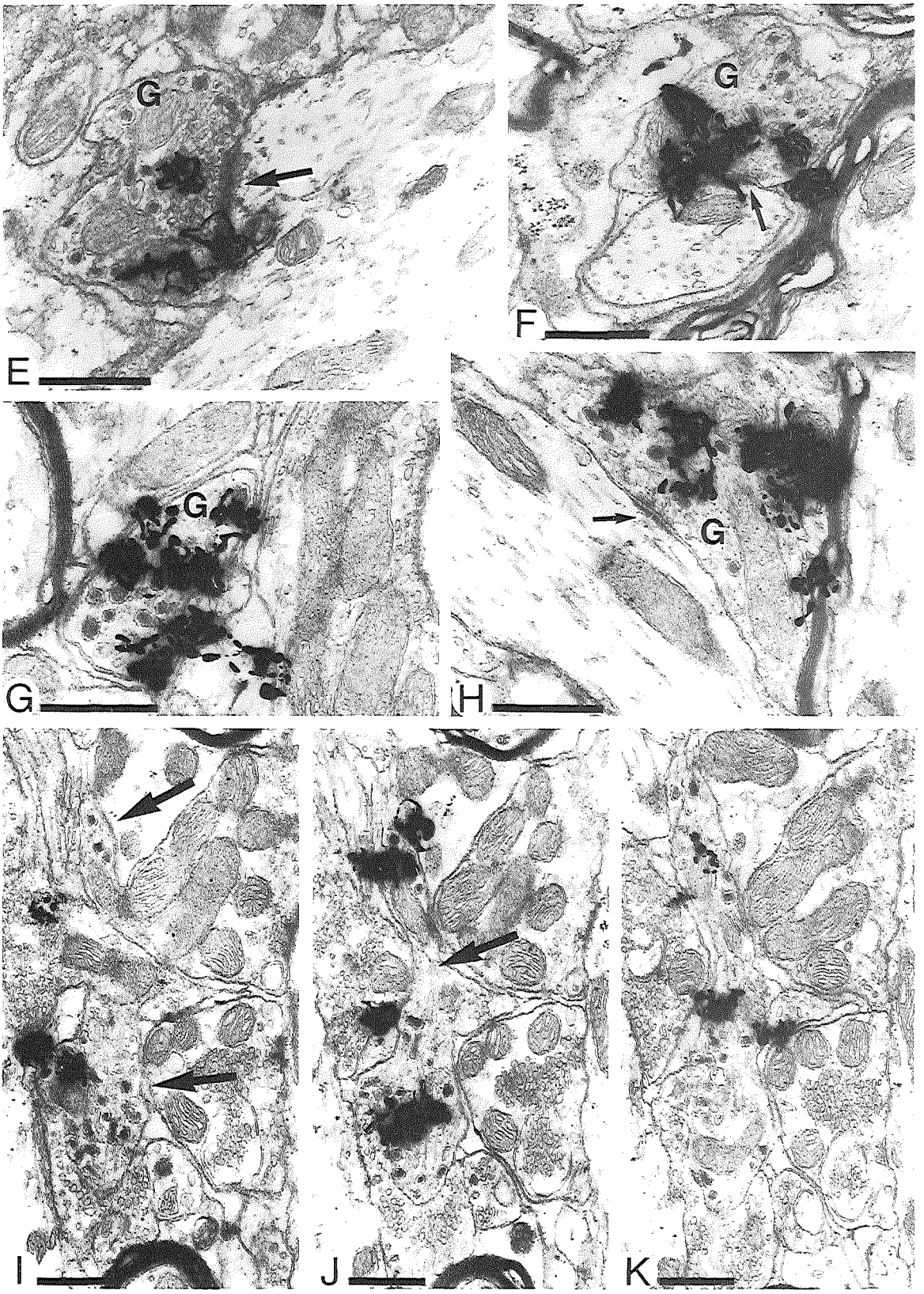
Figure 5

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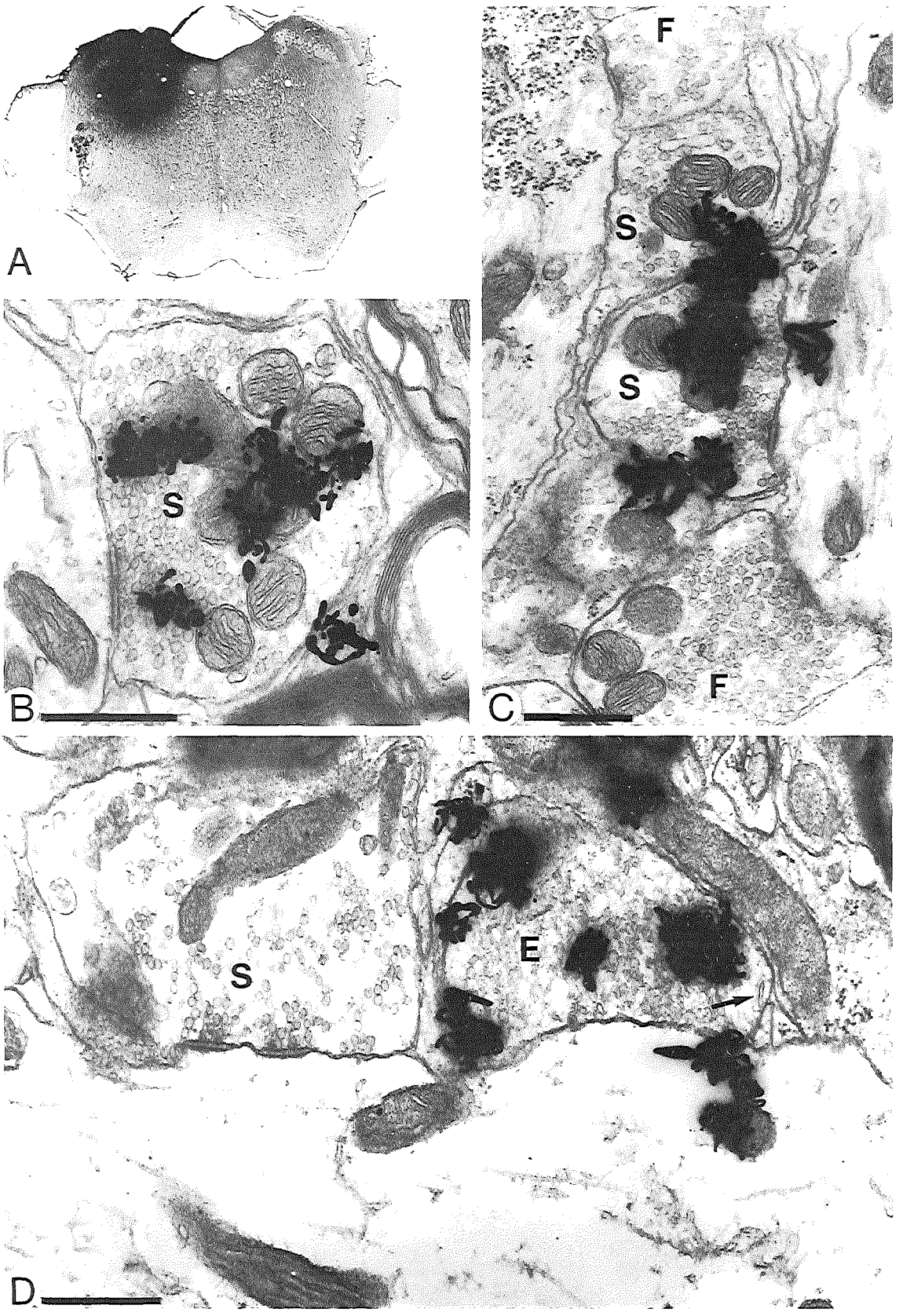

