COLLATERALIZATION OF THE PATHWAYS DESCENDING FROM THE CEREBRAL CORTEX TO BRAIN STEM AND SPINAL CORD IN CAT AND MONKEY

COLLATERALIZATION OF THE PATHWAYS DESCENDING FROM THE CEREBRAL CORTEX TO BRAIN STEM AND SPINAL CORD IN CAT AND MONKEY

An anatomical double-labeling study

Collateralisatie van de afdalende banen van de hersenschors naar hersenstam en ruggemerg in de kat en de aap

Een anatomische dubbel-labeling studie

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We can still endorse the old adage
that to move things is all mankind can do,
and that for such the sole executant is muscle,
whether in whispering a syllable or in felling a forest.
Sir Charles S. Sherrington, 1858-1952

aan Tineke
aan moeder

Contents

PUBLICATIONS

1. Keizer K, Kuypers HGJM, Huisman AM, Dann O (1983) Diamidino Yellow dihydrochloride (DY.2HCL); a new fluorescent retrograde neuronal tracer, which migrates only very slowly out of the cell. Exp Brain Res 51: 179-191

2. Huisman AM, Kuypers HGJM, Conde F, Keizer K (1983) Collaterals of rubrospinal neurons to the cerebellum in rat. A retrograde fluorescent double labeling study. Brain Res 264: 181-196

3. Keizer K, Kuypers HGJM (1984) Distribution of corticospinal neurons with collaterals to lower brain stem reticular formation in cat. Exp brain Res 54: 107-120

4. Fries W, Keizer K, Kuypers HGJM (1985) Large layer VI cells in macaque striate cortex (Meynert cells) project to both superior colliculus and prestriate visual area V5. Exp Brain Res 58: 613-616

5. Keizer K, Kuypers HGJM, Ronday HK (1987) Branching cortical neurons in cat which project to the colliculi and to the pons: a retrograde fluorescent double-labeling study. Exp Brain Res 67: 1-15

6. Keizer K, Kuypers HGJM (1989) Distribution of corticospinal neurons with collaterals to the lower brain stem reticular formation in monkey (Macaca fascicularis) Exp Brain Res 74: 311-318

7. Keizer K, Kuypers HGJM, Vlies van der R, Ronday HK A search for branching neurons in the cat superior colliculus projecting to lower brain stem, spinal cord, thalamus and the pontine grey. In preparation

The publications 1, 3-6 are used in the chapters II-VI of this thesis.

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The gain in brain
is mainly in the stain.

Floyd E. Bloom,
Madrid 1983

Chapter I

General Introduction

I.1. Introduction

The cerebral cortex of mammals contains about ten billion ($10*10^{10}$) neurons (Carpenter 1976). The majority of these cortical neurons have relatively short axons which contribute to the local intracortical circuitry and to the cortico-cortical connections within and between the cerebral hemispheres. A relatively small proportion of cortical neurons have long axons which project to subcortical regions, i.e. the basal ganglia, the thalamus, the brain stem and to the spinal grey matter. It is through the projections to the brain stem and spinal cord, i.e. *the cortical descending pathways*, that the cerebral cortex can exert control over movements and thus effect behaviour.

Animal and human movements are brought about by contractions of striated muscles. The corticofugal descending pathways are the crucial link between the cerebral cortex and the motor apparatus. The study of the anatomy and the physiology of these pathways has provided us with a better insight in nervous control of movements.

The first detailed anatomical studies of the origin, course and termination areas of the cortical descending pathways were made with the now classical technique of the fifties, the anterograde fiber degeneration method (Nauta and Gygax 1954, Chambers and Liu 1957, Kuypers 1958, 1958a, b, c, Kuypers and Lawrence 1967, Fink and Heimer 1967, Nyberg-Hansen 1969).

The findings obtained with this technique were confirmed and further extended by the application of two sensitive anatomical methods which were developed in the early seventies. These modern techniques are based on axonal transport of substances to and from the cell body (Weiss and Hiscoe 1948). It appeared that foreign substances can be taken up by the neuron and transported antero- and retrogradely through the axon.

The anterograde axonal transport technique, combined with autoradiography is used to localize radioactive tracers. Tritiated aminoacids are incorporated into proteins in the cell body and transported anterogradely through the axon to its terminals. The radioactivity is visualized by means of autoradiography, i.e. by applying a photographic emulsion on the section, which is developed after a certain exposure time. This method visualizes the silver grains which will be produced above the radioactive regions. This was found to be a reliable and sensitive technique for demonstrating in detail the course and the termination pattern of axons, including small ones (Lasek et al. 1968, Cowan et al. 1972). This method has the advantage over the degeneration technique that it circumvents the problem caused by damaging passing fibers in degeneration studies. Thus after making a lesion e.g. in the brain stem the degenerating fibers to the spinal cord are derived from the neurons destroyed by the lesion but in part also represent fibers interrupted by the lesion. When

injecting labeled aminoacids, these substances are not transported through the passing axons. In fact, the labeled aminoacids are only transported through axons after the aminoacids have been incorporated into proteins by the metabolic machinery of the individual cells. Thus the radioactively labeled axons are only derived from neurons situated in the injection area. More recently, the anterograde transport of other substances has been used e.g. the Wheat Germ Agglutinin conjugate of HRP, see below (Gonatas et al. 1979) and the plant lectin, Phaseolus vulgaris Leucoagglutinin (PHA-L, Gerfen and Sawchenko 1984).

The Horseradish Peroxidase (HRP) technique makes use of the intra-axonal transport of the enzym HRP (Kristensson and Olsson 1971, Lavail and Lavail 1972, Mesulam 1978). HRP is taken up by cell bodies, damaged fibers and terminals. Subsequently, it is transported through the axon in antero- and retrograde directions. The presence of HRP in the neuron is visualised by its enzymatic activity. The sections are incubated with hydrogen peroxidase and a substrate. Originally, Diamidinobenizidine (DAB) was used as substrate (Graham and Karnovsky 1966). However, because of its carcinogenity it has been replaced by Tetramethylbenzidine (TMB, Mesulam 1978). The labeled neurons are filled by the brown-blueish reaction product. The retrograde HRP method was used by us for demonstrating the precise cortical distribution of the cells of origin of the descending pathways. Recently, the efficiency of the HRP technique has been improved using HRP coupled to the lectin wheat germ agglutinin as a tracer (WGA-HRP, Gonatas et al. 1979).

The autoradiographic and HRP techniques were the start of a rapid expansion of the anatomical knowledge about the descending projections from the cerebral cortex to the spinal cord and the brain stem. This thesis deals especially with the following cortical projections in cat and monkey to: a. the spinal cord (Armand et al. 1974, Armand and Aurenty 1977, Berrevoets and Kuypers 1975, Groos et al. 1978, Cheema et al. 1984, Armand et al. 1985), b. the dorsal column nuclei (Weisberg and Rustioni 1976, 1977, 1979), c. the brain stem reticular formation (Berrevoets and Kuypers 1975, Catsman-Berrevoets and Kuypers 1976), d. the pontine nuclei (cf. Brodal 1982, Bjaalie 1983, 1985, 1986, Glickstein 1985), e. the superior colliculi (Goldman and Nauta 1976, Kawamura and Konno 1979, Fries 1984, Catsman-Berrevoets et al. 1979, Leichnetz et al. 1981, Gilbert 1975), and e. the red nucleus (Catsman-Berrevoets et al. 1979, Hartmann-von Monakow 1979, Leichnetz 1982). The cortical projections to the thalamus and the basal ganglia will not be dealt with.

It appeared from the retrograde HRP studies that the cell bodies of the cortical fibers descending to the brain stem and spinal cord are exclusively derived from deep pyramidal cells in cortical layer V. The distributions of cortical cells which were labeled after injections of HRP in different target areas of these descending cortical fibers overlapped considerably in various cortical areas. In this context, the intriguing question arose whether branching cortical neurons exist, which give rise to more than one descending fiber, which project to different target areas. This notion led to a renewed interest in the study of axon collateralisation.

Unfortunately, in the mid-seventies no suitable anatomical technique for demonstrating divergent axon collaterals was available. In part I.2. of this chapter the different techniques for demonstrating such axon collaterals will be described. The present study is devoted mainly to the use of the retrograde fluorescent double-labeling technique for demonstrating axon collaterals. This method will be described extensively in part I.3. The remainder of chapter I deals with the anatomy and functional aspects of the descending pathways and the cortical motor areas.

I.2. Axoncollaterals

At the turn of the century the great spanish neuroanatomist, S. Ramon Y Cajal (1852-1934) demonstrated the existence of axon collaterals by means of the *Golgi method* (Cajal 1911, see Cajal 1952). This technique encrusts the entire neuron, i.e. the cell soma, the dendritic tree and the axon with a silver compound. For reasons unknown only a small proportion of the neuronal population is visualized in this way. The drawings made by Cajal demonstrate in great detail local axon bifurcations and terminal axon arborisations. He described the recurrent axon collateral of the pyramidal neurons in the cerebral cortex and axon collaterals given off by pyramidal tract fibers, projecting to the tegmentum, to the olivary nuclei and to the pontine grey (Cajal 1952, vol I, pp: 60, 187, 927-929, 967, 968).

It was not possible however to trace the fibers and collaterals over long distances. The precise origin and termination area of the axonal branches therefore could not be determined with the Golgi technique.

Branching neurons can be indentified *electrophysiologically*. Stimulation electrodes are placed in the presumed target areas of the different axon branches and a recording electrode is placed extracellularly near the cells of origin. Cells are considered to be antidromically activated when they respond at a constant latency at all stimulus intensities above threshold, faithfully follow repetition rates of 100 to 200/sec or higher and their failure due to collision block whenever an orthodromically evoked action potential preceeded the antidromically evoked action potential with a time interval less than twice the antidromic latency plus the refractory period (Abzug et al. 1974, Peterson et al. 1975, Shinoda et al. 1976). When a neuron is activated from each target area according to these criteria, it is considered that the cell distributes axon collaterals to both target areas. Further evidence of such an assumption can be obtained by means of the collision test (Shinoda et al. 1976). When a neuron is activated from each electrode, collision of the impulses initiated from each target area is studied by delivering a pair of stimuli. This procedure ascertains that both stimuli activate the same neuron antidromically, and the conduction time between the branching point of the axon and the site of excitation can be calculated. The maximal interval between two stimuli for the blockade of the second impulse should correspond to the sum of the conduction between the two stimulating electrodes and the refractory period of the axon at the second site (see for detailed description Shinoda et al. 1976). In contrast to the Golgi method, these electrophysiological methods can reveal the cells of origin and the distant projection areas of divergent axon collaterals. In this way it was demonstrated electrophysiologically that fibers of the pyramidal tract send collaterals to the red nucleus (Tsukahara et al. 1968), to the pons (Allen et al. 1975), to the dorsal column nuclei (McComas and Wilson 1968, Endo et al. 1973, Atkinson et al. 1974, Humphrey and Corrie 1978) and to the cervial and lumbar spinal grey matter (Shinoda et al. 1976). In this way it was shown also that single cells in the reticular formation emit collaterals both to cervical and lumbar levels of the spinal cord (Peterson 1975, 1979). However, the electrophysiological method has its limitations. The technique favors large neurons above small ones, and the number of neurons which can be investigated is limited. Thus, the total population of branching neurons cannot be visualized completely and their spatial relationship to other neurons is difficult to establish.

I.3. Fluorescent tracers

In the seventies, several attempts were made to develop an anatomical technique which could demonstrate the cells of origin of divergent axon collaterals (Hayes and Rustioni 1979, 1981, Olsson and Kristensson 1978, Cesaro et al. 1979). These so called double-labeling techniques were based on the same basic principle. At least two different neuronal tracers are used, which after being transported retrogradely through both collaterals, could be demonstrated independently in the parent neuronal cell body.

The HRP-tritiated apo-HRP technique (Hayes and Rustioni 1979, 1981) is based on the fact that HRP as well as tritiated, enzymatically inactive apo-HRP, are transported retrogradely to the parent cell body (Kristensson and Olsson 1971, Lavail and Lavail 1972, Geisert 1976). The presence of HRP is visualised histochemically by its enzymatic activity, while the presence of the tritiated-apo-HRP is demonstrated by means of autoradiography. A major disadvantage is, that the autoradiographical technique can only demonstrate the presence of a tritiated label in the upper 3 μm of the section (Sidman 1970). Therefore the number of double-labeled neurons tends to be underestimated (see also Cavada et al 1984).