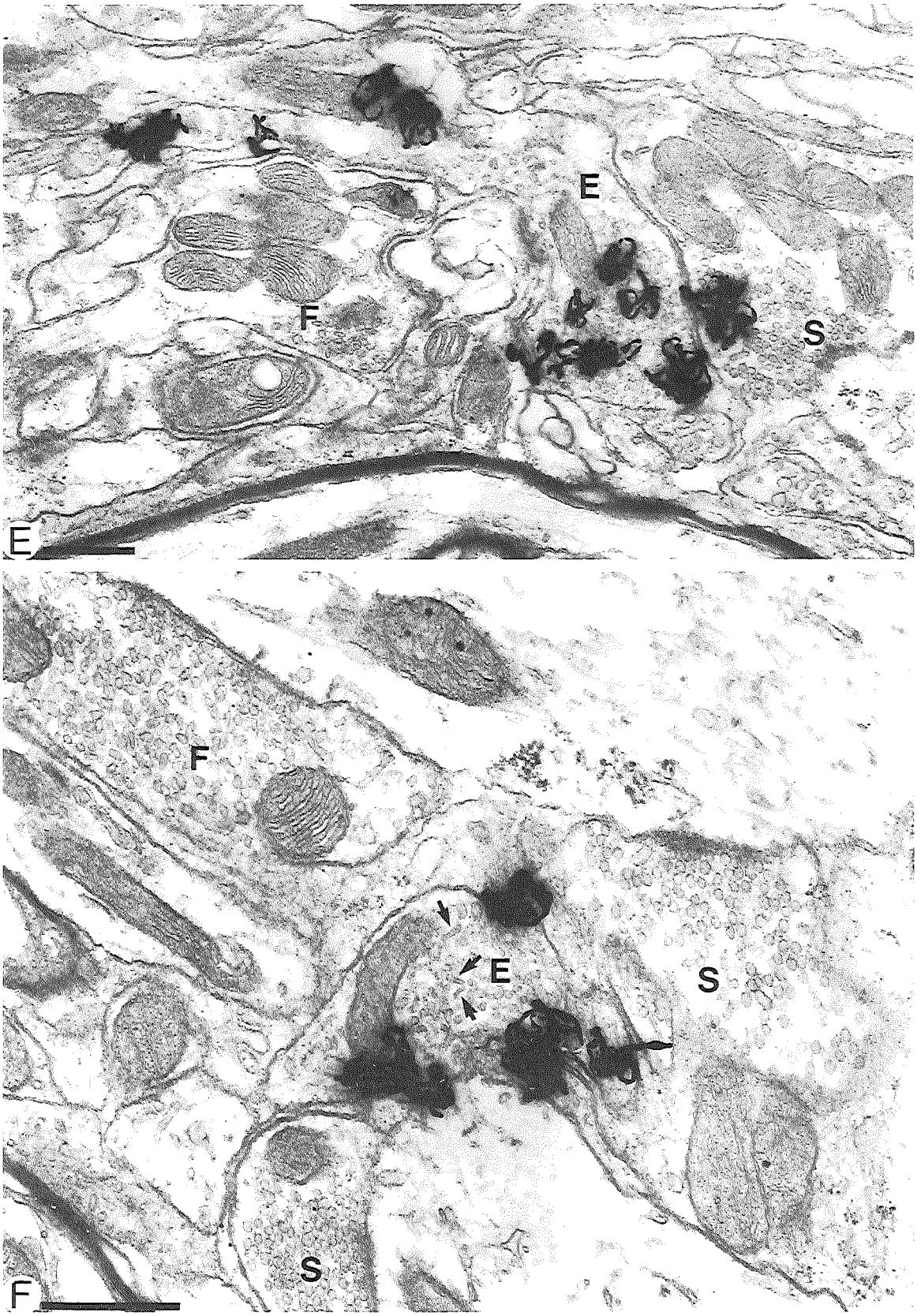
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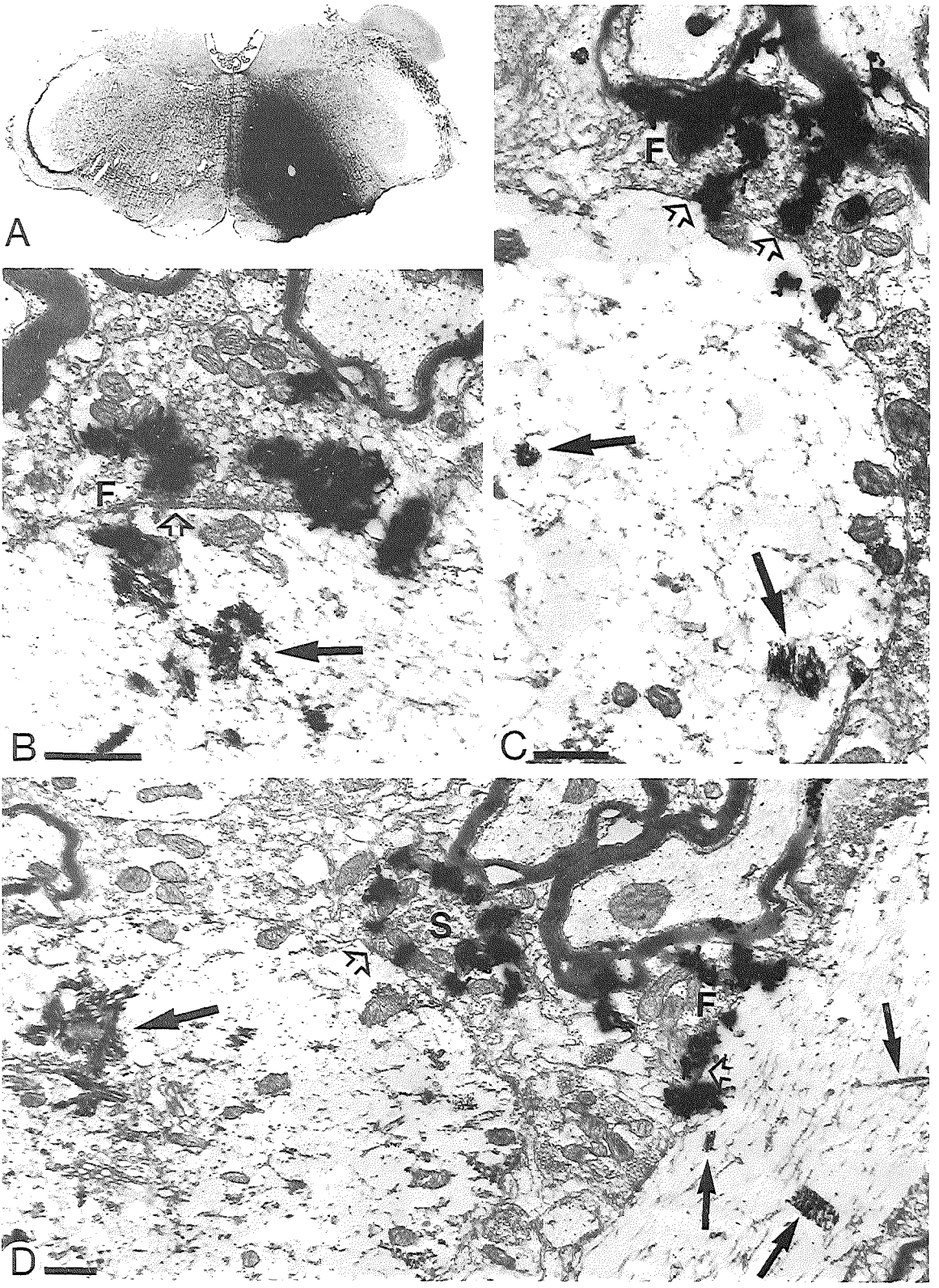
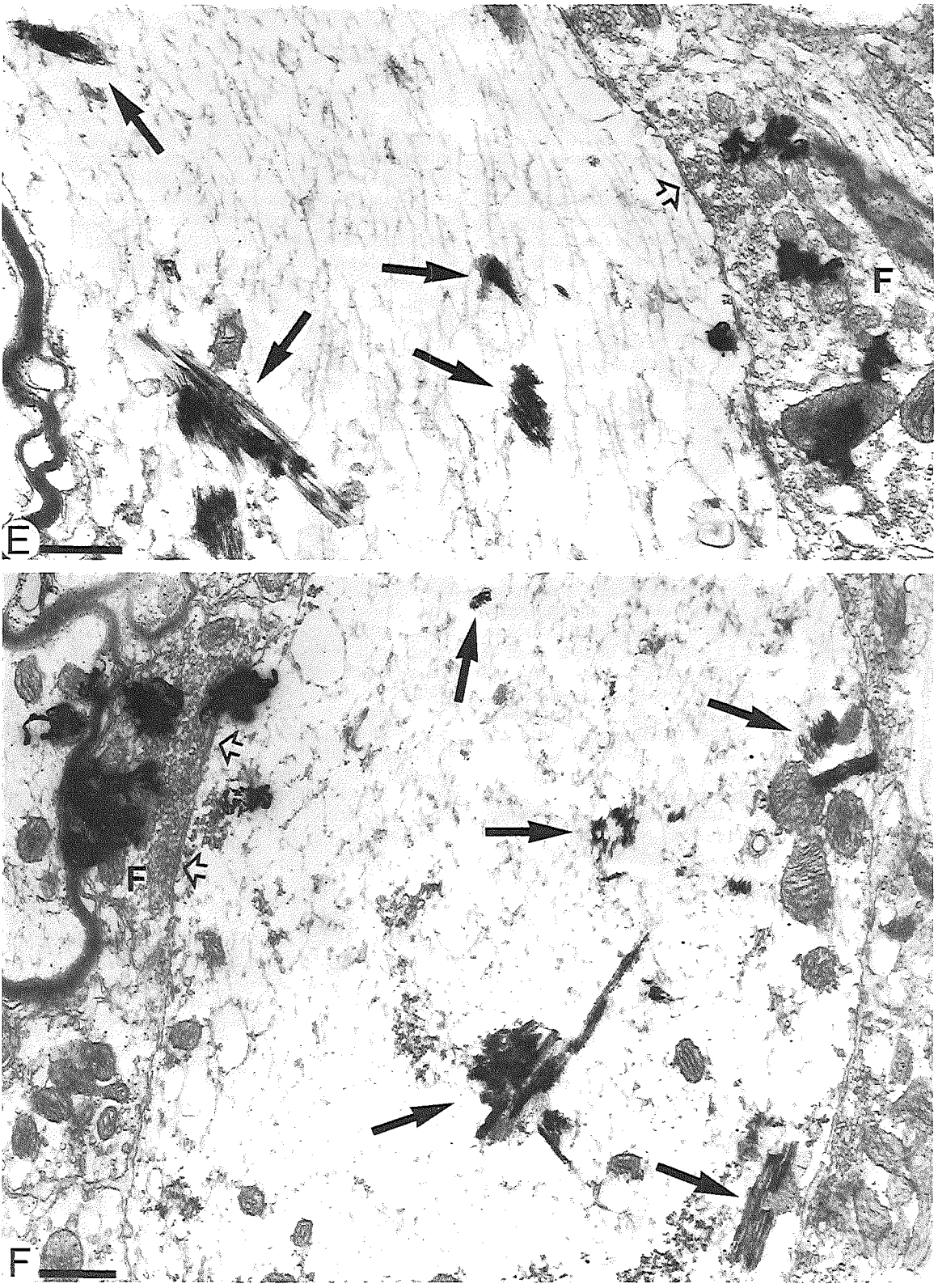
Figure 7

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dorsal part of the MRF, in which case the labelling was restricted to the intermediate zone of the ventral horn without involving the motoneuronal cell groups (c.f. also Martin et al., 1985). It was therefore concluded that the labelled terminals in the motoneuronal cell groups derived their radioactivity directly from neurons in the brainstem injection sites. In light of this the differences in densities of the labelling of the motoneuronal cell groups in the three groups of experiments were interpreted to indicate that projections to lumbar motoneuronal cell groups from the vlMRF, and to a lesser extent those from the raphe nuclei, were much denser than those from the area of the nucleus coeruleus and subcoeruleus (compare also quantities of ^{3}H-leucine and exposure times), which is in keeping with histofluorescent findings (Dahlström and Fuxe, 1965).

The analysis

The distribution of the radioactivity in the lumbar motoneuronal cell groups was studied using both the cluster method and the circle method. The cluster method marked individual structures which contained a large amount of radioactivity. The circle method gave an estimate of the probability that a specific tissue compartment contained radioactivity. On the basis of the findings obtained with the circle method (Fig 1) it was concluded that only terminals (T) and items comprising terminals contained radioactivity. However, according to the cluster method (Table 4) some axons also contained radioactivity. In order to understand this discrepancy it should be realized that the circle method only gave an estimate of the probability that a specific type of tissue compartment contained radioactivity. This was arrived at by comparing the distribution of the real grains over the tissue with that of randomly distributed hypothetical grains over the same tissue. This comparison (Fig. 1) showed that the item terminal (T) and the items comprising T had a high probability of containing radioactivity. On the other hand, axons (i.e. the items Ax and the groups of items Ax/CS-D-R) had a low probability while the remaining items had an even lower one. Therefore, guided by a customary statistical criteria (95% limits of confidence) only the items with T were considered to contain radioactivity and axons were not. However since this statement was based on probability it did not exclude that some axons contained radioactivity, which in fact was demonstrated by their cluster-labelling.

The findings obtained with the two methods were also used to determine whether in the various experiments different types of terminals were preferentially labelled. Since terminal profiles had a much higher probability of containing radioactiviy than the other tissue compartments (Fig. 1), it was assumed that in the items containing a terminal profile as well as other tissue compartments, the terminal profile represented the source of radioactivity. Therefore the type of terminal encountered in the various items was identified. On this basis the preferential distribution of the radioactivity over the different types of terminals in the different groups of experiments was assessed. The cluster method, on the other hand, marked individual terminals and in many cases their type could be identified. Thus also by means of this method a preferential labelling of different types of terminals in the different groups of experiments was determined.

The cluster-labelled terminals in general carried many silvergrains and therefore must have contained a large amount of radioactivity. From this it was inferred that their parent cell bodies probably were located in areas densely filled with radioactivity i.e. the centers of the injection sites. However, other terminals which carried only little radioactivity probably did not carry a cluster but only a few silver grains. These terminals, which could be detected only by the circle method, were possibly derived from parent cell bodies which contained relatively little radioactivity e.g. because they were located in the periphery of the injection site.

The terminal labelling

Earlier studies (Bodian, 1966; McLaughlin, 1972a; Conradi, 1969a; Bernstein and Bernstein, 1976) distinguished five major types of terminals (Table 1) in motoneuronal cell groups. However, in the present material a sixth type of terminal has been distinguished and designated as E-type (Fig. 6D-F). It contained many relatively small vesicles and sometimes also elongated or canaliculi-like structures (c.f. Table 1). This type of terminal was distinguished as a separate category because of its morphology and its pronounced labelling only after injections in the area of the nucleus coeruleus and subcoeruleus (Figs. 2 and 3). It had not been described earlier in the motoneuronal cell groups probably because it

is relatively rare and may be taken for a distorted F-type terminal.

After vlMRF injections the circle method as well as the cluster method showed that the F-type terminals were by far the most frequently labelled. Yet S- and G-types also showed some preferential labelling (Figs. 2 and 3). After injections in the raphe nuclei and the adjoining MRF both methods showed that F- and G-types were preferentially labelled, while according to the circle method S-type terminals also showed prominent labelling. The finding that in both groups of experiments F- and G-type terminals showed preferential labelling may be due to the fact that the injection sites in the vlMRF and in the raphe nuclei partially overlapped. Assuming that all axonal branches of one neuron are fitted with the same type of terminal, the above findings may indicate that the descending projections from the raphe nuclei and the vlMRF to the lumbar motoneuronal cell groups may be derived from at least two sets of neurons, e.g. serotonergic and non-serotonergic neurons (Bowker et al., 1982; Skagerberg and Björklund, 1985).

After raphe injections only the circle method revealed a prominent labelling of S-type terminals probably because their parent cell bodies did not contain a massive amount of radioactivity. This may have resulted from their location at the periphery of the injection site, e.g. in the vlMRF rather than in the raphe nuclei. Conversally the relatively strong preferential cluster-labelling of G-type terminals after raphe injections (Fig. 3) may have resulted from a concentration of their parent cell bodies in the raphe nuclei. According to both the circle and the cluster method vlMRF injections produced labelling of some C-type terminals, while no such labelling occurred after raphe injections. This labelling of C-type terminals was quite striking since they generally have been regarded to be derived from short propriospinal neurons (McLaughlin, 1972b; Matsushita and Ikeda, 1973; Bodian, 1975; Pullen and Sears, 1983).

In the third group of experiments with injections in the area of the nucleus coeruleus and subcoeruleus an entirely different distribution of the radioactivity over the different types of terminals was observed than in the cases of the first two groups (Figs. 2 and 3). Thus in the cases of the third group, according to both the circle method and the cluster method E-type terminals were preferentially labelled. Further, according to the circle method S-types were to some extent preferentially labelled and F-types also showed some labelling. These findings may have been due to the fact that the E-type terminals were derived from neurons in the center of the injection area and perhaps the same might apply to the S-type terminals. The F-type terminals on the other hand may have been derived from neurons in the periphery of the injection site.

Synaptic terminals and their postsynaptic structures

Approximately 40-50% (Tables 2 and 5) of the labelled S- and F-type terminal profiles exhibited a synapse, while few labelled E- and G-types exhibited a synapse. This may have resulted from the fact that they established relatively small sized synaptic contacts or that only few of these terminals established a regular synaptic contact. In the latter case their influence on motoneurons might be exerted in a diffuse non-synaptic manner (Maxwell et al., 1983; Chan-Palay, 1975; Beaudet and Descarries, 1978; Leger and Descarries, 1978; Beaudet and Sotelo, 1981; Wiklund et al., 1981a; Schaffar et al., 1983). The S-, F-, G- and E-types of terminals contacted proximal dendrites much more frequently than distal dendrites and seldom a cell soma. In cat the bulk of the total population of S- and F-type terminals in the lumbar motoneuronal cell groups were found on distal dendrites (Koziol and Tuckwell, 1977). If this also applies to the rat many of the S- and F-type terminals on distal dendrites must originate from other sources than the injection areas.