In *the HRP-Iron dextran technique* (Olsson and Kristensson 1978, Cesaro et al. 1979) the two tracers are demonstrated histochemically in the cell body by processing the section for HRP and subsequently demonstrating the presence of ferric iron by means of the Perl's reaction. In double-labeling experiments, using this technique the number of double-labeled neurons may be underestimated, because the most abundant reaction product of the one tracer tends to mask that of the other.

In the late seventies, Kuypers and co-workers developed the *fluorescent double-labeling technique*, which is based on the retrograde transport of different fluorescent substances. Kristensson and his collaborators had demonstrated (Kristensson 1970, Kristensson et al 1971), that Evans Blue (EB) combined with bovine albumin (BA), when injected in the tongue of a rat, is transported retrogradely through the peripheral nerve to the cell body of the motoneuron (Kristensson 1970, Kristensson et al 1971). The labeled neurons fluoresce red when illuminated with light of 550 nm excitation wavelength. Further experiments showed that EB

without bovine albumine produced the same or even better retrograde labeling of the cells of the substantia nigra after injections of the tracer in the striatum of the rat (Kuypers et al. 1977). An attempt was made to combine EB with HRP in double-labeling experiments. This turned out to be unsatisfactory. Therefore a large number of other substances were tested on the rat nigrostriatal projection (Kuypers et al. 1977).

These tests revealed that 4'-6-diamidino-2-phenylindol 2HCl (DAPI) and Primuline (Pr) could be used as fluorescent tracers. DAPI labels the nucleus and the nucleolus and produces a bright blue fluorescense when illuminated with excitation light of 360 nm. Primuline also fluoresce at 360 nm, but produces golden fluorescent granules in the cytoplasm (Kuypers et al 1977). DAPI and primuline can be injected as a mixture, thus acting as one tracer. Subsequently, it was demonstrated that double-labeling of cells was possible by injecting EB and DAPI/Pr in the respective target areas of two divergent axoncollaterals of a single neuron (Fig. 1). Using this method, the existence of collaterals was demonstrated in the projections from the mammillary body to the thalamus and the mesencephalon (VanderKooy et al. 1978), but also in the ascending raphe and nigral projections (VanderKooy and Kuypers 1979) and in the nigral projections to the tectum and the thalamus (Bentivoglio et al. 1979a). When used in cat, however, these tracers were not very effectively transported over long distances.

Other chemical substances were also tested. Bisbenzimide (Bb) and Propidium Iodide (PI) were found to meet the requirements as retrograde fluorescent tracers. Neurons retrogradely labeled with Bb show a yellow-green granular fluorescence of the nucleus and a pronounced yellow-green ring around the nucleolus, when illuminated with light of 390 nm excitation wavelength. When illuminated with light of 360 nm excitation wavelength the nucleus fluoresced blueish green. Neurons labeled with Propidium Iodide display a brilliant orange-red fluorescence of cell body and proximal dendrites when illuminated with light of 550 nm excitation wavelength. The differences in localisation and fluorescence between Bb (yellow-green fluorescent nucleus at 360 nm) and PI (orange-red fluorescent cytoplasm at 550 nm) suggested that they could be used in double-labeling experiments (Kuypers et al. 1979). However, PI is rather toxic and is not transported over long distances.

Thus, the search for fluorescent tracers was resumed. Because DAPI, a diamidino compound was found to be the most effective retrograde fluorescent tracer, other diamidino compounds were tested, synthetised by Dr. O. Dann (Institute of Pharmacy and Food Chemistry, Friedrich-Alexander-University). These substances possess a high affinity to DNA and RNA. Of the very many substances tested, two seemed very promising: True Blue (TB) and Granular Blue (GB) (Bentivoglio et al. 1979b, Rosina et al. 1980). Of these two tracers TB was found to be a very effective retrograde fluorescent tracer, especially in rats. Cells labeled with TB display a deep blue fluorescence of the cytoplasm and the nucleolus when illuminated with excitation light of 360 nm wavelength. In the rat, it was used satisfactorily in double and triple labeling studies (Olmos and Heimer 1980, Swanson et al. 1980, Swanson and Kuypers 1980a, b).

In the cat, however, the results were disappointing, since TB was not transported over long distances. In that same year, another diamidino compound (Fast Blue (FB), Bentivoglio et al. 1980a) brought the solution. FB is transported effectively over long distances in rat, cat and monkey (Kuypers et al. 1980, Huisman et al. 1982). Neurons retrogradely labeled with FB show a blue fluorescence of the cytoplasm when illuminated with light of 360 nm wavelength. This blue fluorescence of FB is somewhat duller than that obtained with TB. These blue fluorescent tracers, which label preferentially the cytoplasm can be combined with Bb which produces a yellow fluorescence in the nucleus. Moreover, all these tracers can be studied simultaneously at the same excitation wavelength. In that same year, however, another yellow fluorescent tracer (Nuclear Yellow (NY), Bentivoglio et al. 1980a) was found, which in contrast to Bb labels only the nucleus, where it produces a pure golden yellow fluorescence at 360 nm. This tracer is a benzimidazole and was provided by Dr. Loewe of the Hoechst Company. In the subsequent years the tracer combinations NY-TB in rat and NY-FB in cat and monkey were used successfully in retrograde double-labeling experiments (Fig. 1, Bharos et al. 1981, Huisman et al. 1982).

The ideal tracer combinations seemed to be discovered. Yet, another problem turned up. In one of the first studies using NY and TB in rat, it was revealed that NY migrated out of the retrogradely labeled neuron into the surrounding tissue. This was noticed by NY labeling of glial nuclei. Thus, it was concluded that it might also give rise to false labeling of neighbouring

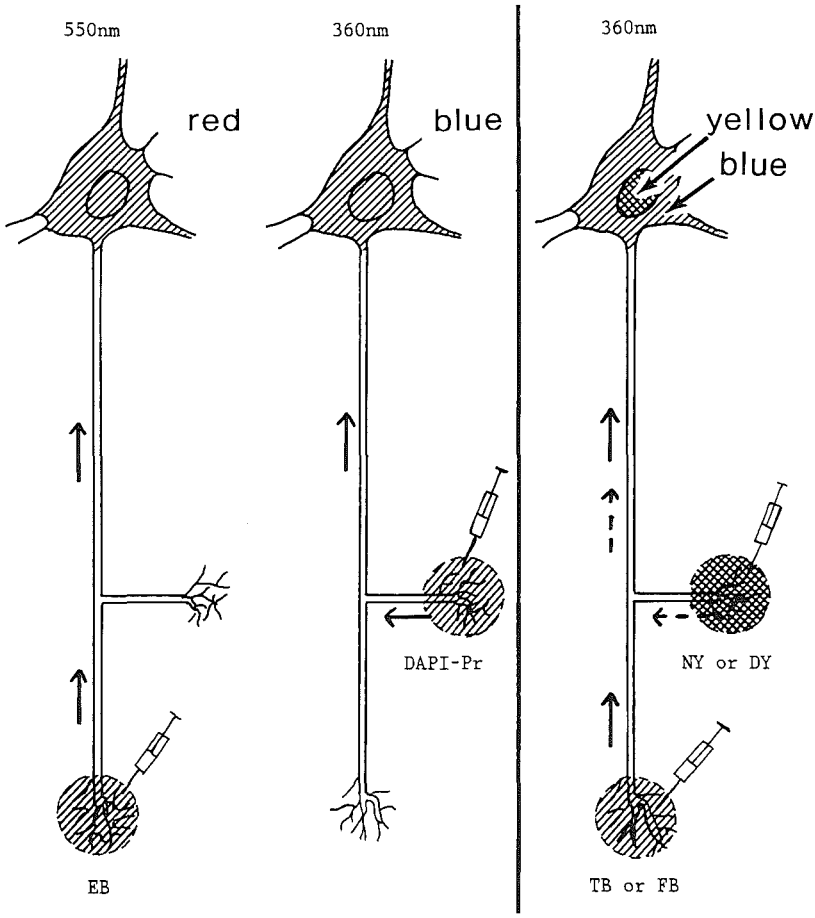

Fig. 1. Diagram showing the retrograde axonal transport and labeling of the neuronal cell body of different fluorescent tracers, which are suitable for use in double labeling experiments. On the left the combination Evans Blue (EB) and DAPI/Primuline (DAPI/Pr) is shown. EB labels the cell body red at 550 nm excitation wavelength and DAPI/Pr labels the cell body blue with golden fluorescent granules in the cytoplasm at 360 nm. On the right the combination of the blue fluorescent tracers True Blue (TB) and Fast Blue (FB) and the yellow fluorescent tracers Nuclear Yellow (NY) and Diamidino Yellow (DY) is shown. TB and FB label the cytoplasm of the cell body blue at 360 nm, and NY and DY label the nucleus of the cell body yellow at the same 360 excitation wavelength.

neurons (Catsman et al. 1980). When studying the migration of NY out of the cell body it became clear that leakage of the tracer out of the cell body could be prevented by restricting the survival time and using lower concentrations of NY (Bentivoglio et al. 1980b). The same holds true for Bb.

The careful titration of the optimal survival time in relation to the distance over which NY had to be transported made it necessary that in double-labeling experiments the two tracers had to be injected at different times during the survival period. As a consequence, in some of the experiments the animals had to be injected or sacrificed at night. Moreover some animals, despite carefully chosen survival periods, showed large numbers of brilliantly labeled glial nuclei. These cases had to be disregarded. Nonetheless, when used properly the tracer combinations NY-TB and NY-FB are reliable and sensitive in double-labeling experiments, demonstrating divergent axoncollaterals (Bharos et al. 1981, Catsman-Berrevoets and Kuypers 1981, Huisman et al. 1981, 1982, Kuypers and Huisman 1984).

The fluorescent double-labeling technique could still be improved, when a tracer was found, which like NY would display a yellow fluorescent nucleus, but which would not leak out of the cell body after relative long survival times.

The ideal tracer should meet the following specifications:
1. It should be taken up and transported effectively through the axon, such that in a short period of time the tracer can accumulate sufficiently in the cell body to be detected by means of the fluorescence microscope, 2. It should preferentially label either the cytoplasm or the nucleus, 3. It should not leak out of the axon or the cell body into the surrounding tissue, where it may label neighbouring neurons (false positive labeling), 4. It must be possible to use it in combination with other fluorescent tracers, and 5. when combined with a second tracer, the combination should fluoresce preferably at the same excitation wavelength, 6. An additional requirement for some kind of experiments is that the tracers should only be taken up by nerve endings and not by fibers of passage, 7. Finally, the tracer should be neither neurotoxic, producing neurological deficits by local tissue necrosis, nor systemic toxic giving inconvenience to the animal or even killing it.

In chapter II the properties of a new fluorescent tracer will be described: *Diamidino Yellow dihydrochloride (DY.2HCl)*. This tracer is effectively transported retrogradely to the cell body, producing a bright yellow fluorescence of the nucleus. After long survival times, it leaks only very slowly out of the cell, and it can be easily combined with the fluorescent tracers, Fast Blue (FB) and True Blue (TB), which label preferentially the cytoplasm (Fig. 1).

After DY became available, two other retrograde fluorescent tracers were reported in the literature. Katz et al. (1984) demonstrated in cat that rhodamine-labeled fluorescent latex microspheres, measuring 0.02-0.2 μm in diameter, were retrogradely transported from axons to the cell body. The labeled neurons showed a red fluorescence of cytoplasm and proximal dendrites. When injected into the brain the microspheres produce small, sharply defined injection areas. After being retrogradely transported, the label persists for at least 10 weeks in the neuronal cell body and without leaking out of the retrogradely labeled cell. Rhodamine labeled microspheres have no obvious cytotoxicity and are very resistant to fading under illumination. The microspheres may be used in combination with other retrograde and anterograde fluorescent tracers and with immunocytochemical procedures (Katz et al. 1984).
Fluoro-Gold described by Schmued and Fallon (1986) is retrogradely transported by the neuron producing golden, intense fluorescent granules in the cytoplasm and dendrites. It does not leak out of retrogradely labeled neurons despit long survival times and it is highly resistant to fading under illumination. Fluoro-gold is not taken up by undamaged fibers of passage. It can be combined with autoradiography, enzyme histochemistry (HRP, PHA-L), immunocyto-chemistry and other retrograde fluorescent tracers. However, in double-labeling experiments using Fluoro-gold in combination with TB, FB and DY, the intense fluorescence of Fluoro-gold tends to mask the other tracers.

I.4. The descending pathways

I.4.1. The spinal cord

The ultimate link between the central nervous system and the muscles is formed by the motoneurons which are situated in the ventral horn of the spinal cord and in the cranial motor nuclei in the brain stem. The axons of the motoneurons reach the muscles via the peripheral nerves. The assembly of a motoneuron and the muscle fibers innervated by it is called "the motorunit". It forms "the final common path" (Sherrington 1858-1952) through which the central nervous systems controls

muscle activity. One way to understand how the brain controls movements is to unravel the descending connections from motor cortex and brain stem to the motoneurons.

The motoneurons in the ventral horn of the spinal grey matter in cat and monkey are grouped into two major longitudinal columns, a medial and lateral one. In transverse sections these columns constitute the medial and lateral motoneuronal cell groups. The medial motoneuronal cell group innervates the short and long muscles attached to the vertebral column. The lateral motoneuronal cell group innervates the muscles of trunk, girdle and extremities (Sprague 1948). In general the more distal muscles are supplied by motoneurons located laterally. The intrinsic muscles of hand and foot are innervated by the motoneurons situated in the most dorsolateral part of the lateral motoneuronal cell group in C8 and T1, and in S1 and S2 respectively (Reed 1940, Sterling and Kuypers 1967).

The intermediate zone of the spinal grey is regarded as comprising the lateral parts of laminae V and VI, as well as laminae VII and VIII according to the nomenclature of Rexed (1952, 1954). The neurons in the intermediate zone give rise to ascending and descending propiospinal fibers as well as to supraspinal projections (Rustioni et al. 1971, Molenaar et al. 1974, Verburgh and Kuypers 1987). The propiospinal neurons may be subdivided according to the length of their fibers into short and long propiospinal neurons. The long propiospinal neurons distribute their fibers bilaterally throughout the length of the spinal cord, and their cell bodies are concentrated in lamina VIII and the adjoining part of lamina VII. Some of the long propiospinal neurons are situated in the lateral parts of laminae IV to VI. The long propiospinal fibers are distributed mainly to the ventromedial part of the intermediate zone and to the medial motoneuronal cell group innervating axial and girdle muscles. In contrast, the short propiospinal neurons display the opposite distribution pattern. The short propiospinal neurons distribute their fibers unilaterally over a distance of five to eigth segments, and terminate preferentially in the dorsolateral parts of the intermediate grey and in the lateral motoneuronal cell group, innervating the limb muscles (Sterling and Kuypers 1968, Rustioni et al. 1971. Molenaar et al. 1974, Verburgh and Kuypers 1987).

According to retrograde fluorescent tracer studies the neurons situated in spinal segments C3-C8, which give rise to short descending (propiospinal) fibers (projecting to T2-T8) are much more numerous than those giving rise to long descending propiospinal fibers (Verburgh and Kuypers 1987). The short propiospinal neurons are located both in the areas containing the long descending propiospinal neurons and the remaining areas, i.e. the medial parts of laminae IV to VI and the central and lateral parts of lamina VII.

About 20% of the C3-C8 neurons projecting to supraspinal levels give rise to a descending propiospinal collateral reaching T2, whereas 8%, 3% and 1% give rise to longer descending collaterals reaching T9, L2 and S1 respectively. About 30% of the C3-C8 neurons projecting to T3 or below are branching neurons which give rise to an ascending supraspinal collateral. This indicates that some spinal neurons are propiospinal cells as well as "tract" cells (spino-thalamic, spinomesencephalic, spinoreticular and spinocerebellar, Verburgh and Kuypers 1987).

The connections of spinal interneurons to the motoneurons can also be visualised by means of the transneuronal transport of WGA-HRP. After injecting WGA-HRP in a peripheral nerve, it is transported to motoneuronal somata and subsequently, transneuronally, to neurons in several spinal segments (Harrison et al. 1984). After injections of WGA-HRP in a hindlimb nerve, transneuronally labeled neurons were present primarely in laminae V to VII ipsilaterally and in lamina VIII contralaterally of spinal segments L3 and L4. The ipsilateral cells were labeled troughout most of the intermediate grey, except for its medial parts (Jankowska and Skoog 1986).

On the basis of their trajectory and their termination in the brain stem and in the spinal grey matter, three groups of descending brain stem pathways can be distinguished:

1. the medial group of descending brain stem pathways,
2. the lateral group of descending brain stem pathways,
3. the descending pathways from the coeruleus/subcoeruleus nuclei, the raphe nuclei and the ventral reticular formation.

The projection of the corticospinal tract is superimposed on these pathways and overlaps their terminal distribution completely.

I.4.2. The medial group of descending brain stem pathways

These pathways are derived from the vestibular complex, the interstitial nucleus of Cajal, the superior colliculus and the medial reticular formation of the medulla oblongata, the pons and the mesencephalon (Nyberg-Hansen 1964, 1966, Petras 1967, Kuypers and Maisky 1975, 1977, Peterson and Coulter 1977, Edwards 1972). These pathways descend medially in the brain stem and proceed in the ventral and ventrolateral funiculi of the spinal cord. They terminate, mostly bilaterally, in the medial parts of the intermediate zone, i.e. in the area of the long propiospinal neurons in the cat (Fig. 2, Holstege and Kuypers 1982, Huerta and Harting 1982), and in opossum (Martin et al. 1979, 1981). Several of the fibers of these pathways distribute collaterals to both the cervical and lumbar grey (Wilson and Peterson 1981). According to retrograde fluorescent double-labeling studies in rat, 50% of the neurons in the interstitial nucleus of Cajal, 68% in the Deiters nucleus, 23% in the medullary vestibular nuclei ipsilaterally and 50% of the neurons contralaterally were double labeled from C5-C8 and T7-T8 (Huisman et al. 84). These findings indicate that the medial descending pathways represent a rather diffuse system. Its fibers give off many collaterals along their trajectory throughout the length of the spinal cord (Martin et al. 1981). The anatomical organisation suggests that the medially descending pathways are involved in steering synergistic body and integrated body-limb movements, as in postural and orienting movements.

Lesion studies in the freely moving monkey are in keeping with this view. When in a bilaterally pyramidotomized monkey, additional lesions are made, which disrupt the medial descending pathways bilaterally at the level of the VI nucleus, the animals had severe problems in maintaining erect posture and equilibrium, in orienting movements of body and head and in directing their course of progression (Kuypers 1964, Lawrence and Kuypers 1968b).

I.4.3. The lateral group of descending brain stem pathways

These pathways are derived from the red nucleus, the dorsally adjoining mesencephalic reticular formation and the ventrolateral pontine tegmentum, and descend contralaterally in the dorsolateral funiculus of the spinal cord (Busch 1961, Staal 1961, Edwards 1972, Kuypers and Maisky 1975, Holstege and Kuypers 1982, Holstege 1988). The main component is formed by the rubrospinal pathway, which terminates preferentially in the dorsolateral part of the spinal intermediate zone (Fig. 2). Some rubrospinal fibers project to motoneurons which innervate distal extremity muscles (Shapovalov 1972, Holstege 1988).

The dorsomedial part of the red nucleus projects to the cervical enlargement, while its ventrolateral part projects to the lumbosacral enlargement (Hayes and Rustioni 1981, Martin et al. 1981, Huisman et al. 1982, Holstege 1988). In the overlapping area neurons are situated which give off collaterals to both enlargements. However, in cat only 2% to 4% of the rubrocervical neurons give off a collateral to the lumbar cord. In sharp contrast with the 66% of the reticulocervical neurons with a collateral to the lumbosacral cord (Peterson et al. 1975, Huisman et al. 1981, 1982, 1984, Wilson and Peterson 1981). These findings indicate that the rubrospinal tract in opossum, rat, cat and monkey is more differentiated than the medial descending pathways. In contrast with the diffuse projections of the medially descending pathways to the spinal cord, the rubrospinal pathway represents a more focussed system, the fibers of which are ditributed to a restricted set of spinal segments.

The fiber connections suggest, that the rubrospinal pathway plays a role in the steering of specific movements of the individual limbs. Moreover, the termination of the rubrospinal fibers in the dorsal and lateral parts of the intermediate zone and in the dorsolateral part of the lateral motoneuronal cell groups suggests that the rubrospinal pathway plays a role especially in steering movements of the distal parts of the extremities. This is in keeping with the findings of lesion experiments in freely moving monkeys (Lawrence and Kuypers 1968b), and more recently in a chronic recording study in red nuclei in conscious monkeys (Kohlerman et al. 1982).

I.4.4. The descending pathways from the coeruleus/subcoeruleus nuclei, the raphe nuclei and the ventral reticular formation

In the late seventies, when the retrograde HRP technique and the anterograde autoradiographic technique became widely available, the projections of the descending brain stem pathways were reinvestigated. Retrograde HRP studies in the cat generally confirmed

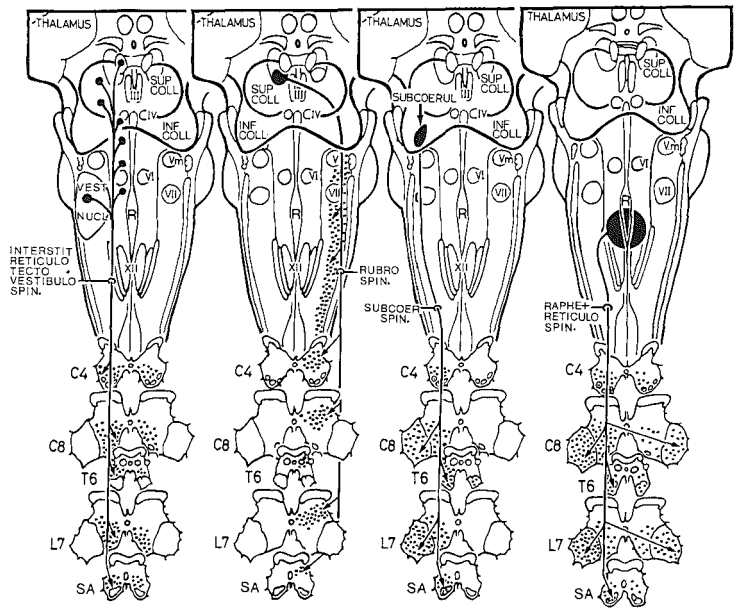

Fig. 2. The two diagrams on the left show the terminal distributions in the intermediate zone of the spinal cord of the medial and lateral group of descending brain stem pathways (see text). Note that the medially descending pathways distribute fibers bilaterally to the ventromedial part of the intermediate zone and ipsilaterally to its dorsolateral part. In contrast, the laterally descending pathway distribute fibers contralaterally to the lateral and dorsal parts of the spinal intermediate zone. The two diagrams on the right show the distribution of the subcoeruleospinal and raphe- reticulospinal fibers to the spinal motoneuronal cell groups. INF COLL, inferior colliculus; R, raphe nuclei; SUP COLL, superior colliculus; VEST NUCL, vestibular nuclei. (Redrawn from Kuypers 1981).

the classical anterograde degeneration studies concerning the projections from the brain stem to the spinal cord (see "the medial and lateral group of descending brain stem pathways"). However, other cell groups such as the locus coeruleus and subcoeruleus, the caudal raphe nuclei and adjoining medial reticular formation were also found to be labeled retrogradely from the spinal cord. These projections were demonstrated in cat (Kuypers and Maisky 1975), in monkey (Kneisley et al. 1978), in rat (Leichnetz et al. 1978), in opossum (Crutcher et al. 1978) and in lower vertebrates (Ten Donkelaar 1982).

The anterograde autoradiographic technique showed that the fibers from the locus coeruleus and subcoeruleus descend mainly ipsilaterally and terminate in all parts of the spinal grey matter including the motoneuronal cell groups throughout the spinal cord (Fig. 2, Basbaum et al. 1978, Holstege et al. 1979, Martin et al. 1979, 1981, Holstege and Kuypers 1982). A differential projection of the nucleus raphe magnus to the dorsal horn, and of the more caudally located nuclei raphe pallidus and obscures and the adjoining medial reticular formation to the intermediate grey and the motoneuronal cell groups of the ventral horn (Fig. 2) could be demonstrated with anterograde and retrograde labeling techniques (Basbaum et al. 1978, Holstege et al. 1979, Martin et al. 1979, Bowker et al. 1982, Holstege and Kuypers 1982). Both the raphe nuclei and the nucleus subcoeruleus also project to spinal autonomic cell groups. The existence of direct projections from

caudal raphe neurons to somatic motoneuronal cell groups was confirmed by electron microscopic autoradiography (Holstege and Kuypers 1987).