The transmitters

The raphe nuclei and the ventral part of the MRF contain many serotonergic neurons (Dahlström and Fuxe, 1964; Wiklund et al., 1981b; Steinbusch, 1981) while the locus coeruleus contains many noradrenergic neurons (Dahlström and Fuxe, 1964; Swanson, 1976; Wiklund et al., 1981b). In motoneuronal cell groups of monkey brainstem (Takeuchi et al., 1983) and chicken spinal cord (Atsumi et al., 1985) serotonin was found in varicosities containing several dense-cored vesicles. In rat ventral horn (Pelletier et al., 1981) serotonin was demonstrated inside dense-cored vesicles of such varicosities. Since these varicosities resembled the G-type terminals of the present study, they were expected to represent sero-

tonergic terminals. This was supported by the fact that these G-type terminals were only labelled after injections in the raphe and in the vlMRF and not after injections in the area of the nucleus coeruleus and subcoeruleus. By the same token, the E-type terminals may represent noradrenergic terminals since they were mainly labelled from the area of the nucleus coeruleus and subcoeruleus. However, this is at variance with other observations (Kojima et al., 1985) that noradrenalin tends to be concentrated in small granular vesicles and in large granules, which both are lacking in the E-type terminal. Moreover in other areas terminals resembling the E-type contained serotonin (Chan-Palay, 1975; Leger and Descarries, 1978; Beaudet and Sotelo, 1981; Beaudet and Descarries, 1981; Wiklund et al., 1981a).

EM autoradiography in combination with HRP-histochemistry

In the EM-autoradiographs of the L4-L6 spinal segments from the two rats with vlMRF injections combined with HRP injections in the ipsilateral hindleg muscles, only the cluster-labelled terminals were taken into account. The distributions (Table 7) were basically the same as in the three rats with only ^{3}H-leucine injections in the vlMRF (Fig. 3, Tables 5 and 6). In the rats with combined injections nearly all cell somata in the motoneuronal cell groups of the hindleg muscles contained TMB crystals and also many proximal dendrites as well as several distal dendrites contained such crystals. Since only HRP and not HRP coupled to wheat germ agglutinin was injected, it was most unlikely that these neurons were transneuronally labelled (Gerfen et al., 1982; Itaya and Van Hoessen, 1982; Ruda and Coulter, 1982; Harrison et al., 1984; Peschansky and Ralston, 1985; Porter et al., 1985; Wiesendanger and Wiesendanger, 1985). The labelled neurons were therefore regarded to represent motoneurons. More than 50% of the cluster-labelled synaptic terminals contacted HRP-labelled motoneurons (Table 7). This percentage should be regarded as a minimum since neither all motoneurons in the selected area were labelled nor all portions of a labelled motoneuron contained TMB crystals.

II.2.

AN ULTRASTRUCTURAL STUDY BY MEANS OF THE ANTEROGRADE TRANSPORT OF WHEAT-GERM AGGLUTININ COUPLED TO HORSERADISH PEROXIDASE AND USING THE TETRAMETHYL BENZIDINE REACTION

II.2.A. INTRODUCTION

Horseradish peroxide (HRP) in combination with the chromogen 3',3'-diaminobenzidine tetrahydrochloride (DAB) has been widely used for anterograde tracing both at the LM and the EM level. For anterograde tracing at the EM level, HRP in combination with DAB is much less efficient than ^{3}H-leucine in combination with the EM autoradiographic technique (Holstege, J.C. and Dekker, 1979). Recently it was demonstrated that the sensitivity of the HRP technique could be enhanced by coupling HRP to wheat-germ agglutinin (WGA) (Gonatas et al., 1979; Trojanowski et al., 1981) and even more so by using 3,3', 5,5'-tetramethyl benzidine (TMB) as a chromogen (Hardy and Heimer, 1977; Mesulam, 1978; 1982). So far TMB has been used in a few HRP studies at the EM level (Stürmer et al, 1981; Schönitzer and Holländer, 1981; Sakumoto et al., 1981; Carson and Mesulam, 1982; Aldes and Boone, 1985; Henry et al., 1985; Lemann and Saper, 1985; Naus et al., 1985; Westman et al., 1986). However, the HRP-TMB reaction product is probably unstable during osmification (Sakumoto et al., 1980; Carson and Mesulam, 1982; Henry et al., 1985) and in aqueous solutions or lower grades of alcohol (Stürmer et al., 1981). As a consequence of HRP-TMB reaction product may disappear when the tissue is processed for electron microscopy. Therefore several investigators still prefer the use of DAB as a chromogen at the EM level in spite of its lesser sensitivity in comparison with TMB.

In the present study an attempt was made to overcome the problems associated with the instability of TMB reaction product by using the method of chemical dehydration (Muller and Jacks, 1975) and by adjusting the temperature and pH of the osmium tetroxide solution (Sakumoto et al., 1980; Carson and Mesulam, 1982). In addition the efficacy of the terminal labelling obtained with the anterograde transport of WGA-HRP in combination with the chromogen TMB (WGA-HRP/TMB) was tested and compared with the efficacy of the terminal labelling obtained in a similar study using ^{3}H-leucine in combination with EM autoradiography (Ch. II.1.).

II.2.B. EXPERIMENTAL PROCEDURES

Six rats were used, five rats received two injections of 0,15 µL 2% WGA-HRP (Sigma ^{R}) in distilled water and one control rat received two injections of 0.15 µL 0,9% NaCl in distilled water. The injections were placed in the ventral part of the medial reticular formation (vlMRF) between the facial and hypoglossal nuclei 1.2 mm off the midline. Three days later the animals were deeply anaesthetized with pentobarbital and transcardially perfused with 0.9% NaCl in cacodylate buffer (pH 7.3, 37°C) followed by a fixation fluid containing 2.5% glutaraldehyde and 1% paraformaldehyde (pH 7.3, 20°C). The lower brainstems containing the WGA-HRP injection areas were kept overnight in the fixation fluid containing 30% sucrose. They were then cut transversely in frozen sections (30 µm) and treated according to the DAB method (Graham and Karnovsky, 1966). The sections were mounted on slides, dehydrated and lightly counterstained with cresyl-violet. Immediately after perfusion the L5 and L6 spinal segments of all rats were cut transversely in slabs (70 µm) on a vibratome and the ventral horn, ipsilateral to the brainstem injections, was dissected and treated according to the TMB method (Mesulam,

1978) except that plain acetate buffer (0.01 M., pH 3.3) was used throughout as a stabilizer. Immediately after the incubation, the dissected slabs were placed in phosphate buffer (pH 6.0, 20°C) for 5-15 min and then placed in 1.5% osmium tetroxide in phosphate buffer (pH 6.0, 45°C) (Sakumoto et al., 1980; Carson and Mesulam, 1982) for 40 minutes. After thorough rinsing in distilled water, they were dehydrated with di-methoxy-propane (Muller and Jacks, 1975) and embedded in Araldite. From these blocks, semithin sections were cut in which the WGA-HRP/TMB labelling was studied, especially in the three rats which showed the most pronounced HRP reaction. The blocks of these three rats and those from the control rat were trimmed to pyramids containing part of the motoneuronal cell groups. From these pyramids ultrathin sections were cut, which were mounted on 200 mesh grids, stained with uranyl acetate and lead citrate and then examined in a Philips 300 electron microscope.

II.2.C. Collection and Analysis of the Data

Three blocks were studied from each of the three rats injected with WGA-HRP and from the control rat. From each block 3-5 non-serial sections were used. A total of 47 sections was studied including 12 sections from the control rat. Each section was completely examined for TMB crystals. When such a crystal was encountered, it was photographed on reversal film, projected and analysed. In each photomicrograph, the tissue compartment containing one or more TMB crystals was registered. When a terminal profile was labelled its type was registered. Furthermore it was determined whether this profile established a synaptic contact in which case the postsynaptic structure was also registered. A total of 825 tissue compartments containing HRP reaction product were analysed. The results of this analysis were expressed for each block as percentages as described in the autoradiographic experiments (Ch. II.1.). In the majority of the cases a variance analysis (Snedecor and Cochran, 1980) performed on the different percentages obtained in each block did not show a statistically significant difference. Only in a few isolated cases an either significant or relatively large ($F > 2$) but not significant difference was found, which did not show a consistent pattern. It was therefore concluded that the variations in the percentages could not be attributed to differences between the animals but could only be attributed to differences between the blocks, resulting from random variations and errors of observation. This made it possible to calculate the 95% confidence limits of the mean of the percentages obtained in the different blocks.

II.2.D. Results

Light microscopy

In the DAB-reacted frozen sections of the lower brainstem from the five rats injected with WGA-HRP, the injection sites were located ipsilaterally in the ventrolateral MRF (Fig. 2A) at the levels between the hypoglossal nucleus and the caudal part of facial nucleus. The injection site included part of the ipsilateral inferior olive. The semithin sections of the L5 and L6 segments from these five rats were studied in the LM with bright field illumination. The HRP reaction products (the TMB precipitate) could be identified as dark brown particles in the neuropil of the motoneuronal cell groups. In several instances the reaction product was observed around a dendrite (Figs 2B, C), on a cell soma (Fig. 2B) or inside a myelinated axon. Occasionally a TMB-labelled cell soma was observed in the intermediate zone just outside the motoneuronal cell groups. In the semithin sections of the control rat no HRP reaction products were observed.

Electron microscopy

Three rats were studied in detail. Examination of the ultrathin sections from these rats showed many electron-dense crystal-like structures representing the TMB precipitate (Figs. 2D-N). In the 35 ultrathin sections studied, a total of 825 structures were labelled by these TMB crystals (Table 1), with an average of ±4.2 labelled structures per grid-square (7225 μm^2). The majority of these structures were terminal profiles (66%) while axons were much less frequently labelled (10%). Dendrites were also observed to contain TMB crystals (10% of the labelled

structures) and in one occasion a crystal was found inside a cell soma. Several labelled structures (14%) could not be identified mostly because they were very small and destroyed by the TMB crystal(s) and occasionally because of fixation artefacts.

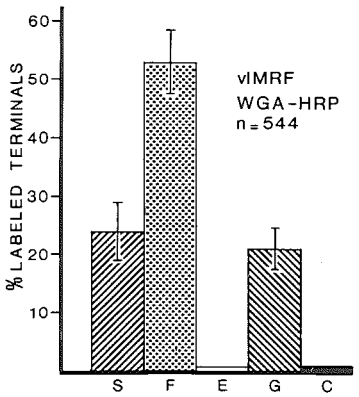
In the lumbar motoneuronal cell groups six major types of terminals were distinguished: F-type (flattened vesicles); S-type (spherical vesicles); E-type (micro-vesicles and some elongated ones); G-type (granular vesicles); C-type (with a subsynaptic cistern) and T-type (with subsynaptic densities, Taxi bodies) (for a detailed description see Ch. I.4 and Ch II.1). Fig. 1 shows that F-type terminals were most frequently labelled (53%) while S- and G-type terminals were labelled much less frequently (24% and 21% respectively). E- and C-type terminals were only seldom labelled (1% for both), while T-type terminals were never found to be labelled. Approximately 50% of the labelled F- and S-type terminals exhibited a synaptic contact mostly with proximal dendrites and to a much lesser extent dendrites. The few labelled E-type terminals which were observed did not show a synaptic contact and the C-type terminals always established a synapse and contacted exclusively proximal dendrites (Table 2). Labelled terminal profiles were never observed to be engaged in axo-axonic contacts. In the 12 ultrathin sections from the one control rat TMB-crystals were not encountered.

Table 1 Frequency of the different TMB-labelled structures.

	(%)	(n)
Terminal	66 ± 4	544
Axon	10 ± 4	84
Dendrite	10 ± 5	81
Cell Soma	<1 ± -	1

Values given are mean percentages (±95% confidence limits) and total numbers (n). See also Collection and Analysis of the Data.

Fig. 1. Frequency of the different TMB-labelled types of terminals.