The existence of direct projections from the caudal brain stem to spinal motoneurons was also demonstrated in cat using retrograde transneuronal transport of WGA-HRP from spinal nerves, through the motoneurons, to neurons in the caudal raphe and the adjoining medial reticular formation (Alstermark et al. 1987).

The small calibre of these fibers probably explains why these projections escaped detection by means of the classical fiber degeneration techniques.

Double-labeling studies using retrograde fluorescent tracers demonstrated that the coeruleo- and raphespinal fibers give off many collaterals along their trajectory throughout the spinal cord. In cat 55%-60% of the raphecervical neurons in the nucleus raphe magnus appeared to be double-labeled after combined injections in the cervical cord and at more caudal spinal levels (Huisman et al. 1982). In monkey about 40% of the raphespinal neurons were doublelabeled. These high percentages occured more or less independently of the level of the caudal spinal injection. These findings are in keeping with those in rat (40%) (Huisman et al. 1981, 1984) and with those in opossum (Martin et al. 1981).

The raphespinal neurons resemble the reticulospinal neurons in this respect, since according to an electrophysiological study (Peterson et al. 1975), 66% of the reticulocervical neurons distribute collaterals to the segment caudal to L1. The high degree of collateralization of the raphe- and reticulospinal systems stands in sharp contrast with the very limited degree of collateralization of the rubrospinal fibers especially from the caudal part of the red nucleus (see "the lateral group of descending pathways").

The existence of descending pathways from the raphe nuclei and the locus coeruleus was already demonstrated in the early sixties by means of the aldehyde-induced fluorescence of monoamines developed by Falck and Hillarp (Falck et al. 1962). A first mapping of the monoamine containing neurons and fibers revealed that the nucleus coeruleus and the nucleus subcoeruleus are noradrenergic while the raphe nuclei are serotonergic (Dahlstrom and Fuxe 1964, 1965). However, recent studies demonstrated the existence of parallel non-monoaminergic spinal projections from the raphe nuclei, ventro-medial reticular formation and from the locus coeruleus and subcoeruleus (Skagerberg and Bjorklund 1985, Stevens et al. 1985).

Immunohistochemical studies revealed the presence of several peptides, i.e. substance P, thyrotropin releasing hormone (TRH), enkephalin-like substances and cholescystokinin in the raphe- and reticulospinal projections. Each of these peptides may coexist to a variable extent with serotonine (Hokfelt 1978, 1979, Bowker et al. 1983, see for review Holstege and Kuypers 1987). In addition, according to an immunohistochemical study most of the non-serotonergic raphespinal neurons were found to contain GABA (Millhorn et al. 1987).

The demonstration of direct connections of these descending brain stem pathways with somatic motoneurons was unexpected, because direct motoneuronal connections were considered as the exclusive property of the corticospinal tract in primates (Lawrence and Kuypers 1968a, b). The electrophysiological findings in the subsequent years indicated that the coeruleo- and raphespinal pathways had a diffuse, facilitatory influence on motoneurons (Conway et al. 1988, Hounsgaard et al. 1988, see for review Kuypers and Huisman 1982, Holstege and Kuypers 1987). It has therefore been suggested that these pathways function as a gain setting system (McCall and Aghjanian 1979), which determines the overall responsiveness of the motor apparatus. Neurons in the raphe magnus which project to the spinal dorsal horn, however, exert an inhibitory influence on pain transmission (Willis 1982, Jones and Gebhart 1986, Mokha et al. 1986). It seems likely that the activity of these systems determines the responsiveness of the organism under extreme circumstances, as in fight and flight, which require readiness in motor control and raising of the pain threshold.

It is of interest that the nucleus coeruleus and subcoeruleus as well as the raphe nuclei receive major projections from the amygdala, the periaquaductal grey matter and the medial and lateral hypothalamus (see for review Holstege 1987). Thus, pathways exist from the limbic system which could influence spinal motor activity and the transmission of sensory information from the spinal cord to supraspinal levels.

I.4.5. The corticospinal pathway

The corticospinal pathway in cat and monkey originates from the sensorimotor cortex and descends ipsilaterally

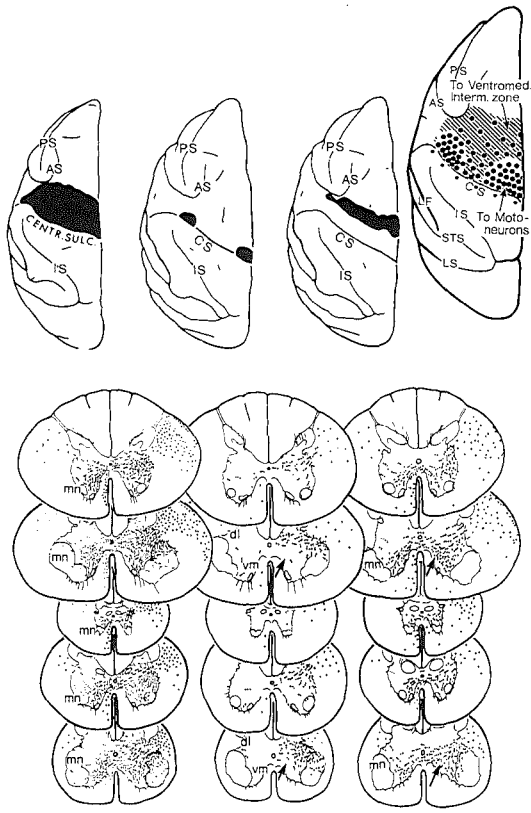

Fig. 3. Distributions of corticospinal fiber degeneration in ventromedial (vm) and dorsolateral (dl) parts of intermediate zone and motoneuronal (mn) cell groups in macaque after lesions in different parts of precentral corticospinal area. Note: distributions to dorsolateral intermediate zone and motoneuronal cell groups after lesions of hand and food areas, and to ventromedial part of intermediate zone bilaterally after rostral lesion. Upper right: diagram of precentral origin of projections to ventromedial intermediate zone and motoneuronal cell groups. (Adapted from Kuypers 1981).

through the internal capsule to the ventral surface of the brain stem. Most of the corticospinal fibers decussate at the transition of the medulla oblongata with the spinal cord and descend in the contralateral dorsolateral funiculus of the spinal cord. The remaining, uncrossed fibers descend in the ventral funiculus, adjacent to the ventral fissure and the dorsolateral funiculus. In cat and monkey the corticospinal fibers are distributed throughout the lenght of the spinal cord.

In monkey and especially in higher primates such as the chimpanzee and man, the corticospinal fibers have a much wider distribution than in the cat. It was demonstrated in monkeys by the anterograde degeneration technique that the precentral motor cortex projects to the dorsal and lateral parts of the intermediate zone (i.e. laminae V and VI, and lateral part of lamina VII) mainly contralaterally (Fig. 3), to the ventromedial parts of the inter-mediate zone (lamina VIII and medial part

of lamina VII) bilaterally and to the motoneuronal cell groups of distal extremity muscles mainly contralaterally (Fig. 3, Kuypers 1960, Nyberg-Hansen and Brodal 1963, Kuypers and Brinkman 1970, Liu and Chambers 1965). These findings were confirmed by autoradiography and anterograde axonal transport of WGA-HRP (Coulter and Jones 1977, Cheema et al. 1984). It was also shown that the corticospinal fibers originating from somatosensory areas 3b, 1 and 2, project to the dorsal horn Kuypers 1960), i.e. to laminae III, IV and V as well as to the laminae I and II (Cheema et al. 1984). Laminae I and II play an important role in nociception. According to anterograde and retrograde HRP transport studies the pericentral cortex also projects to the dorsal column nuclei (Catsman-Berrevoets and Kuypers 1976, Weisberg and Rustioni 1977, Cheema et al. 1985). Electrophysiological studies in cat suggested that the cortical projections to the dorsal column nuclei are at least partly sustained by corticospinal fibers (Endo et al.1973, Atkinson et al. 1974). This assumption could be confirmed anatomically in monkey by a retrograde fluorescent double-labeling study, which demonstrated that 60% of the cortico-cuneate neurons are branching neurons which project to the cuneate nucleus as well as to the spinal cord (Bentivoglio and Rustioni 1986). These branching neurons were present in the caudal part of area 4 but were predominant in somatosensory areas 1 and 2.

In higher primates the distribution of cortical fibers to the intermediate zone follows the same pattern as in the monkey. However, the direct projection to the motoneuronal cell groups is relatively more extensive and also involves the more medially situated motoneurons innervating girdle and proximal extremity muscles (Tower 1944, Kuypers 1964, and Schoen 1964 in man).

Lesion experiments showed (Fig. 3), that different parts of the precentral corticospinal area projects to different levels of the spinal cord and to different parts of the spinal grey matter (Kuypers and Brinkman 1970). The projections of the "hand area" of the motor cortex (in the caudal part of the precentral corticospinal area) to the contralateral spinal grey matter extends from C2 to T4 and involves the dorsal and lateral parts of the intermediate zone and the dorsal parts of the lateral motoneuronal cell groups in C7, C8 and T1 (Fig. 3). Similarly, the projections of the "foot area" on the convexity of the hemisphere and on its medial aspect to the dorsal and lateral parts of the intermediate zone extend from T10 to S1 and involve the dorsal parts of the motor neuronal cell groups in L5, L6, L7 and S1. The cortical projections of the hand and foot area to the intermediate grey of the spinal cord largely coincide with the terminations of the rubrospinal fibers in this area (Kuypers 1981).

The rostral part of the precentral corticospinal area, together with a portion of the cortex along the central sulcus at the level of the precentral dimple (i.e., the area interposed between the hand and foot representations) projects bilaterally to the ventromedial parts of the intermediate zone, throughout the lenght of the spinal cord. Only a few of these fibers terminate in the motoneuronal cell groups. These rostral precentral projections to the spinal cord coincide largely with the terminations of the medial descending brain stem pathways (Kuypers 1981).

The rostral and caudal parts of the motor cortex also differ with respect to their projections to the brain stem nuclei which give rise to the descending brain stem pathways. The rostral part of the precentral corticospinal area and the rostrally adjoining area 6 are the main source of cortical afferents of the bulbar medial reticular formation which gives rise to a major component of the medial descending brain stem pathways (Kuypers and Lawrence 1967, Catsman-Berrevoets and Kuypers 1976). The caudal part of the precentral corticospinal area, including the hand and foot representation, projects somatotopically to the ipsilateral magnocellular red nucleus, which in turn projects to the dorsolateral parts of the spinal intermediate grey matter (Kuypers and Lawrence 1967, Hartmann-von Monakow et al. 1979).

In cat the termination pattern of the corticospinal fibers from areas 4 and 6 in the intermediate grey matter of the spinal cord is very similar to that in monkey (Fig. 4, Cheema et al. 1984, Armand et al. 1985). However, in contrast with the findings obtained in monkey the corticospinal fibers in cat do not establish direct connections with the motoneuronal cell groups. Recently, however, very sparse anterograde labeling of corticospinal fibers was demonstrated in the motoneuronal cell groups using the anterograde transport of WGA-HRP (Cheema et al. 1984). The corticospinal motor area in cat comprises area 4 and the medially adjoining lateral part of area 6 (Catsman-Berrevoets and Kuypers 1976, Keizer and Kuypers 1984). The motor cortex in cat may be divided in a specific zone and a

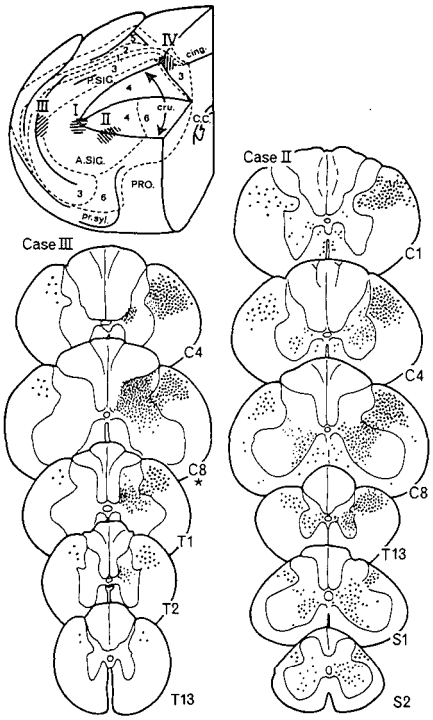

Fig. 4. Differential distribution of radioactivity in cat spinal white and grey matter after injections of tritiated leucine in different parts of sensori-motor cortex. On the right the spinal distribution of labeled fibers in case II with injection in rostral part of area 4. On the left the spinal distribution in case III with injection in lateral part of area 4 and in area 3. Note that in case II labeled fibers are distributed bilaterally throughout the spinal cord and directed to the ventromedial parts of the spinal intermediate zone. Whereas in case III they are restricted to the contra-lateral cervical enlargement and directed to the dorsal part of the intermediate zone and the dorsal horn, including laminae I and II. Cruciate sulcus (cru.) is opened; A. Sig., anterior sigmoid gyrus; cing., cingulate sulcus; C.C., corpus callosum; pr. sylv., presylvian sulcus; P. Sig., posterior sigmoid gyrus; PRO, gyrus proreus. (Redrawn from Armand et al. 1982).

common zone (Armand and Kuypers 1980). The specific zone consists of the hand and foot representations, which are situated in the medial and the lateral parts of the motor cortex, respectively (Fig. 5, Nieoullon and Rispal-Padel 1976). These two regions project only contralaterally to the cervical and lumbar grey matter (Fig. 4, Armand and Kuypers 1980, Armand et al. 1985). The common zone is situated in between the two specific zones and extends rostrally next to area 6. The common zone projects bilaterally to both spinal enlargements (Fig 4). The projection of the specific zone is restricted to the dorsal and lateral parts of the intermediate grey matter, whereas the common zone typically distributes fibers bilaterally to the ventromedial part of the intermediate grey matter (Armand et al. 1985). It sends also some fibers to the dorsal and lateral parts of the intermediate zone.