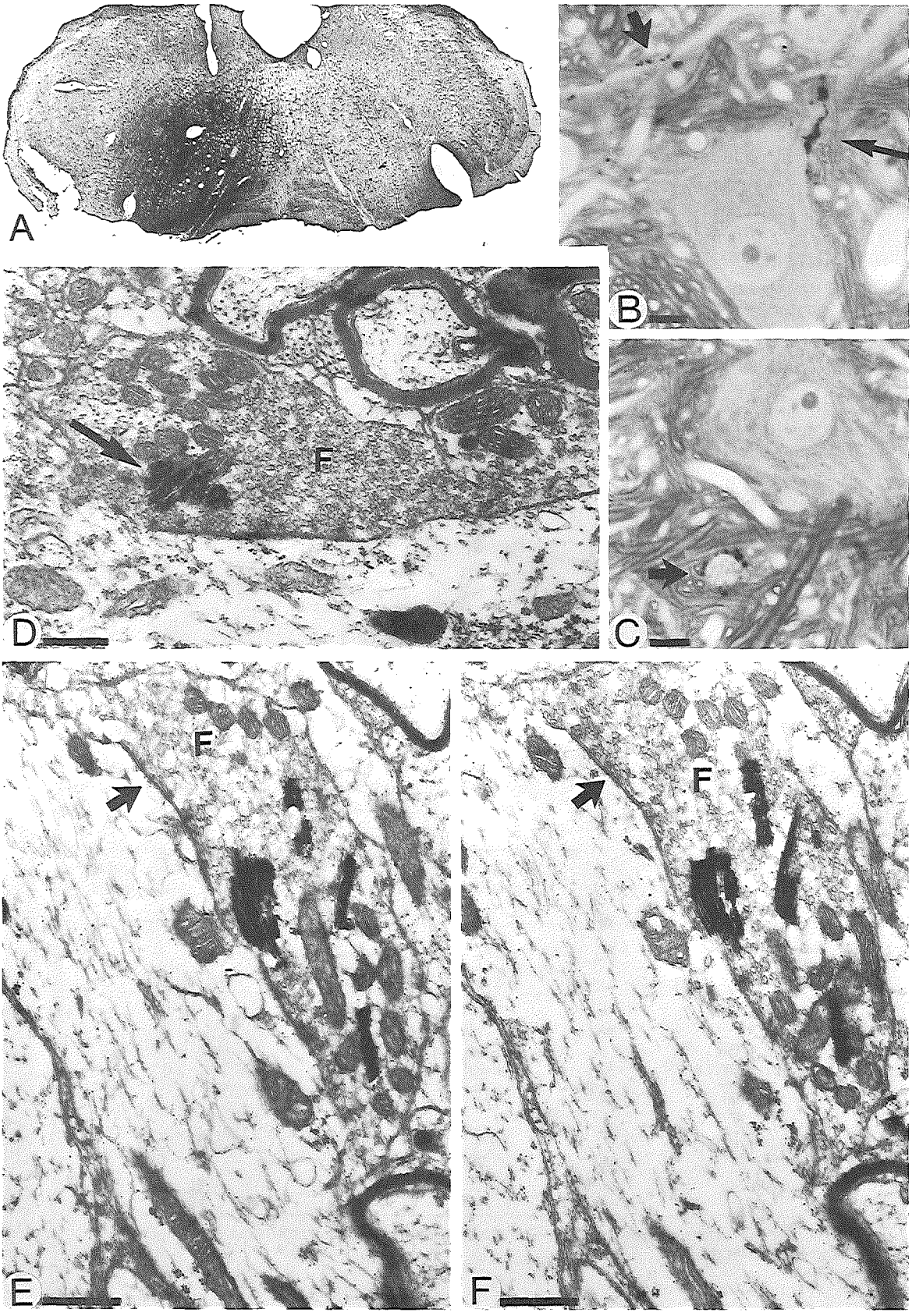
Values indicated are mean percentages (±95% confidence limits) and total number (n). See also Collection and Analysis of the Data.

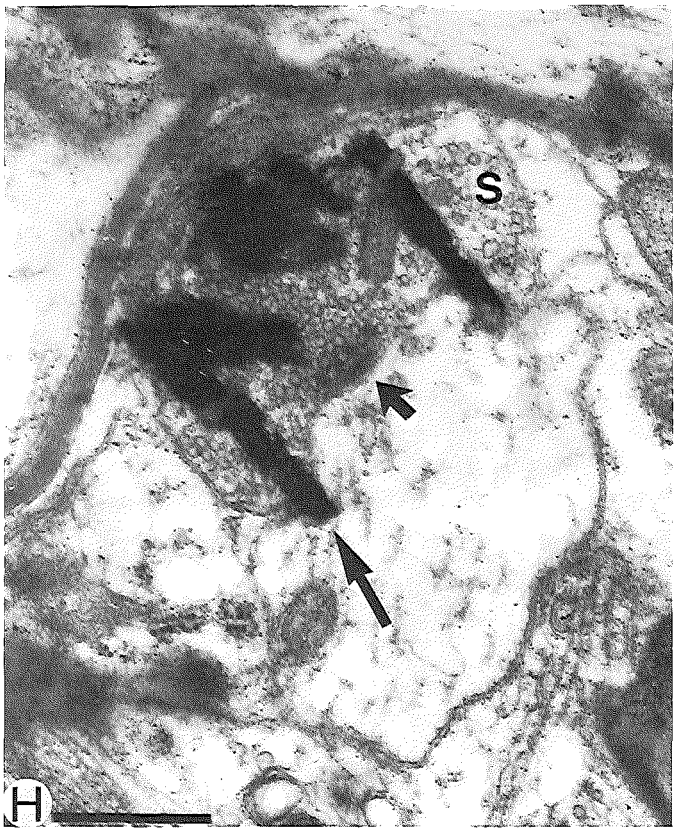
Table 2 Frequency of the different 3,3',5,5'-tetramethylbenzidine-labelled types of terminals (1), of the proportion showing synaptic contacts (2) and of the different types of terminals contacting the various postsynaptic structures (3).

(1) Type of terminal	(2) Percentage synaptic terminals	(3) Postsynaptic structure		
		Cell soma	proximal dendrites	distal dendrites
S-type (24%) ($n = 128$)	50% ± 7 ($n = 63$)	5% ± — ($n = 3$)	80% ± 13 ($n = 50$)	15% ± 10 ($n = 10$)
F-type (53%) ($n = 289$)	47% ± 6 ($n = 134$)	6% ± — ($n = 8$)	84% ± 9 ($n = 113$)	10% ± — ($n = 13$)
E-type (1%) ($n = 4$)	0% ($n = 0$)			
G-type (21%) ($n = 117$)	9% ± 6 ($n = 9$)	0% ($n = 0$)	89% ± — ($n = 8$)	11% ± — ($n = 1$)
C-type (1%) ($n = 5$)	100% ($n = 5$)	0% ($n = 0$)	100% ($n = 5$)	0% ($n = 0$)

Values given are mean percentages (± 95% confidence limits) and total numbers (n). See also Collection and Analysis of the Data.

Figure 2




S
H


F
I


F
G

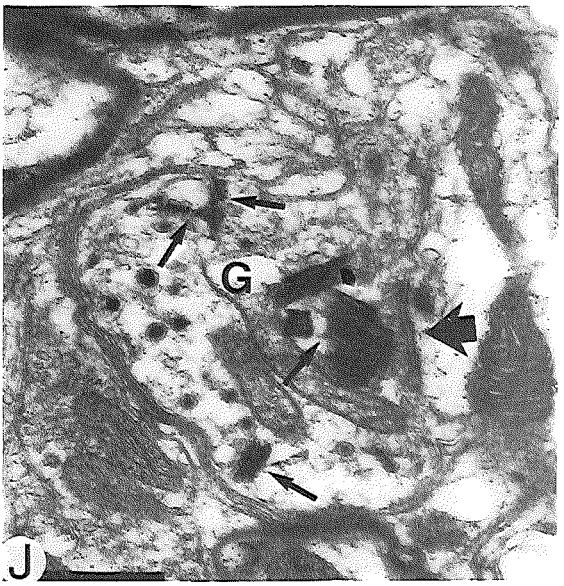

G
J

Figure 2

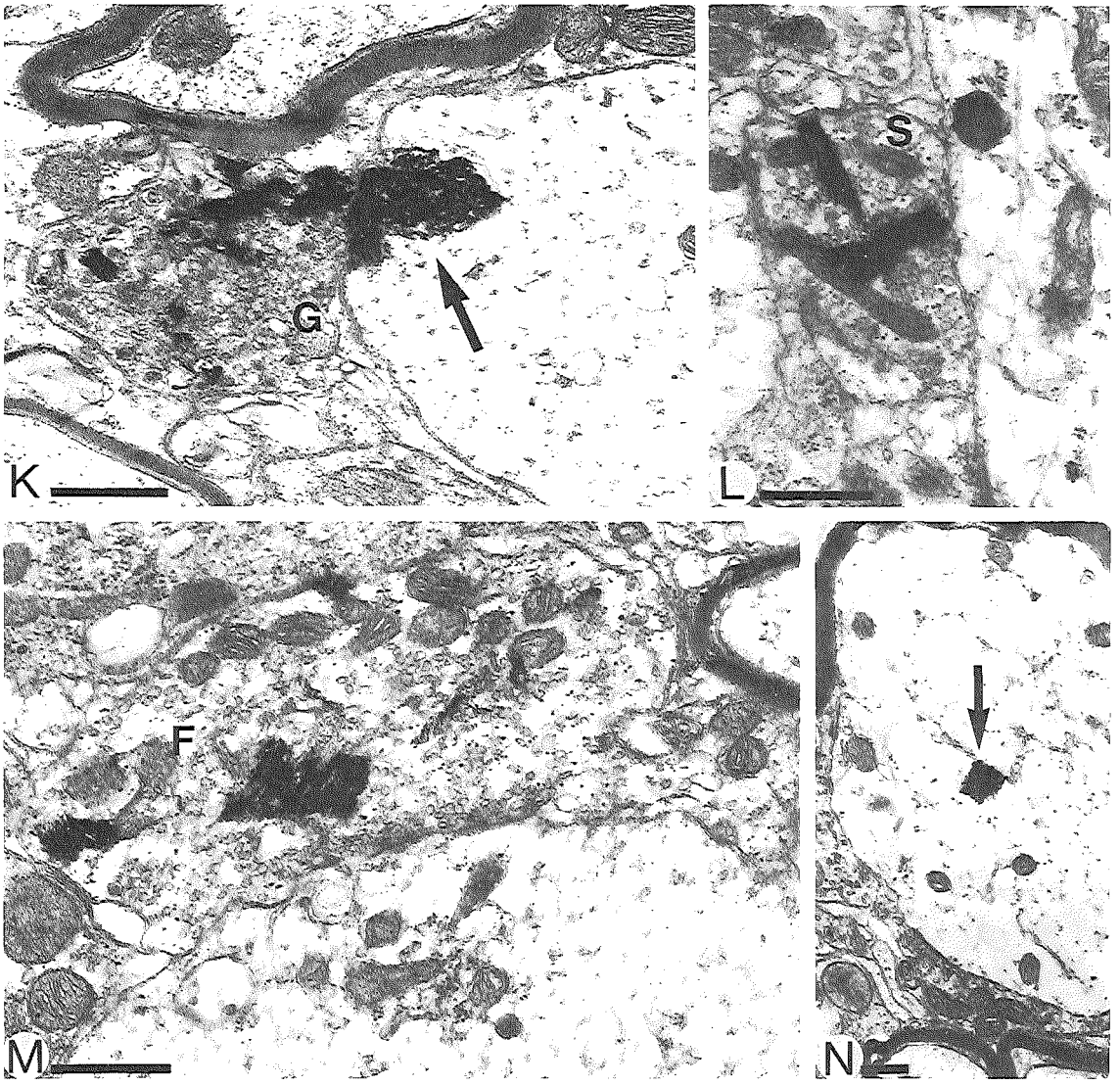


Fig. 2. (A.) Lightmicrograph of the WGA-HRP injection area in the MRF at the level of the inferior olive. Tissue reacted with DAB. (B-C.) Lightmicrographs of semithin sections (3 μm) from the L5 and L6 lateral motoneuronal cell groups after injections of WGA-HRP in the MRF. The tissue was reacted with TMB. Note the dark WGA-HRP reaction product around dendrites (short arrows) and alongside a cell soma and proximal dendrite (long arrows) Bar = 10 μm. (D-N.) Electronmicrographs of the L5 and L6 lateral motoneuronal cell groups after injection of WGA-HRP in the MRF. Tissue reacted with TMB. Bar = 0,5 μm. (D.) A labelled F-type terminal synapsing on a cell soma. Arrow indicates a TMB crystal which represents the WGA-HRP reaction product. (E-G.) A serial sectioned F-type terminal containing several TMB crystals and establishing a synaptic contact (arrows) with a proximal dendrite. Depicted are three profiles from a row of 6. H. An S-type terminal labelled with several TMB crystals and establishing a synapse (short arrow) on a distal dendrite. One TMB crystal (long arrow) has slightly protruded out of the terminal. (I.) An F-type terminal labelled with a large TMB crystal. (J.) A G-type terminal containing several TMB crystals (small arrows) and establishing a synaptic contact (large arrow) with a distal dendrite. (K.) A G-type terminal containing several TMB crystals. One crystal (arrow) is largely located within a dendrite but has a small portion inside the terminal. Possibly this dendritic labelling resulted from protrusion of the TMB crystal from the terminal into the dendrite. (L.) An S-type terminal containing two TMB crystals. A synaptic specialization is not visible. (M.) A TMB-labelled F-type terminal synapsing on a proximal dendrite. (N.) A small TMB crystal (arrow) located within a dendrite.

II.2.E. DISCUSSION

The findings obtained in the present ultrastructural study after WGA-HRP injections in the vlMRF using TMB as a chromogen clearly demonstrated the existence of descending projections to the lumbar motoneuronal cell groups, which is in keeping with the findings in the EM autoradiographic experiments (Ch. II.1.). In a similar study (Holstege, J.C., unpublished observations), using DAB as a chromogen, these projections could not be demonstrated, re-emphasizing the superior sensitivity of the chromogen TMB as compared with DAB.

Light microscopy

The frozen sections containing the WGA-HRP injection sites were incubated with DAB (Graham and Karnovsky, 1966). Since this method is less sensitive than the TMB method, it will result in a relatively smaller injection site. However the area where the injected WGA-HRP was taken up and from which it was subsequently transported is probably shown more accurately when using the DAB method. The location and extent of the WGA-HRP injection sites in the vlMRF (Fig. 2A) were almost identical to the ^{3}H-leucine injection sites in the vlMRF in the autoradiographic experiments (Ch. II.1.). This made it possible to compare the results obtained with the two different techniques.