The projections from the motor cortex to the brain stem nuclei in cat show a similar organisation as in monkey. For example, the caudal parts of the pericruciate motor cortex (specific zone) project to the rubrospinal part of the red nucleus, whereas the rostral and medial parts of the motor cortex (common zone), including area 6 project to the bulbar medial reticular formation (Kuypers 1958, 1964, Armand et al. 1985).

The cortical projections to the dorsal column nuclei (DCN) originate mainly from areas 4 and 3a (Weisberg and Rustioni 1979). According to a double-labeling study using the HRP-tritiated apo-HRP technique only a small proportion of the cortico-DCN neurons in cat represented branching neurons which project to the dorsal column nuclei and to the spinal cord (Rustioni and Hayes 1981). This is in sharp contrast with the findings obtained in monkey (Bentivoglio and Rustioni 1986). Moreover, in cat the branching neurons were concentrated in area 3a, while in monkey they were concentrated in areas 1 and 2. These findings were confirmed in a retrograde fluorescent double-labeling study, in which it was demonstrated that 14-16% of the cortico-cuneate neurons are branching corticospinal neurons (Bentivoglio and Rustioni 1986).

The rostral part of the corticospinal area in monkey and the rostral and medial parts of the motor cortex in cat (common zone) project to the intermediate grey of the spinal cord as well as to the medial reticular formation of the lower brain stem. In a fluorescent double-labeling study in rat and cat it has been demonstrated that the

corticospinal neurons situated in the rostro-medial part of the motor cortex distribute collaterals to the mesencephalon (Catsman-Berrevoets and Kuypers 1981). Therefore the question arose whether corticospinal neurons in the rostral part of the motor cortex also distribute collaterals to the medial reticular formation of the lower brain stem. An attempt has been made to clarify this question in cat and monkey, using the retrograde fluorescent double-labeling technique. The results of these studies are presented in chapters III and IV.

I.5. The motor areas of the cerebral cortex

The discovery of the motor cortex is usually attributed to Fritsch and Hitzig 1870 and Ferrier 1886 (referred to by Phillips 1987) who obtained movements by electrical stimulation of a particular region of the convexity of the hemispheres. Jackson in the 1860's and 1870's already introduced the idea of a motor cortex, on the basis of his clinical observations of motor deficits and in focal motor epilepsia. In the pioneering studies of Fritsch and Hitzig 1870 and Ferrier 1886, movements could be elicited from large areas of the frontal lobe, including its medial aspect and from postcentral parietal regions.

At the turn of the century, Leyton and Sherrington (1917, referred to by Phillips 1987) reinvestigated the motor cortex in primates using near-threshold stimuli. They found the electrically excitable motor areas to be restricted to the precentral cortex. In 1905 the first major cytoarchitectonic maps of the cerebral cortex were published independently by Campbell (1905) in England and by Brodmann (1905) in Germany. According to Brodmann the precentral cortex in man comprises of areas 4 and 6 which are relatively thick and agranular, whereas the postcentral cortex can be divided into the areas 3a,b, 1 and 2, which are thinner and of a granular type.

In the caudal part of the precentral cortex, corresponding to area 4, movements can be elicited with the lowest threshold. This region was called the primary motor cortex (Leyton and Sherrington 1917). It is somatotopically organized. In the rostrally adjoining cortical region, corresponding to area 6, movements can also be elicited but at a higher stimulus threshold. This region is called the premotor cortex (Fulton 1935). In the 1950's Woolsey in monkey (Woolsey et al. 1952, Woolsey 1958), and Penfield in man (Penfield and

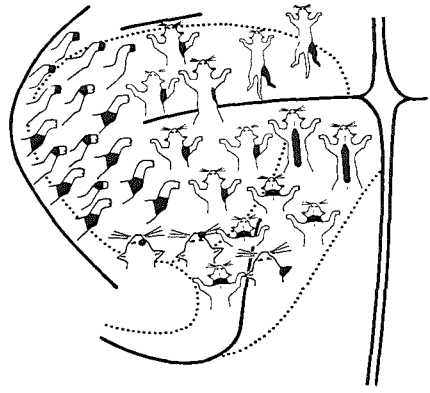

Fig. 5. Diagram showing the somatotopic organization in the cat motor cortex. Each figurine is an image of one part of the musculature activated by stimulation of the cortical areas on which it lies. Note that the representations of neck, back and shoulder movements are situated in the rostromedial part of the pericruciate motor cortex, whereas movements of fore- and hindlimb are represented in the lateral and medial parts of the pericruciate cortex respectively. (After Nieoullon and Rispal-Padel 1976).

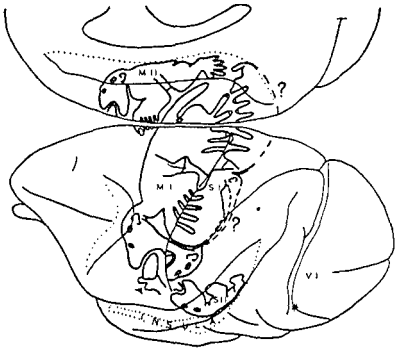

Fig. 6. Diagram of monkey cerebral cortex showing the somatotopic localization of parts of the body in the precentral primary motor cortex (M I) on the convexity of the hemisphere and in the supplementary motor cortex (M II) on the medial aspect of the hemisphere. Note that the representations of neck, back and shoulder movements are situated in the rostral part of the motor cortex, partly overlapping with the premotor cortex. (After Woolsey 1958).

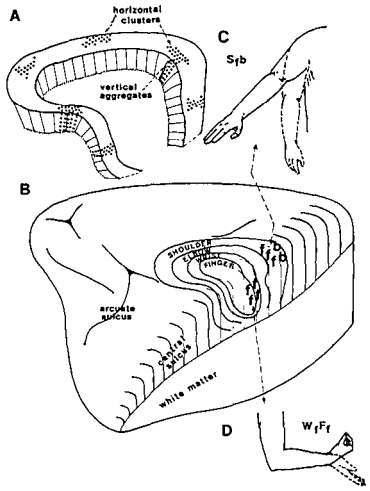

Fig. 7. A. Diagram illustrating basic components of a joint zone B: a three-dimensional drawing of the left frontal lobe of a monkey showing the spatial organization of joint zones, i.e. finger, wrist, elbow, shoulder within the precentral forelimb area. In the labeled joint zone, f and b represent flexion and abduction clusters respectively. Activation of neighbouring clusters within or across contiguous joint zones can produce combination movements, shoulder flexion-abduction (Sfb) in C and wrist flexion-finger flexion WfFf) in D. (After Murphy et al. 1978).

Welch 1951) described a second somatotopically organized motor area on the medial aspect of the hemisphere (Fig. 6), i.e. in the medial part of area 6.

Thus in the precentral cortex we may recognize the primary motor cortex (roughly corresponding with area 4), the premotor cortex (lateral area 6) and the supplementary motor area (SMA, medial area 6).

I.5.1. The primary motor cortex

In monkey the primary motor cortex is situated in the anterior bank of the central sulcus and the adjoining caudal part of the frontal lobe. It corresponds roughly with Brodmann's area 4, which together with area 6 comprises the agranular cortex. The pyramidal cells in these areas are large and numerous. The fourth, internal granular layer is lacking. Area 4 contains the giant pyramidal cells (so-called "Betz-cells") in cortical layer V, the deep layer of pyramidal cells. These Betz cells gradually diminish in number and size when moving anteriorly towards area 6. Contrary to general beliefs, the border between area 4 and 6 can not easily be distinguished (see Wise 1984). There is no abrupt disappearance of the Betz cells.

According to electrical mapping studies the motor cortex is somatotopically organized (Woolsey et al. 1952, Woolsey 1958), Penfield and Welch 1951). Movements of the hand and independent movements of the fingers are represented laterally on the convexity of the precentral gyrus (Fig 6). Lateral to this region the face is represented. Medial to it, hidden on the medial surface of the hemisphere, the representation of the foot is situated (Fig. 6). The distal extremities occupy relatively large areas within the motor cortex. This results in a distorted representation of the body (Fig. 6). Murphy and Kwan who studied the representation in the

forelimb area using intracortical microstimulation described a concentric organization (Fig. 7), where the cortical region which controls movements of a more distal joint was surrounded by the regions controlling more proximal joints (Murphy et al. 1978, Kwan et al. 1978). Thus, for the entire forelimb, shoulder, elbow, wrist, and fingers would be represented in this order, with the shoulder region at the periphery and the finger region in the center (Fig. 7). This crescent-shaped organization is at variance with the simiusculi organization of Woolsey (1958, Fig. 6). This might be due to difference in methodology, since in Woolsey's study surface stimulation was used in anesthesized monkeys, whereas in the latter studies intracortical stimulation was used in awake monkeys. In a chronic recording study of the motor cortex in monkey (Lemon 1981) also a distinct central zone was observed in which only neurons with inputs from the hand and fingers were found. This zone extended deep into the bank of the central sulcus and had its rostral border on the convexity of the gyrus immediately rostral to the sulcus. It was bounded rostrally and medially by neurons with inputs from wrist, elbow and shoulder regions. However, on the edge of this central hand and finger zone there was extensive intermingling of hand-input neurons with neurons with more proximal inputs, particularly from the wrist. Hand-input neurons were also found scattered at a distance from the central hand or finger zone. In addition, there were cell groups with intermingling of inputs from different parts of the limb and multiple representations of inputs from the digits were found (Lemon 1981). These findings are in keeping with those obtained using "spike triggered averaging" of the rectified electromyogram, which revealed that clusters of output neurons facilitate the same muscle, and each muscle is represented many times over in the motor cortex (Lemon 1988).

The somatotopical organization of the primary motor cortex is also demonstrated by its connections with the spinal cord (see "the corticospinal pathway"). In monkey and especially in primates and man the corticospinal neurons in the primary motor cortex establish direct cortico-motoneuronal connections.

Ablation of the primary motor cortex in monkey resulted in a temporarely paresis of the contralateral half of the body. In time the monkey was able to walk ,climb and jump again, using its extremities in integrated limb-body movements. However, lesion studies in which the pyramidal tract was transsected on both sides demonstrated undisputably that the capacity to execute highly fractionated movements of the the distal limbs is lost for ever (Lawrence and Kuypers 1968a).

The primary motor cortex is reciprocally connected with somatosensory areas 1 and 2, and with parietal area 5 and the premotor areas (Jones and Powell 1969, Pandya and Kuypers 1969, Pandya and Vignolo 1971, Strick and Kim 1978, Muakassa and Strick 1979).

The main projection from the thalamus to the primary motor cortex (area 4) arises from the VLp nucleus of the thalamus. This nucleus is the recipient of fibers from the deep cerebellar nuclei (Strick 1976, Kievit and Kuypers 1977, Kievit 1979, Asanuma et al. 1983a, b). Moreover, anatomical findings suggest that the motor cortex is the only target of the cerebello-thalamo-cortical pathway (Friedman and Jones 1981, Jones 1983). However, other anatomical findings indicated that the supplementary cortex also receives a cerebellar input (Wiesendanger 1987).

Cerebral blood flow studies in humans show that the primary motor cortex is activated unilaterally whenever voluntary movements are executed. In circumstances in which the person is asked to imagine a particular movement without actually performing it, the primary motor cortex does not show an increase in activity. These findings suggest that the primary motor cortex is the executive locus for voluntary movements (Olesen 1971, Risberg and Ingvar 1973, Roland and Larsen 1976, Roland et al. 1980a, b, Roland and Friberg 1985).

I.5.2. The premotor cortex

In monkey the premotor cortex is situated rostral to the motor cortex (area 4) on the convexity of the hemisphere. The premotor cortex corresponds roughly with lateral area 6. It is of an agranular type but lacks the giant Betz cells. There is some confusion about the precise border between areas 4 and 6. In this thesis the transition from the primary motor cortex to premotor cortex will be presumed to be gradual (Wise 1984).

The caudal part of area 6, which corresponds with the rostral part of the corticospinal area, projects bilaterally to the ventro-medial part of the spinal intermediate grey (Kuypers 1964, Kuypers and Brinkman 1970). In contrast with the primary motor cortex, the premotor cortex does not establish direct cortico-motoneuronal connections. It projects heavily and bilaterally to the bulbar medial reticular formation, which is a major contributant to the medial descending brain stem

pathway, steering especially axial and proximal movements (see "the medial group of descending brain stem pathways"). Electrical stimulation of the premotor cortex evoked movements at higher stimulus thresholds as compared with the motor cortex (Bucy 1933, Fulton 1935, Woolsey 1952, Asanuma and Rosen 1972, Kwan et al. 1978, Weinrich and Wise 1982, Sessle and Wiesendanger 1982). Moreover, the movements were often slower and more complex involving axial, proximal and integrated limb-body movements.

The premotor cortex receives afferents from prefrontal regions and the parietal and temporal regions, which in turn are connected with the visual, somatosensory and acoustic cortical areas (Kuypers et al. 1965, Pandya and Kuypers 1969, Pandya and Vignolo 1971, Jones et al. 1978, Barbas and Pandya 1987). Most of the cortico-cortical connections are reciprocal. Retrograde HRP experiments of Muakassa and Strick (1979) showed that the following four regions within the frontal cortex project to the hand area of the motor cortex, i.e. 1. the surface and caudal bank of the arcuate sulcus, 2. the lateral bank of the superior precentral sulcus, 3. the SMA and 4. the ventral bank of the cingulate sulcus.

Anatomical findings suggest that visual information reaches the motor cortex via connections with the premotor cortex (Kuypers et al. 1965, Pandya and Kuypers 1969, Chavis and Pandya 1976, Muakassa and Strick 1979, Godschalk et al. 1984). Such an organisation has also been suggested by lesion studies in monkey (Haaxma and Kuypers 1975). After ablations of the premotor cortex, located medial to the arcuate sulcus and including the SMA, the monkeys had severe problems in visually guided hand and independent finger movements (Moll and Kuypers 1977). After bilateral ablation of the cortex situated medial to the upper limb of the arcuate sulcus, including the tissue lying in the posterior bank of its upper and lower limbs, monkeys had severe problems in relearning a visual conditional motor task (Passingham 1985). Although they could make either of the movements, they were slow to relearn which movement to make in respons to a visual cue.

Chronic recording studies in monkeys revealed that neurons in the postarcuate region of the premotor cortex modulate their activity upon presentation of a visual cue, prompting a visually guided arm and hand movement, but before that movement started (Godschalk et al. 1981, 1985). The premotor cortex also receives connections from other sensory areas (Bignall and Imbert 1969, Weinrich and Wise 1982). These findings suggest that the postarcuate premotor cortex is involved in processing polysensory information required for cueing and guiding limb movements in space.

The premotor cortex is one of the main recipients of pallidal input via the VLa nucleus of the thalamus (DeVito and Anderson 1982, Schell and StricK 1984).

Cerebral blood flow studies in humans demonstrated, that the premotor cortex showed an increase in activity whenever the person had to perform non-routine voluntary movements, which had to be executed under visual, somatosensory and auditory guidance (Roland and Larsen 1976, Ingvar and Philipson 1977, Roland et al. 1980b).

I.5.3. The supplementary motor area (SMA)

The SMA is situated on the medial aspect of the hemisphere, deeply hidden in the interhemispheric fissure. It corresponds roughly with the medial part of area 6. Stimulation experiments demonstrated that the SMA contains a complete, somatotopically organized representation of the body. It was called the supplementary motor cortex by Penfield (Penfield and Welch 1951 in humans, and Woolsey et al. 1952 in monkey). In a microstimulation study in monkey such a somatotopic organization in the SMA could not be observed (Macpherson et al. 1982). However, in a more recent intracortical microstimulation study a clear rostrocaudal organization in the SMA was demonstrated, such that from rostral to caudal the representations of orofacial movements, forelimb movements and hindlimb movements were observed. The latter two representations showing some overlap (Mitz and Wise 1987). However, the representations of proximal and distal limb movements were intermingled in the SMA, in contrast with the proximal to distal representation of the limbs in the precentral motor cortex.

Recently it was demonstrated that the SMA projects bilaterally to the intermediate zone of the spinal cord. The spinal projections of the limb areas were found to be less dense than those of the motor cortex and direct cortico-motoneuronal connections could not be demonstrated anatomically (Murray and Coulter 1981, Brinkman 1982, Cheema et al. 1983). Electrophysiological findings are suggestive of some monosynaptic connections to spinal motoneurons (Wiesendanger 1987), but this projection is very sparse.

The SMA is reciprocally connected with area 4 and lateral area 6 and parietal areas 1, 2 and 5 and 7 (Jurgens

1984). The SMA receives a major contingent of afferents from the thalamic VLo nucleus, which is the pallidal thalamic nucleus, receiving pallidal fibers (Schell and Strick 1984).

In patients with lesions of the SMA a severe reduction of spontaneous motor activity was observed, more pronounced contralaterally than ipsilaterally (Laplane et al. 1977, Talairach and Bancaud 1966). These symptoms were accompanied by a reduction of speech and impairment of bimanual coordination. However, when prompted by questions they were able to speak. This "akinesia" receded after two weeks. Longlasting symptoms were scarce.

Cerebral blood flow studies in humans show that the SMA is activated during the preparation and execution of a voluntary motor tasks that consist of complicated movement sequences (Roland et al. 1980a). Well learnt sequences of movements for which no sensory information is necessary, including speech, activate SMA but not the premotor cortex (Roland et al. 1976, 1980a, 1982, 1985, Larsen et al. 1978, 1979). In contrast to the primary motor cortex, the SMA is always activated bilaterally.

I.6. The corticopontine and corticotectal projections

The connections from the cerebral cortex to the cerebellum by way of the pontine nuclei have been suggested to represent part of a circuitry which subserve comparison of the peripheral inputs with central instructions. In an attempt to clarify the anatomical aspects of the corticopontine projections, we looked also to the collateralization of corticopontine fibers. For that purpose we had a closer look at the collateralization of fibers from the visual and auditory cortices.

In cat, anterograde tracing and degeneration studies demonstrated that widespread cortical areas project to the basal pons (Sanides et al. 1978, Chiba 1980, see for review Brodal 1982) and in monkey (Sunderland 1940, Kuypers and Lawrence 1967, Dhanarajan et al. 1977, Brodal 1978, Wiesendanger et al. 1979, Vilensky and Van Hoesen 1981, Brodal 1982). According to these studies corticopontine neurons originate from frontal, sensorimotor, parietal, occipital and temporal cortex. However, anterograde studies are less appropiate to study all cortical regions which project to the pons. Moreover, the relative contributions of the various cortical areas to the pontine projection can not be established.

Retrograde HRP studies confirmed most of the anterograde tracing findings, such that the cells of origin of the corticopontine fibers were present in widespread cortical regions in cat (Kawamura and Chiba 1979, Albus et al. 1981, Brodal 1982, Bjaalie and Brodal 1983, Bjaalie 1985, 1986), in monkey (Glickstein 1985) and rat (Wyss and Sripanidkulchai 1984). In cat corticopontine fibers originate mainly from sensorimotor (areas 6, 4, 3, 1, 2), parietal (area 5 and rostral area 7), and cingulate cortex. The projections from visual areas 17, 18 and 19 and premotor area 6 are less dense than from the other sensorimotor and parietal areas. The second auditory area (AII) in cat also projects to the basal pons (Brodal 1972).

In monkey the cortical origin of corticopontine fibers seems to be more restricted. Anterograde tracing and degeneration findings (Brodal 1982, Wiesendanger et al. 1979) and retrograde HRP findings (Glickstein 1985) showed that area 4, the motor cortex is a major source of corticopontine fibers. According to the anterograde studies the projections from area 6 are less dense than from area 4, whereas the density of the labeling of cells with retrograde HRP in area 4 and 6 was found to be equal. However, after injections of HRP in the basal pons, the corticopontine as well as the corticobulbar and corticospinal neurons will be labeled in layer V of areas 4 and 6. These two populations of labeled corticospinal and corticopontine neurons cannot be distinguished from each other, and a reliable comparison between the number of labeled corticopontine neurons in areas 4 and 6, therefore, cannot be made. The anterograde studies demonstrated a moderate to sparse projection from areas 5 and 7 in monkey and a heavy projection arising from somatosensory areas 3, 1 and 2. In contrast, the distribution of retrogradely HRP-labeled neurons showed that there are considerably fewer labeled corticopontine neurons in the primary somatosensory areas than in the rostrally adjoining areas 4 and 6 and the caudally adjoining areas 5 and 7. The retrograde HRP findings also demonstrate that the cingulate cortex in monkey is a major source of corticopontine fibers. Moreover, anterograde as well as retrograde studies have demonstrated corticopontine projections arising from the frontal areas 8 and 9. In the visual areas a moderate amount of labeled neurons was present in area 19, some in area 18 and only a small number in area 17, which were restricted to the upper bank of the calcarine fissure (Glickstein 1985). None were present in the temporal cortex.

All retrograde HRP studies demonstrated that in cat and monkey the cells of origin of the corticopontine fibers are situated exclusively in cortical layer V and are pyramidal in shape. Cortical layer V also contains the cells of origin of the cortical descending pathways to the brain stem and the spinal cord.

The corticotectal neurons distribute fibers to the colliculi. The colliculi are the origin of the tectobulbar and tectospinal fibers, which are a major component of the medial group of descending brain stem pathways (Hollander 1974, Berrevoets and Kuypers 1975, Maghelaes-Castro et al. 1975, Gilbert and Kelly 1975, Kawamura and Konno 1979, Tortelli et al. 1980, Catsman-Berrevoets and Kuypers 1981, Fries 1984). Anterograde tracing and degeneration studies demonstrated cortical projections to the colliculi originating from occipital, temporal, parietal and frontal regions (Whitlock and Nauta 1956, Kuypers and Lawrence 1968, Goldman and Nauta 1976, Kunzle et al. 1976, Kunzle and Akert 1977, Hartmann-von Monakow et al 1979, leichnetz et al. 1981). These findings were confirmed in retrograde HRP studies (Lund et al. 1975, Leichnetz et al. 1981, Fries 1984).

The cortical distributions of the corticopontine and the corticotectal neurons are suggestive of a common origin of some of the corticopontine and corticotectal fibers from the same parent cells. In a related study, which was performed in our laboratory, it was demonstrated by means of the retrograde transport of Fast Blue, that the corticospinal and pyramidal fibers distribute collaterals to the pontine grey (Ugolini and Kuypers 1986). Thus the corticopontine fibers originating from the sensorimotor area are at least in part derived from branching corticobulbar and corticospinal neurons. An electrophysiological study in cat demonstrated that corticopontine fibers from visual area 18 and lateral suprasylvian cortex represented collaterals of corticotectal fibers (Baker et al. 1983). In light of these findings the intriqueing question arose whether the corticopontine fibers originating from the various cortical areas would be derived from branching cortical neurons which distribute collaterals to the pontine grey and to the superior colliculi. An attempt has been made to clarify this point by means of the retrograde fluorescent double-labeling technique, these experiments are presented in chapter V.

I.7. Meynert cells

The solitary cells of Meynert (Meynert 1867) form a class of large neurons in primate striate cortex that are located, in the macaque, in layer VI (Le Gros Clark 1942, Lund 1973, Chan-Palay et al. 1974). Retrograde tracer studies have demonstrated that Meynert cells project to the posterior bank of the superior temporal sulcus (Lund et al. 1975, Maunsell and Van Essen 1983, Fries and Zeki 1983), which contains a visual area concerned with the analysis of visual movement that was originally known as the "motion area of the STS" and later indicated as area V5 (Zeki 1974, Zeki 1978).