In the semithin sections a dark-brown reaction product was identified in the lumbar motoneuronal cell groups (Figs. 2B, C). The density of this TMB labelling was far less than the density of the silvergrains in the same area obtained in the autoradiographic experiments. WGA-HRP labelled neurons which were occasionally observed in the ipsilateral intermediate zone probably resulted from retrograde labelling from passing fibers or from terminals in the injected area e.g. the inferior olive (Swenson and Castro, 1983) or the ventral MRF (including the lateral reticular nucleus) (Hrycyshyn and Flumerfelt, 1981; Shokunbi et al., 1985). It cannot be excluded that the labelling of neurons in the intermediate zone was the result of transneuronal transport of the WGA-HRP, a phenomenon which has recently been shown to occur (Gerfen et al., 1982; Itaya and Van Hoessen, 1982; Ruda and Coulter, 1982; Harrison et al., 1984; Peschansky and Ralston, 1985).

Electron microscopy

The method employed in the present study for processing the TMB-reacted slabs resulted in an acceptable preservation of the TMB crystals within the tissue, while the ultrastructure was only occasionally affected. Post-staining of the thin sections with uranyl-acetate and lead-citrate greatly improved the contrast in the tissue whereas the reaction product could still be easily recognized (Figs. 2D-N).

Examination of the ultrathin sections from the selected areas in the motoneuronal cell groups showed an average of ±4.2 TMB-labelled structures per gridsquare (7225 μm^2). This is somewhat less efficient in comparison with the number of structures which were labelled by a cluster of 6 or more silver grains in the EM autoradiographic experiments (Ch. II.1.) (average of ±7.2 clusters per gridsquare of 7225 μm^2), but far less efficient in comparison with the total number of silvergrains per gridsquare (calculated average of ±300 silver grains per gridsquare). This implies that for the anterograde tracing of terminals the general efficacy of the EM autoradiographic technique is superior to the WGA-HRP/TMB technique.

In respect to the distribution of the TMB crystals over the various tissue compartments it was found that the majority (66%) was located in terminal profiles, while 10% was located in axons and 14% in profiles which could not be identified (see Table I). This is in general agreement with the results obtained in the EM autoradiographic experiments. However, 10% of the TMB crystals was found to be located inside dendritic profiles (Fig. 2N). This finding could not be attributed to "background crystals" since the control experiment showed that no crystals were formed in the absence of HRP. It must therefore be assumed that these labelled dendrites belonged to neurons in the intermediate zone which were labelled retrogradely from the injection area (Hrycyshyn and Flumerfelt, 1981; Swenson and Castro, 1983; Shokunbi et al., 1985) or, more likely, that the WGA-HRP had been transported transsynaptically to motoneuronal dendrites. Finally the presence of TMB crystals inside dendrites may be explained by protrusion of crystals from terminals into dendrites (Fig. 2K) above or below the plane of section.

The distribution of the TMB crystals over the different types of terminals (Fig. 1) was basically similar to the distribution of the radioactivity over the different types of terminals obtained in the autoradiographic

experiments (Ch. II.1). This also holds true for the findings obtained for the percentages of "synaptic terminals" and the postsynaptic structures contacted by these terminals (Table 2). For a more detailed discussion of findings similar to those obtained in the present study see Ch. II.1.

II.2.F. CONCLUSION

The present study clearly shows that WGA-HRP in combination with TMB as a chromogen can be reliably used at the EM level for anterograde tracing of fiber systems and the identification of the different types of terminals and with a much better efficacy than can be obtained when using DAB as a chromogen. It is also shown that chemical dehydration of the tissue, which was used in order to preserve a maximum number of TMB crystals during processing, resulted in good preservation of the ultrastructure and also in a number of TMB crystals which was sufficient for quantitative analysis.

The WGA-HRP/TMB technique is much less time consuming and less complicated than the EM autoradiographic technique. In addition the analysis of the EM sections is less elaborate since most crystals are confined within one structure, while "background crystals" are virtually absent. The EM autoradiographic technique on the other hand has some major advantages over the WGA-HRP/TMB technique since a larger number of terminals can be labelled and a better preservation of the tissue can be obtained. Furthermore it is of importance to note that ^{3}H-leucine is taken up by cell somata only, while on the contrary WGA-HRP is taken up and transported from terminals and fibers of passage and may also be transported trans-neuronally (Gerfen et al., 1982; Itaya and Van Hoesen, 1982; Ruda and Coulter, 1982; Harrisson et al., 1984; Peschanski and Ralston, 1985).

It may thus be concluded that the autoradiographic technique as well as the WGA-HRP technique in combination with TMB as a chromogen can be reliably used for anterograde tracing at the EM level. A choice between the two methods should be determined for each individual experiment separately, guided by the advantages and the disadvantages of each method.

CHAPTER III

GENERAL DISCUSSION AND CONCLUSIONS

III.1.

ANTEROGRADE TRACING AT THE ULTRASTRUCTURAL LEVEL: AUTORADIOGRAPHY AND HRP HISTOCHEMISTRY COMPARED

III.1.A. INTRODUCTION

In the experiments described in Ch. II the projection from the brainstem medial reticular formation to spinal motoneurons in the rat were studied at the ultrastructural level. In these studies both ^{3}H-leucine (in combination with electron-microscopic (EM) autoradiography) and WGA-HRP (in combination with tetramethyl-benzidine (TMB) histochemistry) were used in the same neuronal system and the data were collected using a similar procedure. This makes it possible to compare the two anterograde tracing techniques with respect to three important aspects: reliability, analysis and efficiency.

III.1.B. RELIABILITY, ANALYSIS AND EFFICIENCY

The experiments described in Ch. II showed that with either tracing technique the majority of the labelling was located in terminals and to a lesser extent in axons. Moreover the same types of terminals were labelled. In the WGA-HRP experiments it was also found that 10% of the labelled structures were dendrites. This indicates that the WGA-HRP had been transported either transneuronally through the labelled terminals or retrogradely from the brainstem injection site to dendrites belonging to interneurons provided with ascending axons. Labelling of dendrites could not be demonstrated in the autoradiographic experiments using ^{3}H-leucine. As a whole the findings demonstrate that ^{3}H-leucine and WGA-HRP can be reliably used for anterograde tracing of terminals. They also show the different properties of the two tracers. ^{3}H-leucine is taken up by cell somata only (and not by passing fibers or terminals) and transported in an anterograde direction without transneuronal transport. WGA-HRP on the other hand is taken up by cell somata, injured fibers and by terminals and it is subsequently transported in anterograde and/or retrograde direction and may also be transported transneuronally.

The type of analysis which may be used on the data obtained with each technique has been described in Ch. I.3. With respect to the EM autoradiographic tracing technique it has been shown that, when single grains are analysed, the results are expressed in terms of probability and that it cannot be determined whether individual structures actually contain radioactivity. In addition there is a denomination problem which further complicates the single grain analysis. These problems can be partially overcome by using the cluster analysis. In the experiments described in Ch. II the results of the single grain analysis and the cluster analysis were basically similar. A few differences occurred, which were probably due to the fact that the cluster analysis can only detect heavily labelled structures. The analysis of the WGA-HRP material is far less complicated. Every structure containing one or more crystals is considered to be labelled, especially because it has been shown that TMB crystals are not formed in the absence of WGA-HRP. It should be kept in mind, however, that some parts of the brain contain endogenous HRP. This is not the case with spinal motoneurons or interneurons.

An indication of the efficiency of an anterograde tracing technique may be obtained by determining the average number of terminal profiles which are labelled in (non-serial) ultrathin sections. However, in case of the EM autoradiographic tracing technique, when using single grain analysis, it is not possible to determine the number of labelled profiles, but only the number of silvergrains which are observed. In contrast, in the cluster analysis the efficiency can be expressed as the number

TABLE 1

A COMPARISON OF THE DIFFERENT ASPECTS OF THE AUTORADIOGRAPHIC TECHNIQUE AND THE WGA-HRP TECHNIQUE AT THE ULTRASTRUCTURAL LEVEL.

	TRACER	
	^{3}H-leucine	WGA-HRP
Characteristics of tracer:	Anterograde transport only	Anterograde and retrograde transport
	Uptake by cell bodies only	Uptake by cell bodies, teminals and (damaged) axons
	Virtually no transneuronal transport	Significant transneuronal transport
Procedure:	Autoradiography	WGA-HRP histochemistry using TMB
Characteristics of procedure:	Relatively complicated	Relatively easy
	Time consuming (2 months up to 1 year)	Fast (1 week)
Ultrastructure:	Optimal	Some artifacts
Visualization:	Silver grains (size and shape depending on developer	TMB crystals
Resolution:	Depending on geometrical and photographic error	Depending on histochemical error
Background:	Present (variable)	Virtually absent
Analysis:	Single grains: complicated Clusters: relatively easy	Relatively easy
Efficiency:	Single grains: high Custers: relatively low	Relatively low

of labelled profiles that are observed and the same holds true for the analysis of the WGA-HRP material. In our experiments (Ch. II) an average of approximately 300 silvergrains and an average of 7 cluster-labelled structures per gridsquare (7225 μm^2) were found, whereas an average of 4 TMB-labelled structures per gridsquare was found in the WGA-HRP material. Since many of the parameters in these experiments were similar, the above findings may imply that the efficiency of the EM autoradiographic technique (with either method of analysis) is higher than the efficiency of the WGA-HRP technique. However, it should be noted that the EM autoradiographic technique is rather complicated and time consuming (with exposure times ranging from two months to more than one year), while the WGA-HRP technique is much easier to perform and results are obtained much faster (within one week). This difference may be of crucial importance in deciding which technique to use.

III.1.C. CONCLUSION

The various properties of the EM autoradiographic tracing technique and the WGA-HRP tracing technique are summarized in Table 1. The method to be used for anterograde tracing of terminals at the ultrastructural level largely depends on the system to be studied. It may be concluded that the EM autoradiographic technique with ^{3}H-leucine is most suitable for studying connections with a relatively weak projection or when retrograde transport and the existence of many passing fibers may interfere with the interpretation of the results. In other cases the WGA-HRP technique using TMB as a chromogen is to be preferred.

III.2.

DESCENDING BRAINSTEM PROJECTIONS TO SPINAL MOTONEURONS

III.2.A. INTRODUCTION

Anterograde degeneration findings showed that the descending projections to the spinal cord, which are involved in motor control, are derived from the motor cortex and from several nuclei in the brainstem (Kuypers, 1981; c.f. Ch. I.5). The descending projections from the motor cortex were found to terminate in a somatotopic manner mainly in the intermediate zone but, especially in higher primates, also in the motoneuronal cell groups (Kuypers, 1960; Schoen, 1964; Petras, 1967; Martin et al., 1975). According to the degeneration findings the descending brainstem projections, which largely parallel the cortical projections, also terminate primarily in the intermediate zone (Nyberg-Hansen, 1966b; Kuypers et al., 1972; Martin et al., 1975). On the basis of their termination in the intermediate zone the brainstem projections have been divided into a medial and a lateral system (Kuypers, 1964). The introduction of axonally transported tracers (see Ch. I.2) prompted a reinvestigation of the descending brainstem projections. Thus, after hemi-infiltration of cat spinal cord with HRP (Kuypers and Maisky, 1975) many retrogradely labelled neurons could be demonstrated in various brainstem areas. The majority of these areas were already known from anterograde degeneration studies to project to the spinal cord. However, several other areas such as the nucleus retroambiguus, the dorsal column nuclei, the locus coeruleus and subcoeruleus, the lateral pontine tegmentum including the nucleus of Koelliker-Fuse and even some hypothalamic areas were also found to project directly to the spinal cord. Similar findings were obtained in monkey (Kneisley et al., 1978), rat (Leichnetz et al., 1978), opossum (Crutcher et al., 1978), bird (Cabot et al., 1982) and lower vertebrates (Ten Donkelaar, 1982). Since HRP is taken up not only by terminals but also by damaged fibers of passage (Brodal et al., 1983), the exact terminations of these fiber systems in the spinal grey matter was difficult to determine by means of this technique. For this purpose anterograde transport techniques were used, which demonstrated that, in contrast to the earlier degeneration findings, the brainstem fibers to the spinal cord also terminate extensively in the motoneuronal cell groups. These axonal transport findings finally confirmed the much earlier histofluorescent findings of Dahlström and Fuxe (1965), which showed the existence of serotonergic and noradrenergic brainstem projections to the motoneuronal cell groups. The following paragraphs will discuss the various aspects of the descending projections from the nucleus coeruleus and subcoeruleus, the caudal raphe nuclei and the ventral part of the medial reticular formation to spinal motoneurons.