According to retrograde HRP studies, Meynert cells were also found to project to the superior colliculus (Fries and Distel 1983, Fries 1984). Meynert cells are easy recognizable in Nissl stained sections because of their size and the ovoid shape of their cell bodies. This feature made it possible to demonstrate, that after injections of HRP in the posterior bank of the superior temporal sulcus and in the superior colliculus that in either experiment, almost all Meynert cells were found to be labeled. These findings suggested that Meynert cells project by means of axoncollaterals to visual area V5 as well as to the superior colliculus.

An attempt has been made to clarify this point by means of the retrograde double-labeling technique (chapter VI).

Chapter II

Diamidino Yellow dihydrochloride (DY.2HCl); a New Fluorescent Retrograde Neuronal Tracer, Which Migrates Only Very Slowly Out of the Cell

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Summary. Earlier studies showed that Nuclear Yellow (NY), True Blue (TB) and Fast Blue (FB) are transported retrogradely through axons to their parent cell bodies. NY produces a yellow fluorescent labeling of the neuronal nucleus at 360 nm excitation wavelength, while TB and FB produce a blue fluorescence of the cytoplasm at this same wavelength. Therefore, NY may be combined with TB or FB in double-labeling experiments demonstrating the existence of axon collaterals. However, retrograde neuronal labeling with TB or FB requires a relatively long survival time, while NY requires a short survival time since NY migrates rapidly out of the retrogradely labeled neurons. This complicates double-labeling experiments since TB and FB must be injected first and NY later, a short time before the animal is sacrificed. We report a new yellow fluorescent tracer which labels mainly the nucleus and migrates much more slowly out of the retrogradely labeled neurons than NY. This new tracer can be used instead of NY in combination with TB or FB in double-labeling experiments and unlike NY can be injected at the same time as TB or FB. The new tracer is a diamidino compound (no. 28826) which is commercially[3] available. It will be referred to as Diamidino Yellow Dihydrochloride (DY·2HCl). According to the present study DY·2HCl is transported over long distances in rat and cat, and produces a yellow fluorescence of the neuronal nucleus at 360 nm excitation wavelength, resembling that obtained with NY. When combined with TB or FB, DY·2HCl is as effective as NY in double labeling of neurons by way of divergent axon collaterals.

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Introduction

In previous studies (Bentivoglio et al. 1980b), Nuclear Yellow (NY) was found to be transported retrogradely through axons to their parent cell bodies, which results in a yellow fluorescent labeling of the nucleus. However, after long survival times NY migrates out of retrogradely labeled neurons, which may produce false neuronal labeling (Kuzuhara et al. 1980; Bentivoglio et al. 1980a). This can be prevented by restricting the NY survival time (Bentivoglio et al. 1980a). With this precaution, NY may be combined successfully with True Blue (TB) in rat (Bentivoglio et al. 1979a; Kuypers et al. 1980; Huisman et al. 1981) and Fast Blue (FB) in cat (Bentivoglio et al. 1980b; Kuypers et al. 1980; Catsman-Berrevoets and Kuypers 1981; Huisman et al. 1982). However, the TB and FB survival times (Bentivoglio et al. 1980b) are much longer than the NY survivial time (Bentivoglio et al. 1980a). Therefore, TB or FB must be injected first and NY later, i.e. a short time before the animal is sacrificed (Kuypers et al. 1980). This complication can be avoided if NY is replaced by a new retrograde tracer called Diamidino Yellow Dihydrochloride (DY·2HCl) which is now commercially available[4].

The retrograde neuronal labeling obtained with DY·2HCl in several neuronal systems in the brain of rat and cat will be reported in the present study. The findings may be summarized as follows; DY·2HCl produces retrograde neuronal labeling resembling that obtained with NY, but DY·2HCl migrates much

more slowly out of the retrogradely labeled neurons than NY. DY·2HCl is transported retrogradely over long distances in rat as well as in cat. Effective retrograde DY·2HCl labeling requires a survival time comparable to that of TB and FB. DY·2HCl is also transported anterogradely through axons, which results in blue fluorescence of glial nuclei along these axons and in their termination area. However, this anterograde labeling of glial nuclei has a much longer time course than the retrograde neuronal labeling. In rat sensorimotor cortex the callosal and the corticospinal neurons form two separate populations (Catsman-Berrevoets et al. 1980). No false DY·2HCl retrograde labeling occurs from DY·2HCl-labeled callosal neurons to adjoining TB-labeled corticospinal neurons, even after long survival times. In several neuronal systems in the brain, the double labeling of neurons by way of axon collaterals with DY·2HCl in combination with TB or FB is as effective as with NY combined with TB or FB. Therefore, DY·2HCl may be used instead of NY in double-labeling experiments, thereby minimizing the risk of false double labeling and making it possible to inject the two tracers at the same time.

Material and Methods

The retrograde DY·2HCl labeling was studied in five groups of experiments (Table 1).

Group A. In 8 rats 0.4 µl 2% DY·2HCl dissolved in water was injected unilaterally in caudate-putamen. For comparison, in 4 of the rats in addition, 0.4 µl 1% NY was injected in caudate-putamen on the other side. The animals were killed after 2 to 6 days.

Group B. In 4 rats multiple 2% DY·2HCl injections were made in the spinal cord at C2 (1.8 µl) and in 2 rats at L1–L2 (2.0 µl). The animals were allowed to survive from 3 to 21 days. For comparison in 2 rats 1% NY injections were made at C2 (2.0 µl) followed by 3 days survival. In 2 cats multiple 2% DY·2HCl injections were made unilaterally in the spinal white and grey matter, in the first cat at C5–C8 (10.8 µl) and in the second cat at L7–S1 (9.0 µl). The first cat survived 3 weeks and the second 4 weeks.

Group C. In 4 rats 2.4 µl 2% DY·2HCl and in 4 other rats 2.4 µl 1% NY was injected unilaterally in the sensorimotor cortex (24 penetrations). These animals survived from 2 to 6 days.

Group D. In 3 rats 3.0 µl 2% DY·2HCl was injected in sensorimotor cortex (30 penetrations) and 1.0 µl 2% TB was injected ipsilaterally in C2 followed by 6 days survival.

Group E.

1. In 3 rats 0.4 µl 2% TB was injected in ventral thalamus and in the same session 0.6 µl 2% DY·2HCl was injected ipsilaterally in superior colliculus. In one additional rat the tracers were reversed. Each rat survived for 4 days.

2. In 4 rats 0.5 µl 2% TB was injected in spinal dorsal grey at C5–C8 (5 penetrations). In two of these rats, in the same session 1.0 µl 2% DY·2HCl was injected in white and grey matter, in one rat at T7–T8 (5 penetrations) and in the other at T13–L1 (5 penetrations). Both rats were allowed to survive for 7 days. In the 2 remaining rats 7 days after the TB injection, 1.0 µl 1% NY was injected, in one rat at T7–T8 and in the other at T13–L1; these animals were killed 40 h and 43 h later.

3. In 4 rats 2.2 µl 2% TB was injected unilaterally in cerebellar interpositus nucleus (6 penetrations). In 2 of these rats in the same session 2.0 µl 2% DY·2HCl was injected ipsilaterally in white and grey matter at C5–C8 followed by 7 days survival. In the 2 other rats, 7 days after the TB injections, 2.0 µl 1% NY dissolved in a 2% solution of dimethylsulfoxide (DMSO) in water was injected in the white and grey matter at C5–C8 ipsilateral to the TB interpositus injections. These animals were killed 24 h after the NY injections.

4. In 2 cats 8.2 µl and 9.0 µl 3% FB in 2% DMSO in water was injected unilaterally into white and grey matter at C2 (25 penetrations). In the same session the first cat was injected with 1.8 µl 2% DY·2HCl ipsilaterally in the medial reticular formation of the medulla oblongata and caudal pons (6 penetrations). This animal survived for 4 weeks. In the second cat, 4 weeks after the FB injections, 1.8 µl 1% NY was injected ipsilaterally in these brain stem areas (6 penetrations). This animal was killed 36 h after the NY injection.

All the injections were made under Nembutal anaesthesia. The tracers were injected either by means of a glass micropipette connected to an oil filled pressure system or by a Hamilton microsyringe equipped with a 22 G needle. 2% DY·2HCl in 0.2 M phosphate buffer pH 7.2 forms a suspension of a viscosity resembling that of 2% TB and, when injected most of it stays within the tissue without fountaining out of the needle track.

After the appropriate survival times the animals were deeply anaesthetized with Nembutal and perfused transcardially with saline followed by cacodylate buffered formalin (pH 7.2). In the rats 1.5% saline and in the cats 2.7% saline was used. The rats were perfused with 10% formalin, but the cats were perfused with 30% formalin, which increases the blue fluorescence of the FB-labeled neurons. In the rats the brain and spinal cord were placed overnight in cacodylate buffered 30% sucrose (pH 7.2) at 4° C and then cut transversely in frozen sections (30 µm). In later experiments for reasons of economy, the cacodylate buffer was replaced by a citrate buffer (pH 7.2). In the cats the perfusion with formalin was followed by a perfusion with cacodylate buffered 8% sucrose (pH 7.2), after which the material was immediately cut into frozen secitons. The frozen sections were mounted from distilled water on slides and air dried, but were not coverslipped. For tissue containing NY, each section was mounted immediately after cutting in order to prevent in vitro migration of NY out of the retrogradely labeled neurons (Bentivoglio et al. 1980a). When DY·2HCl is used, the sections should not be kept in water for longer than 6 h, otherwise fluorescent glial nuclei appear around the retrogradely labeled neurons. Such glial labeling also occurred after some months of dry storage of the mounted sections at 4° C. All mounted sections were stored in the dark at 4° C. They were studied under a Leitz Ploemopack fluorescence microscope equipped with filter-mirror systems A and D, providing excitation light of 360 nm and 390 nm wavelengths. In the cases of group E the distributions of labeled neurons, including the double-labeled ones, were charted with the aid of an X-Y plotter, which was connected with transducers attached to the microscope stage. In certain cell groups the single- and double-labeled neurons were counted and the ratios between the single- and double-labeled neurons were computed (see Table 2).

Table 1

Group A: NY and DY labeling in nigro-striatal system					
Animals	% Tracer µl	Injection site	% Tracer µl	Injection site	Survival time
4 Rats	2% DY 0.4	Left Caud-put			3 Days
4 Rats	2% DY 0.4	Left Caud-put	1% NY 0.4	Right Caud-put	2, 3 (2×), 6 days
Group B: DY labeling relative to transport distance and survival time					
Animals	% Tracer µl	Injection site			Survival time
4 Rats	2% DY 1.8	C2			3, 7, 14, 21 days
2 Rats	1% NY 2.0	C2 For comparison			3 Days
2 Rats	2% DY 2.0	L1–L2			14 Days
1 Cat	2% DY 10.8	C5–C8			21 Days
1 Cat	2% DY 9.0	L7–S1			28 Days
Group C: NY and DY 'anterograde' glial labeling in thalamus from cortex					
Animals	% Tracer µl	Injection site			Survival time
4 Rats	2% DY 2.4	Sens.mot. cortex			2, 4 (2×), 6 days
4 Rats	1% NY 2.4	Sens.mot. cortex			2, 4 (2×), 6 days
Group D: Test for false retrograde DY-labeling from neighbouring neurons					
Animals	% Tracer µl	Injection site	% Tracer µl	Injection site	Survival time
3 Rats	2% DY 3.0	Sens.mot. cortex	2% TB 1.0	C2	6 days
Group E: Comparison of DY and NY for double labeling in combination with TB or FB					
Animals	% Tracer µl	Injection site	% Tracer µl	Injection site	Surv. time/surv. time
1. 3 Rats	2% TB 0.4	Ventr. thal.	2% DY 0.6	Sup. coll.	4 Days
1 Rat	2% TB 0.6	Sup. coll.	2% DY 0.4	Ventr. thal.	4 Days
2. 1 Rat	2% TB 0.5	Dors grey C5–C8	2% DY 1.0	T7–T8	7 Days
1 Rat	2% TB 0.5	Dors grey C5–C8	1% NY 1.0	T7–T8	TB: 7 D NY: 40 h
1 Rat	2% TB 0.5	Dors grey C5–C8	2% DY 1.0	T13–L1	7 Days
1 Rat	2% TB 0.5	Dors grey C5–C8	1% NY 1.0	T13–L1	TB: 7 D NY: 43 h
3. 2 Rats	2% TB 2.2	Cereb. interp. N	2% DY 2.0	C5–C8	7 Days
2 Rats	2% TB 2.2	Cereb. interp. N	1% NY 2.0	C5–C8	TB: 7 D NY: 24 h
4. 1 Cat	3% FB 8.2	C2	2% DY 1.8	Med. R.F.	4 Weeks
1 Cat	3% FB 9.0	C2	1% NY 1.8	Brainstem	FB: 4 w NY: 36 h

Results

Group A

In the rats of this group with injections in the caudate-putamen the DY·2HCl and NY retrograde labeling of nigrostriatal neurons was compared (see Table 1).