III.2.B. COERULEO-, RAPHE- AND MEDIAL RETICULO-SPINAL PROJECTIONS TO SPINAL MOTONEURONS

Anterograde transport techniques using tritiated amino acids in combination with light microscopy autoradiography showed that fibers from the locus coeruleus and subcoeruleus descended mainly ipsilaterally in the spinal white matter. These fibers are situated in the most peripheral parts of the lateral and ventral funiculi and terminated in all parts of the grey matter including the motoneuronal cell groups throughout the spinal cord (Holstege, G. et al., 1979; Martin et al., 1979; Westlund and Coulter, 1980; Holstege, G. and Kuypers, 1982; Jones and Yang, 1985). The caudal raphe nuclei and the adjoining medial reticular formation were also found to project to all parts of the spinal grey matter (Holstege,

G. et al., 1979; Martin et al., 1979; 1985; Holstege, J.C. and Kuypers, 1980; Holstege, G. and Kuypers, 1982; Jones and Yang, 1985), but these projections are much more intense than those from the nucleus coeruleus and subcoeruleus (Fig. 2). Retrograde and anterograde labelling studies showed that the raphe magnus projects mainly to the dorsal horn via the dorso-lateral funiculus, whereas the more caudally located raphe pallidus and obscurus and the adjoining medial reticular formation project mainly to the intermediate zone and the motoneuronal cell groups via the lateral and ventral funiculi (Basbaum et al., 1978; Martin et al., 1978; Holstege, G. and Kuypers, 1982). The existence of direct projections from the caudal brainstem to spinal motoneurons was recently confirmed in cat by means of retrograde transneuronal transport of WGA-HRP from spinal nerves through the corresponding spinal motoneurons to neurons in the caudal raphe and the adjoining medial reticular formation (Alstermark et al. 1987). Retrograde double labelling studies (Huisman et al., 1980; Hayes and Rustioni, 1981; Martin et al., 1981; Huisman et al., 1984) showed that the coeruleo- and raphe-spinal pathways gave off several collaterals along their trajectory in the spinal cord, indicating a relatively diffuse manner of projection. A further insight into the coeruleo-, raphe- and medial reticulospinal projections to the motoneuronal cell groups was provided by ultrastructural autoradiographic studies (Ch. II; Holstege, J.C. and Kuypers, 1982), which showed that after injection of ^{3}H-leucine in the respective brainstem areas the bulk of the radioactivity in the lumbar motoneuronal cell groups was located in terminals which contacted mostly proximal dendrites. After coeruleus and subcoeruleus injections two types of terminals were radioactively labelled (Fig 1), whereas after injections in the raphe pallidus and obscurus or the ventro-lateral part of the medial reticular formation three types of terminals were labelled, one of which contained many granular vesicles (Fig. 2) (for a description of the different types of terminals in the spinal motoneuronal cell groups see Ch. I.4). In some experiments the anterograde transport of ^{3}H-leucine from the ventro-lateral part of the medial reticular formation was combined with the retrograde transport of HRP from the hindleg muscles (Ch II). In the material from these rats many autoradiographically labelled terminals were observed to establish synaptic contacts mainly with HRP-labelled proximal dendrites. This clearly demonstrated the existence of direct contacts between neurons in the medial reticular formation and lumbar motoneurons. Many physiological studies have shown connections between brainstem neurons and spinal motoneurons mostly via interneurons in the intermediate zone (for reviews see Peterson et al., 1979; Wilson and Peterson, 1981). However, some electrophysiological studies also indicated the existence of monosynaptic connections with motoneurons. Some of these connections were inhibitory (Llinas and Terzuolo, 1964) (cf. Magoun and Rhines, 1946) while others were excitatory (Grillner and Lund, 1968; Wilson and Yoshida, 1969). These studies further suggested that the neurons exerting an inhibitory influence on motoneurons were located caudally in the medullary medial reticular formation at the level of the inferior olive, whereas those exerting an excitatory influence were located more rostrally. These pathways are probably non-serotonergic, since the iontophoretic application of serotonin by itself or in combination with peptides does not seem to elicit postsynaptic potentials in motoneurons (see below).

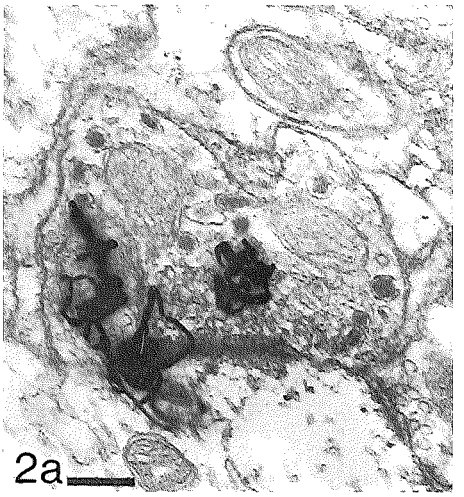
III.2.C. The Transmitters

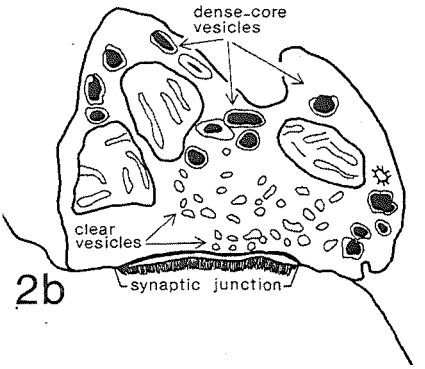
The existence of direct brainstem projections to spinal motoneurons was already shown in a histofluorescent study of Dahlström and Fuxe (1965). This study and those of others (Nygren and Olson, 1977; Commissiong et al., 1978; Bowker et al., 1982; Westlund et al., 1983) showed that spinal projections from the nucleus coeruleus and subcoeruleus were noradrenergic and those from the caudal raphe nuclei and adjoining ventral medial reticular formation were serotonergic. However recent studies demonstrated that there also exists a major non-serotonergic projection from the caudal raphe nuclei and ventral medial reticular formation to the spinal cord (Bowker et al., 1982; Skagerberg and Björklund, 1985) and a non-noradrenergic spinal projection from the locus coeruleus and subcoeruleus (Lai and Barnes, 1985; Stevens et al., 1985). Immunohistochemical studies combined with retrograde tracing or lesion techniques showed that several peptides were present in spinal projec-

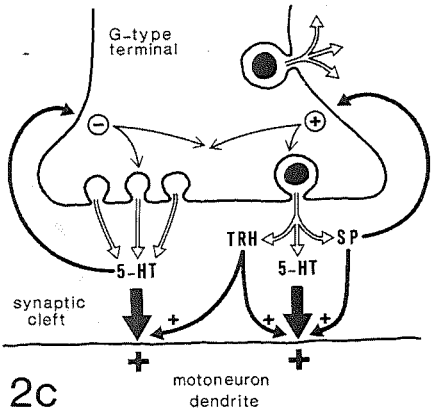
ting neurons of the caudal raphe nuclei and the adjoining ventral medial reticular formation. These peptides include substance P (Bowker et al., 1983), thyrotropin releasing hormone (TRH) (Bowker et al., 1983; Helke et al., 1986), enkephalin-like substances (Hökfelt et al., 1979; Bowker et al., 1983) and cholecystokinin (Mantyh and Hunt, 1984). Each of these peptides has been shown to coexist to a variable extent with serotonin (Chan-Palay et al., 1978; Hökfelt et al., 1978; Johansson et al., 1981; Hunt and Lovick, 1982; Mantyh and Hunt, 1984; Léger et al., 1986). The coexistence of serotonin with both substance P and TRH in one and the same neuron has also been reported (Johansson et al., 1981). Immunohistochemical and histofluorescent studies (Dahlström and Fuxe, 1964; Gibson et al., 1981; Steinbusch, 1981; Mantyh and Hunt, 1984) (for reviews see Hunt, 1983; Gibson and Polak, 1986; Tohyama and Shiotani, 1986) showed that these four peptides as well as serotonin and noradrenalin were also present in fibers and terminals in the spinal ventral horn; several of these findings were confirmed at the ultrastructural level (Johansson et al., 1980; Vacca et al., 1982; Atsumi et al., 1985; Kojima et al., 1985; Ulfhake et al., 1987). Immunohistochemical studies both at light (Wessendorf and Elde, 1985; Bowker, 1986) and electron microscopical (Pelletier et al., 1981) levels also demonstrated the coexistence of serotonin and substance P in some of these terminals. These findings are in keeping with the fact that destruction of serotonergic neurons by means of neurotoxins such as 5,6- or 5,7-dihydroxytryptamine (Johansson et al., 1981; Gilbert et al., 1982) resulted not only in serotonin depletion from the ventral horn but also produced depletion of substance P and TRH. It has therefore been assumed (Hökfelt et al., 1984) that some of the serotonergic terminals in the ventral horn may contain both substance P and TRH, as do the neuronal cell bodies in the caudal raphe nuclei and the ventral part of the medial reticular formation (Johansson et al., 1981), from which these terminals -in all likelihood- are derived. This is in agreement with the finding described in Ch. II that after ^{3}H-leucine injections in the raphe pallidus and obscurus or the ventro-lateral part of the medial reticular formation one type of radioactively labelled terminal in the ventral horn (Fig. 2a) displayed a morphology similar to that of the terminals in the ventral horn, which contain either serotonin (Atsumi et al., 1985; Ulfhake et al.,1987) or substance P (Vacca et al., 1982; Ulfhake et al., 1987) or both these substances (Pelletier et al., 1981) or TRH (Johansson et al., 1980; Ulfhake et al., 1987). The morphology of these terminals is characterized by the presence of a large number of dense core vesicles. Hökfelt and his collaborators have put forward the hypothesis (Hökfelt et al., 1984, 1986) that these dense core vesicles store serotonin together with substance P and TRH whereas the clear vesicles contain serotonin only. In addition, it has recently been shown in rat caudal trigeminal nucleus (Zhu et al., 1986) that the content of dense core vesicles may be released by exocytosis at non-synaptic sites of the terminal membrane. If this would also apply to the dense core vesicles in the terminals in the ventral horn, the different putative transmitters, contained in the dense core vesicles, might exert their effect on motoneurons beyond those which are reached through regular synaptic release. This hypothesis is further strengthened by the finding, obtained after serial sectioning of G-type terminals (Ulfhake et al., 1987), that some of the G-type terminals lack synaptic specializations. Fig. 2a shows an electron-micrograph of a radioactively labelled terminal profile, containing several dense core vesicles, after ^{3}H-leucine injections in the raphe pallidus and obscurus (Fig.2b). The events which may take place at such a terminal are shown in Fig. 2c.

The experiments described in Ch. II showed that, apart from the G-type terminals, F- and S-type terminals were also labelled from the caudal raphe nuclei and, more prominantly, from the ventro-lateral part of the medial reticular formation. These terminals may represent the non-serotonergic brainstem projection to the spinal motoneurons. Indeed it was shown very recently, by combining WGA-HRP anterograde tracing with GABA immuno-cytochemistry at the ultrastructural level, that some of the F-types, derived from the ventro-medial part of the lower brainstem, contained GABA (Holstege, J.C., 1989). It was also shown in this study that some of the (presumed serotonergic) G-type terminals derived from the lower brainstem, contained GABA. This finding is in agreement with studies which showed coexistence of serotonin and GABA in neurons of the caudal raphe nuclei and the adjoining reticular formation, which project to the spinal cord (Milhorn et al., 1987). The transmitter contained within


1


2a


dense-core
vesicles
clear
vesicles
synaptic junction
2b


G-type
terminal
5-HT
TRH
5-HT
SP
synaptic
cleft
motoneuron
dendrite
2c

the S-type terminals is still unknown. Based on the morphology of these terminals (i.e. their content of spherical vesicles and their asymmetric synaptic junctions), it may be speculated that they contain an excitatory transmitter. There is evidence for the existence of a cholinergic pathway from the lower brainstem to the spinal cord (Bowker et al., 1983; Jones et al., 1986). Since acetyl-choline is generally considered an excitatory transmitter, the S-type terminals derived from the lower brainstem may contain acetyl-choline.

III.2.D. Functional Implications

The functional effects of the descending serotonergic projections to motoneurons have been investigated using various experimental designs (see e.g. Jacobs, 1976; Barbeau and Bédard, 1981; Hansen et al., 1983). These studies generally showed that serotonergic pathways had a facilitatory effect on motoneurons and further indicated that substance P and TRH also had a facilitatory effect, possibly by modulating the effect of serotonin on the motoneuronal membrane (Tremblay et al., 1986) and, in case of substance P, also by auto feedback on the terminal (Mitchell and Fleetwood-Walker, 1981) (fig. 2c). Studies using iontophoretic application of various putative transmitter substances on motoneurons in vivo, also showed that serotonin, substance P and TRH enhanced the excitability of motoneurons, either for glutamate and aspartate (White and Neuman, 1980; White and Neuman, 1983; White, 1985) or for inputs from red nucleus and cortex (McCall and Aghajanian, 1979). The same studies showed that similar facilitatory effects were obtained after iontophoretic application of noradrenalin. Thus the coeruleo- and raphe-spinal pathways appear to mediate an overall facilitation of motoneurons. This was confirmed by physiological studies using electrical stimulation in the locus coeruleus and subcoeruleus (Fung and Barnes, 1981; Chan et al., 1986) or the raphe nuclei (Cardona and Rudomin, 1983; Roberts et al., 1988). In addition these facilitatory effects could be blocked by antagonists of noradrenaline or serotonin respectively. The various data provide support for the hypothesis that the serotonin containing fibers from raphe pallidus and obscurus, which project in a diffuse manner to the spinal ventral horn, act as a gain-setting system (McCall and Aghajanian, 1979), enhancing the overall responsiveness of motoneurons. Neurons in the raphe magnus which project to the spinal dorsal horn exert an inhibitory influence on pain transmission (Willis, 1982; Basbaum and Fields, 1984). If these two raphe systems, projecting to the ventral and the dorsal horn, would act simultaneously, it would lead to an enhanced responsiveness of the motor system accompanied by a decreased experience of pain; a state well known from circumstances of fight and flight (Kuypers and Huisman, 1982). The

Fig. 1 Electronmicrograph of an autoradiographically labelled terminal in the rat L5 motoneuronal cell groups after a ^{3}H-leucine injection in the area of the nucleus coeruleus and subcoeruleus. Note the small, relatively elongated vesicles in the labelled terminal (E). Unlabelled terminals show spherical (S) or pleiomorphic (F) vesicles. Bar = 0,2 μm.; 9.5 months exposure time.

Fig. 2a Electron micrograph of an autoradiographically labelled terminal in the L5 motoneuronal cell groups after a ^{3}H-leucine injection in the raphe pallidus and obscurus. Note the large number of dense core vesicles. Arrow points at a coated vesicle. Bar = 0,2 μm., 4 months exposure time.

Fig. 2b Schematic representation of the terminal depicted in fig. 5a.

Fig. 2c Schematic representation (modified after Hökfelt, 1986) of the events which may take place at terminals in the ventral horn with a morphology similar to the terminal shown in 5a and b. Open arrows indicate exocytotic release of 5-HT (serotonin) or 5-HT, substance P and TRH. Large black arrows indicate facilitatory effects of 5-HT on motoneuron excitability. Small black arrows indicate a similar effect on the motoneural dendrite or auto feedback on the terminal which is either negative (5-HT) or positive (substance P), in the latter case possibly by blocking the 5-HT autoreceptor. Exocytotic release from dense core vesicles at non-synaptic sites of the terminal membrane is indicated on the right. For details see text. SP= substance P, 5-HT= 5-hydroxytryptamine (serotonin), TRH= thyrotropin releasing hormone.

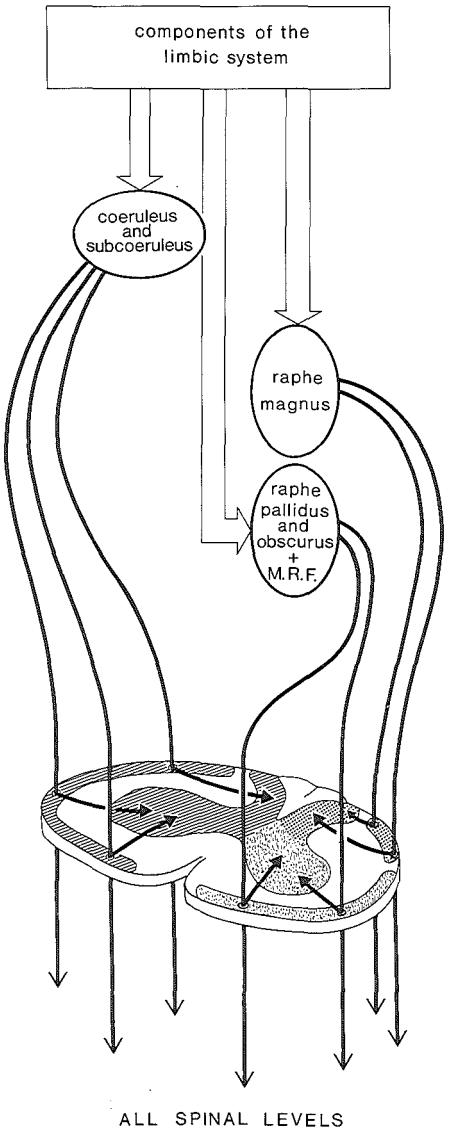


Fig. 3 Schematic representation of the projections from the locus coeruleus and subcoeruleus (on the left) and the caudal raphe nuclei plus the ventral part of the adjoining medial reticular formation (on the right) to all spinal levels by means of several collaterals. The position of the descending fibers in the white matter and the area of termination in the gray matter are shown by the different shadings. Projections from the limbic system to these brainstem areas are also indicated. M.R.F.= ventral part of the medial reticular formation.

same appears to apply to the spinal projections from the locus coeruleus and subcoeruleus, since electrical stimulation in this area produced a facilitatory effect on spinal motoneurons (Fung and Barnes, 1981; Chan et al., 1986) and an inhibitory effect on pain transmission in the dorsal horn (Jones and Gebhart, 1986; Mokha et al., 1986).

The role of the GABA-ergic fibers which project from the ventro-medial part of the reticular formation to spinal motoneurons is less clear. The GABA-ergic projections are derived from the same area in the lower brainstem as the serotonergic fibers. Since many of the spinal projecting neurons in this area give off several collaterals in the spinal cord, it seems likely that the GABA-ergic projections are also highly collateralized. Thus, in analogy with the serotonergic projection, the descending GABA-ergic pathways probably exert a more general influence on motoneurons in the spinal cord and may not be involved in the execution of specific motor tasks. GABA is known as an inhibitory transmitter (Krnjévic and Schwarts, 1966). It is therefore to be expected the GABA-ergic projection will counteract the facilitatory role of serotonin. The balance between the activity in the GABA-ergic and the serotonergic fibers may set the level of excitability of the motoneurons. Physiological studies (Hounsgaard et al., 1988; Crone et al, 1988) have shown that spinal motoneurons display a bistable behaviour, i.e. the motoneurons can be "switched" to a more excitable level. This phenomenon disappeared after spinal transection, but reappeared following intravenous injection of the serotonin precursor 5-hydroxytryptophan, suggesting the involvement of descending serotonergic projections. It was also found that short inhibitory impulses could reset the excitability of the motoneurons to its "normal" level. This effect may be achieved by the descending GABA-ergic projections. The fact that GABA is located in presumed serotonergic terminals in the motoneuronal cell groups may further indicate the intimate relation between GABA and serotonin in this system. During sleep (see e.g. Morrison, 1983) and "relaxed states" of waking it seems likely that the balance between the serotonergic and the GABA-ergic fibers is shifted to the GABA-ergic side with less activity of the serotonergic fibers. Indeed recordings from serotonergic neurons in the lower brainstem of unrestrained animals (for a review, see Jacobs, 1986) have shown that these neurons

are silent during active sleep (a sleep phase during which there exists a complete muscle atonia). When the animal passes through the various states of sleep towards quiet and active waking, the serotonergic neurons become more active.

Taken together, it seems likely that the GABA-ergic and the serotonergic fibers from the lower brainstem, which bypass the interneurons, directly control the excitability of the spinal motoneurons. This system may provide an important mechanism by which the brain can adjust motor behaviour according to the state of arousal of the organism. In view of the above it is of interest to note that the brainstem areas in question receive an important projection from several limbic structures. Thus the locus coeruleus and subcoeruleus receive afferents from the medial and lateral hypothalamus (Hosoya and Matsushita, 1981; Holstege, G., 1987), the bed nucleus of the stria terminalis (Holstege, G. et al., 1985) and the amygdala (Hopkins and Holstege, G., 1978). It should be noted that -in contrast to its surrounding structures like the nucleus subcoeruleus and the parabrachial nuclei- the locus coeruleus proper seems to receive only a minor projection from limbic areas, while its main input is derived from the ventral part of the reticular formation in the lower brainstem and the nucleus prepositus hypoglossi (Aston-Jones et al., 1986). The caudal raphe nuclei and adjacent reticular formation receive a strong input from the medial hypothalamus and the peri-aquaductal grey (Basbaum and Fields, 1984; Hosoya, 1985; Holstege, G., 1987). By way of the various connections mentioned above (fig. 6), the limbic system could have access to all regions of the spinal cord (Kuypers and Huisman, 1982). This would imply that the emotional brain exerts a powerful control over both sensory input and motor output.

III.2.E. CONCLUSIONS

1. The existence of direct projections to spinal motoneurons and interneurons from the raphe pallidus and obscurus, the adjoining ventral medial reticular formation and the locus coeruleus and subcoeruleus is now well substantiated by various anatomical techniques.
2. The spinal projections from the raphe nuclei and the adjoining medial reticular formation contain serotonergic and GABA-ergic fibers. They also contain various peptides several of which are contained within the serotonergic fibers. Whether still other transmitter substances (e.g. acetylcholine or glycine) are present in the various descending brainstem projections to motoneurons remains to be determined.
3. The spinal projections from the locus coeruleus and subcoeruleus are mainly noradrenergic, but there also exists a non-noradrenergic spinal projection.
4. Pharmacological, physiological and behavioural studies indicate an overall facilitatory action of noradrenaline and serotonin (including several peptides) on motoneurons. This may lead to an enhanced susceptibility for excitatory inputs from other sources.
5. The action of GABA is generally considered as inhibitory. Therefore the balance between the facilitatory action of serotonin and the inhibitory action of GABA, may determine the responsiveness of the motoneurons in the spinal cord.
6. The brainstem areas in question receive an important projection from several components of the limbic system. This suggests that the emotional brain can exert a powerful influence on all regions of the spinal cord and may thus control both its sensory input and motor output.

SUMMARY

This thesis deals with an ultrastructural study in rat on the descending projections from the ventro-lateral medullary medial reticular formation, the medullary raphe nuclei and the area of the nucleus coeruleus and subcoeruleus to the motoneuronal cell groups in the lumbar spinal cord. Since these projections terminate not only in the intermediate zone, but also directly on motoneurons, they constitute a special subgroup of the descending pathways to the spinal cord (reviewed briefly in Ch I.5).

Three techniques were used in this study: 1. The anterograde transport of ^{3}H-leucine, visualized by means of electron microscopy (EM) autoradiography; 2. the anterograde transport of wheat germ agglutinin coupled to horseradish peroxidase (WGA-HRP), visualized by means of tetramethyl benzidine (TMB) histochemistry; and 3. the anterograde transport of ^{3}H-leucine combined in the same animal with the retrograde transport of HRP. Details on axonal transport and the various techniques used for tracing connections in the brain are given in Ch I.1, I.2, and I.3.

The actual studies on the descending brainstem projections to spinal motoneurons are described in Ch. 2. The following results were obtained:

1. After ^{3}H-leucine injections in the ventrolateral medullary medial reticular formation, the medullary raphe nuclei and the area of the nucleus coeruleus and subcoeruleus, numerous silvergrains were observed in the EM autoradiographs. On these silvergrains two types of analysis were performed: A. the circle method, a statistical approach in which the probability that a specific type of tissue compartment (terminals, axons, dendrites, etc.) contains radioactivity is determined; and B. the cluster method, which is based on the assumption that those profiles, which carry a cluster of 6 or more silvergrains, are radioactively labelled by the transported ^{3}H-leucine. In all cases both types of analysis revealed that the majority of the radioactivity was located in terminals. In the analysis of the labelled terminals, five different types of terminals were distinguished: S-type terminals (containing mainly spherical vesicles), F-type terminals (containing many flattened vesicles), G-type terminals (containing granular vesicles), C-type terminals (with a subsynaptic cistern), P-type terminals (presynaptic to other terminals). A detailed description of the different types of terminals is given in Ch. I.4. A sixth type, the E-type (containing very small and some elongated vesicles), emerged from this study and had not been described before in the motoneuronal cell groups.

After injections in the ventrolateral medullary medial reticular formation both types of analysis showed that F-type terminals were most frequently labelled (±60%), whereas much fewer S- and G-type terminals were labelled (both ±20%). Approximately 2% of the labelling was found in C-type terminals. After ^{3}H-leucine injections in the medullary raphe nuclei the same types of terminals (except for the C-type terminal) were labelled. However, with the cluster analysis, G-type terminals were more frequently labelled (60%) than F-type terminals (33%), whereas with the circle analysis they were labelled equally frequent (both 37%). S-type terminals were especially labelled in the circle analysis (17%) and much less in the cluster analysis (5%). These differences between the circle and the cluster method may be attributed to the fact that the cluster method can only detect heavily labelled terminals, possibly originating from the center of the injection site, whereas the circle method may also detect terminals with less labelling, possibly originating from the periphery of the injection site. Thus the G-type terminals may originate mainly from the medullary raphe nuclei, whereas the F- and S-type terminals may originate mainly from the ventral part of the medial reticular formation.

After ^{3}H-leucine injections in the area of the nucleus coeruleus and subcoeruleus the labelling was especially located in E-type terminals and (in the cluster analysis to a much lesser extent) in S-type terminals. F-type terminals were also labelled, but only in the circle analysis, possibly indicating that these terminals originate from the periphery of the injection site.

After all injections approximately 40% to 50% of the labelled S- and F-type terminal profiles established synaptic contacts, but only approximately 10% of the labelled E- and G-types did so. In all cases these synaptic contacts were established mainly with proximal dendrites (±75%) and much less with distal dendrites (±20%) and cell somata (±5%).

2. After injections of WGA-HRP in the ventro-lateral part of the medullary medial reticular formation, the labelling (the TMB reaction products) was located mainly in terminals (66%) and to a lesser extent in axons (10%) and dendrites (10%). The same types of terminals (i.e. F-, G-, S-and C-type terminals) were labelled and with approximately the same frequency as found after ^{3}H-leucine injections in the same area of the lower brainstem. This was also true for the percentages of the various labelled types of terminals, which exhibited a synaptic contact and their postsynaptic structures. It is concluded that the horseradish peroxidase tracing technique employed in the present study can be reliably used for anterograde tracing at the ultrastructural level. Its efficiency, advantages and disadvantages in comparison with the electron microscopical autoradiographic technique are discussed in Ch. II.2. and III.1.

3. In two rats ^{3}H-leucine injections in the ventro-lateral medial reticular formation were combined with HRP injections in the ipsilateral hindleg muscles, resulting in retrograde labelling of the corresponding motoneurons as visualized by the TMB reaction products. Only the cluster-labelled terminals were studied. It was found that more than 50% of the postsynaptic structures which were contacted by cluster-labelled terminal profiles contained TMB reaction products. Since not all motoneurons, nor all parts of a motoneuron can be expected to contain reaction products, it was concluded that the large majority of the terminals originating from neurons in the medial reticular formation actually contacted motoneurons.

In Ch II.1 and especially in Ch. III.2 the findings are discussed with a focus on the transmitters which may be present in the different types of terminals originating from the lower brainstem. It is argued that at least two, but probably three different sets of neurons, located in the medullary raphe nuclei and the adjoining ventro-medial part of the medial reticular formation, project to motoneurons in the lumbar spinal cord. The three different sets of neurons are provided with different types of terminals and may contain different transmitters: the G-type terminals probably are serotonergic, the F-type terminals (but probably not all of them) may contain GABA, whereas the transmitter, present in the S-type terminal is still unclear. Similarly the descending projections from the area of the locus coeruleus and subcoeruleus may originate from two different sets of neurons, provided with E- and S-type terminals respectively. One of these types, possibly the E-type, may contain noradrenalin as a transmitter.

With respect to the functional meaning of the brainstem projections to spinal motoneurons, it is proposed that serotonin and noradrenalin exert a facilatory influence on spinal motoneurons, by increasing their susceptibility for other excitatory inputs. These effects may in turn be counteracted by the presumed GABA-ergic projection from the lower brainstem. Parts of the limbic system directly infuence the neurons in the brainstem, which give rise to the descending projections to motoneurons. Therefore it seems likely that the control exerted by the limbic system on motor behaviour is, at least partly, mediated by these brainstem neurons.

SAMENVATTING

Dit proefschrift beschrijft een electronen microscopisch (EM) onderzoek van de afdalende banen vanuit de hersenstam naar de motoneuronale cel-groepen in het lumbale ruggemerg van de rat. Deze verbindingen ontspringen aan neuronen in het ventro-laterale deel van de mediale reticulaire formatie, de raphe kernen en de nucleus coeruleus en subcoeruleus. Aangezien deze projecties niet alleen in de intermediaire zone van het ruggemerg eindigen maar ook direct op motoneuronen, vormen zij een speciale subgroep van de afdalende banen naar het ruggemerg (voor een kort overzicht zie Hfdst. I.5).

In deze studie werden 3 technieken gebruikt: 1) het anterograde transport van ^{3}H-leucine, dat werd aangetoond m.b.v. EM autoradiografie, 2) het anterograde transport van mierikswortel enzym (horseradish peroxidase) gekoppeld aan wheat-germ agglutinin (WGA-HRP), aangetoond d.m.v. incubatie met tetramethyl benzidine en 3) het anterograde transport van ^{3}H-leucine gecombineerd met het retrograde transport van HRP. Details aangaande het axonaal transport en de verschillende technieken die worden gebruikt voor het aantonen van verbindingen in de hersenen werden beschreven in Hfdst.I.1., I.2. en I.3.

Het onderzoek van de afdalende banen naar motoneuronen in het ruggemerg wordt beschreven in Hfdst. II. De volgende resultaten werden verkregen.

1) Na ^{3}H-leucine injecties in het ventro-laterale deel van de mediale reticulaire formatie in de lage hersenstam, in de raphe kernen aldaar en in het gebied van de locus coeruleus en subcoeruleus, werden vele zilverkorrels (grains) gezien in de EM autoradiografische coupes. Deze grains werden op 2 manieren geanalyseerd: a) met de cirkel methode, waarbij via een statistische benadering wordt bepaald hoe groot de waarschijnlijkheid is dat een bepaalde weefselstructuur (eindigingen, dendrieten, axonen etc.) radioactiviteit bevatten; en b) met de "cluster" methode, waarbij ervan uit wordt gegaan dat de structuren, waarboven een groep van 6 of meer grains is gelegen, radioactief gelabeld zijn door het getransporteerde ^{3}H-leucine. In alle gevallen en bij beide manieren van analyse, bleek dat het grootste deel van de radioactieve labelling zich in eindigingen bevond. Bij de verdere analyse van de gelabelde eindigingen werden vijf verschillende typen onderscheiden: S-type eindigingen (met voornamelijk ronde blaasjes), F-type eindigingen (met voornamelijk platte blaasjes), G-type eindigingen (met granulaire blaasjes), C-type eindigingen (met een subsynaptische cisterne), en P-type eindigingen (presynaptisch voor andere eindigingen). Een gedetailleerde beschrijving van de verschillende typen eindigingen wordt gegeven in Hfdst. I.4. Een 6e type eindiging, het E-type (met veelal erg kleine en verschillende langwerpige blaasjes, kwam naar voren in de loop van dit onderzoek en was nog niet eerder beschreven in de motoneuronale celgroepen.

Na injecties in het ventro-laterale deel van de mediale reticulaire formatie in de hersenstam bleek dat, bij beide manieren van analyse, de F-type eindigingen het meest gelabeld waren (±60%), terwijl veel minder S- en G-type eindigingen waren gelabeld (beide ±20%). Ongeveer 2% van de labelling bevond zich in C-type eindigingen. Na ^{3}H-leucine injecties in de raphe kernen werden dezelfde typen eindigingen gelabeld (met uitzondering van het C-type). Echter bij de cluster analyse was het G-type vaker gelabeld (60%) dan het F-type (33%), terwijl met de cirkel analyse beide typen even vaak gelabeld waren (beide 37%). S-type eindigingen waren met name gelabeld bij de cirkel analyse (17%) en veel minder bij de cluster analyse. Deze verschillen tussen de cirkel en cluster methode zijn mogelijkerwijs het gevolg van het feit dat met de cluster methode uitsluitend "hard" gelabelde structuren worden gevonden, die mogelijk uit het centrum van de injectieplaats afkomstig zijn. Daarentegen kunnen met de cirkel methode ook eindigingen worden gevonden die minder hard zijn gelabeld en mogelijk afkomstig zijn van neuronen in de rand van de injectieplaats. Het is daarom waarschijnlijk dat G-type eindigingen voornamelijk afkomstig zijn uit de raphe kernen terwijl de F- en S-type eindigingen voornamelijk afkomstig zijn van neuronen in het ventrale deel van de mediale reticulaire formatie.

Na ^{3}H-leucine injecties in het gebied van de nucleus coeruleus en subcoeruleus bevond de labelling zich met name in E-type eindi-

gingen en (bij de cluster analyse in veel mindere mate) in S-type eindigingen. F-type eindigingen werden ook gelabeld, maar alleen bij de cirkel analyse, hetgeen er mogelijk op wijst dat die eindigingen afkomstig zijn van neuronen in de rand van de injectieplaats.

Na alle injecties bleek dat in de geanalyseerde coupes ongeveer 40-50% van de gelabelde S- en F-type eindigingen een synaptisch contact maakten, en slechts ongeveer 10% van de gelabelde E- en F-typen. In alle gevallen werd voornamelijk synaptisch contact gemaakt met proximale dendrieten (± 75%) en in veel mindere mate met cellichamen en distale dendrieten (± 20%).

2). Na injecties van WGA-HRP in het ventro-laterale deel van de mediale reticulaire formatie in de lage hersenstam werd de labeling (het TMB reactieproduct) voornamelijk gevonden in eindigingen en, in mindere mate, in axonen (10%) en dendrieten (10%). Zoals bij de EM autoradiografische experimenten na injectie van ^{3}H-leucine in de mediale reticulaire formatie werd de labelling ook nu in F-, G-, C- en S-type eindigingen gevonden met gelijksoortige percentages. Dit gold ook voor de percentages van synaptische contacten en hun postsynaptische structuren. Er wordt geconcludeerd dat voor het aantonen van verbindingen in de hersenen op electronen microscopisch niveau, zoals gebruikt in dit onderzoek, de horseradish peroxidase techniek een betrouwbare methode is. De efficiëntie, voor- en nadelen in vergelijking met de EM autoradiografie techniek worden besproken in Hfdst. II.2. en III.1.

3.) In twee ratten werden ^{3}H-leucine injecties in het ventro-mediale deel van de mediale reticulaire formatie gecombineerd met HRP injecties in de spieren van de achterpoot, waardoor de motoneuronen, die deze spieren innerveren, werden gelabeld. Alleen de cluster gelabelde eindigingen werden bestudeerd waarbij werd gevonden dat meer dan 50% van de structuren, waarmee de gelabelde eindigingen een contact maakten, gelabeld waren met HRP reactie producten. Ervan uitgaande dat niet alle delen van een gelabeld motoneuron reactie product bevatten en dat niet alle motoneuronen werden gelabeld, werd geconcludeerd dat het overgrote deel van de eindigingen afkomstig van neuronen in de mediale reticulaire formatie, contact maakten met motoneuronen in het lumbale ruggemerg.

In Hfdst. II.1 en in Hfdst. III.2 werden de resultaten besproken van het onderzoek, met name wat betreft de transmitters die mogelijk aanwezig zijn in de verschillende typen eindigingen. Er wordt beargumenteerd dat, afgezien van de neuronen met C-type eindigingen, tenminste 2 maar waarschijnlijk 3 verschillende groepen neuronen in de raphe kernen en de aangrenzende mediale reticulaire formatie naar de motoneuronen in het lumbale ruggemerg projecteren. Deze 3 groepen neuronen en hun verschillende typen eidigingen bevatten mogelijk verschillende transmitters: het G-type is waarschijnlijk serotonerg, het F-type (echter niet allemaal) bevat gamma-amino-boterzuur (GABA), terwijl het nog onduidelijk is welke transmitter in de S-type eindigingen aanwezig is. Het is eveneens waarschijnlijk dat de projecties naar lumbale motoneuronen vanuit het gebied van de locus coeruleus en subcoeruleus afkomstig zijn van 2 verschillende groepen neuronen gekarakteriseerd door 2 typen eindigingen, het E- en S-type. Eén van deze typen eindigingen. waarschijnlijk het E-type, bevat noradrenaline als transmitter.

Wat betreft de functionele betekenis van de afdalende banen naar motoneuronen in het ruggemerg, wordt het volgende beargumenteerd: serotonine en noradrenaline hebben een faciliterende invloed op motoneuronen door hun gevoeligheid voor andere exciterende prikkels te verhogen. Deze effecten kunnen mogelijk op hun beurt worden tegengewerkt door de GABA projecties naar motoneuronen. De neuronen in de hersenstam, die de verschillende afdalende projecties geven naar motoneuronen staan zelf onder invloed van delen van het limbische systeem. Het lijkt daarom waarschijnlijk dat de controle van het limbische systeem op de motoriek (deels) wordt uitgeoefend via deze verschillende groepen neuronen in de lage hersenstam.

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Curriculum Vitae

De schrijver van dit proefschrift werd op 17 juli 1954 te Warnsveld geboren. Hij bezocht het openbaar lyceum in Zutphen en legde in 1972 het eindexamen HBS B met goed gevolg af. In datzelfde jaar begon hij de studie geneeskunde aan de Medische Faculteit te Rotterdam, waar hij in 1977 het doctoraal examen behaalde.

Na zijn kennismaking met de neurowetenschappen en de electronen microscopie op de afdeling neuroanatomie van de Erasmus Universiteit te Rotterdam onder leiding van Prof.Dr. H.G.J.M. Kuypers, volgde een keuze-practicum in de U.S.A. (Albert Einstein College of Medicine, New York) en een student-assistentschap onder begeleiding van Dr. J.J. Dekker

Vanaf maart 1978 tot december 1983 was hij werkzaam als wetenschappelijk medwerker op de afdeling op basis van een drie en een half jarige poolplaats. Onder leiding van Prof.Dr. H.G.J.M. Kuypers werd een electronen microscopisch onderzoek verricht naar de afdalende banen van de hersenstam naar de motoneuronen in het lumbale ruggemerg van de rat. De resultaten hiervan zijn beschreven in dit proefschrift. In deze periode werden tevens senior-coschappen gelopen (artsexamen april 1983). Na zijn militaire dienst als assistent chirurg in het Militair Hospitaal te Utrecht volgde in juni 1985 na het vertrek van Prof.Dr. H.G.J.M. Kuypers een tijdelijke aanstelling op de afdeling Anatomie onder leiding van Prof.Dr. J. Voogd. In november 1987 werd een vaste aanstelling verkregen.