The NY injection area in caudate-putamen consisted of 3 concentric zones surrounding the needle track. The first zone was very narrow and showed yellow tissue fluorescence and a dense accumulation of bright yellow-white glial nuclei (filter-mirror system A, 360 nm excitation wavelength). The second zone was much wider and also displayed yellow tissue fluorescence, but contained fewer fluorescent glial and neuronal nuclei. The third zone showed no tissue fluorescence and contained only dull fluorescent glial and neuronal nuclei. The inner part of the injection area (i.e. zones I and II) measured 2 mm in diameter.

The DY·2HCl injection area contained in its center a mass of brown-yellow fluorescent material, presumably consisting of DY·2HCl combined with some necrotic tissue. This area was surrounded by a zone which displayed blue tissue fluorescence (filter-mirror system A, 360 nm excitation wavelength) and contained a dense accumulation of fluorescent glial nuclei and neurons with fluorescent nuclei. This zone in turn was surrounded by a zone containing only fluorescent glial and neuronal nuclei. The inner part

of this injection area, comprising the mass of fluorescent material together with the zone displaying tissue fluorescence, was smaller than in the NY injection area and measured only 1 mm in diameter.

In the 4 rats with NY injections followed by 2, 3 and 6 days survival, many NY-labeled neurons were present in the substantia nigra pars compacta (SNC), on the side of the NY injection. They showed nuclear and some cytoplasmic labeling (cf. Bentivoglio et al. 1980a, b). Many fluorescent glial nuclei were present around retrogradely labeled neurons in the SNC (Fig. 1A) and in the substantia nigra pars reticulata (SNR) (cf. Bentivoglio et al. 1980a). The latter presumably were labeled with NY from striato-nigral fibers to the SNR through which NY had been transported anterogradely (Fig. 1A).

In all 8 rats the SNC on the DY·2HCl injection side contained many DY·2HCl-labeled neurons. They showed a yellow fluorescent nucleus and a diffuse yellow fluorescence of the cytoplasm of cell body and proximal dendrites (Fig. 1B), which was more pronounced than in the NY-labeled neurons. The nuclear fluorescence was more diffuse and less granular than that of the NY-labeled neurons and the ring around the nucleolus was less bright. Moreover, the nuclei tended to be smaller than those labeled with NY. The DY·2HCl-labeled neurons showed a bright green fluorescence when using filter mirror system D (390 nm excitation wavelength), and showed a slightly duller yellow fluorescence when using filter mirror system A (360 nm excitation wavelength). In the cases with 2 and 3 days survival no fluorescent glial nuclei were present around the retrogradely labeled neurons (Fig. 1B), but after 6 days some dull yellow fluorescent glial nuclei occurred. However, in none of these cases were DY·2HCl-fluorescent glial nuclei present in the SNR.

In conclusion, DY·2HCl produces retrograde neuronal labeling which resembles that obtained with NY. At the injection site DY·2HCl spreads through the tissue much less than NY. DY·2HCl migrates much more slowly out of the retrogradely labeled neurons than NY (cf. Bentivoglio et al. 1980a).

Group B

In this group the time course and the long distance retrograde transport of DY·2HCl were studied in rat and cat (see Table 1).

In 4 rats with DY·2HCl spinal injections at C2 followed by survival times of 3, 7, 14 or 21 days the following findings were obtained. After 3 days the brain stem nuclei of the descending spinal pathways (Kuypers and Maisky 1975) and the sensorimotor cortex contained DY·2HCl-labeled neurons but no fluorescent glial nuclei. In contrast, in the two rats injected with NY, followed by 3 days survival, many fluorescent glial nuclei were present around retrogradely labeled neurons (Fig. 1C). The DY·2HCl-labeled brain stem neurons showed a nuclear as well as a cytoplasmic labeling (cf. group A). Some also showed yellow granules in the cytoplasm. The labeled neurons in the cortex showed only nuclear labeling. After 7 days the neuronal labeling was much more pronounced than after 3 days and the large neurons in red nucleus showed a brilliantly fluorescent cytoplasm (Fig. 2A). After 7 days the labeled cortical neurons were more numerous and some showed cytoplasmic labeling. However, no fluorescent glial nuclei were present around the retrogradely labeled neurons in brain stem and cortex. After 14 days some dull yellow fluorescent glial nuclei were present around labeled neurons in brain stem and cortex, but especially in red nucleus. In addition, some faintly blue fluorescent glial nuclei were present in the ventrolateral and dorsal funiculi of the lower medulla oblongata. These glial nuclei were probably 'anterogradely' labeled. They were blueish fluorescent when viewed with filter-mirror system A (360 nm excitation wavelength), but were greenish fluorescent when viewed with filter-mirror system D (390 nm excitation wavelength). Some blue fluorescent glial nuclei were also present in the area of the red nucleus. After 21 days the fluorescent glial nuclei around retrogradely labeled neurons as well as those in the medullary funiculi were more numerous and more brilliantly fluorescent than after 14 days. In

Fig. 1A-G Photomicrographs of NY- and DY·2HCl-labeled neurons taken with filter-mirror system A (360 nm excitation wavelength). **A, B** Retrograde labeling by *NY* (**A**) and *DY*·2HCl (**B**) of neurons in the left (**A**) and the right (**B**) substantia nigra pars compacta (SNC) of a rat, 3 days after *NY* and *DY*·2HCl injections in the left and the right caudate-putamen. Note the brilliantly fluorescent *NY*-labeled glial nuclei in **A**, in both pars compacta and pars reticulata, and the absence of them in **B**. **C, D** Corticospinal neurons labeled retrogradely by *NY* (**C**) and *DY*·2HCl (**D**) in rat 3 days after NY injections at C2 (**C**) and 14 days after *DY*·2HCl injections at L1 (**D**). **E** Corticospinal neuron labeled retrogradely by *DY*.2HCl in cat 21 days after DY.2HCl injections at C5–C8. Note brilliantly fluorescent *NY* glial nuclei in C only. Note also some cytoplasmic *DY*.2HCl labeling in large corticospinal neuron in **E. F, G** Neurons labeled retrogradely by *NY* (**F**) and *DY*·2HCl (**G**) in rat thalamus 4 days after *NY* and *DY*·2HCl injections in sensorimotor cortex. Note presence of numerous fluorescent glial nuclei after *NY* injections (**F**) and their absence after *DY*·2HCl injections (**G**)

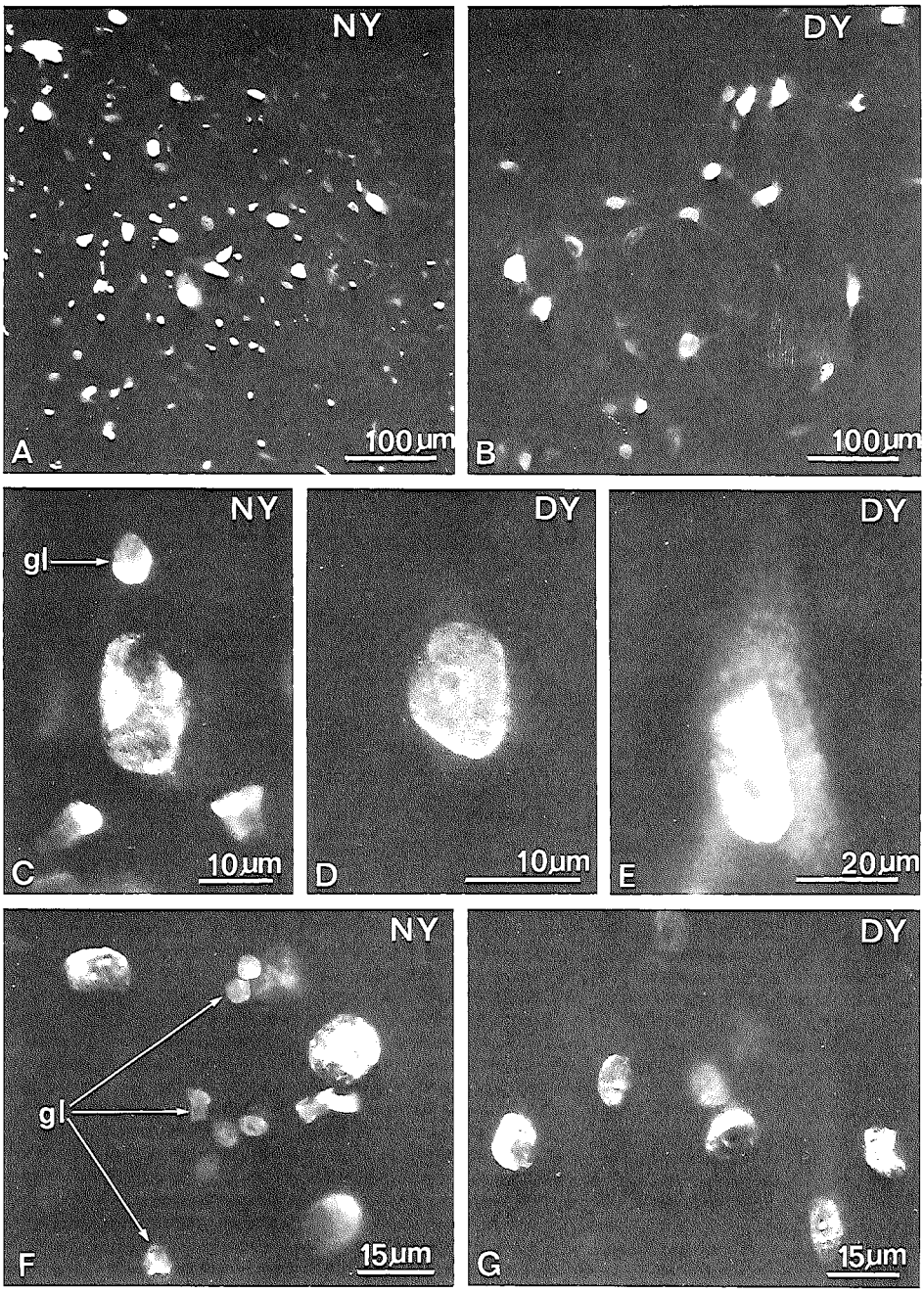
NY
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Fig. 1A–G

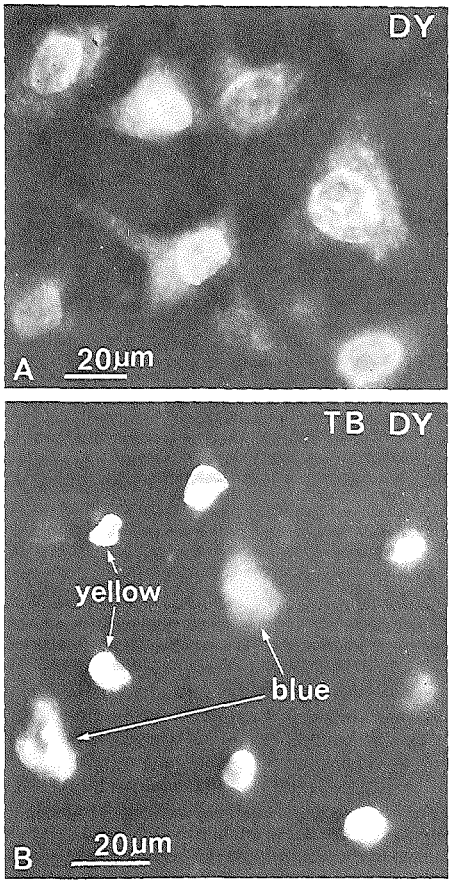

Fig. 2A, B. Photomicrographs of DY·2HCl- and TB-labeled neurons taken with filter-mirror system A (360 nm excitation wavelength). **A** Neurons labeled retrogradely by *DY*·2HCl in red nucleus of rat after *DY*·2HCl injections in spinal cord at C5–C8 (7 days survival). Note the nuclear and cytoplasmic *DY*·2HCl labeling. **B** Retrogradely *TB*-labeled corticospinal neurons and *DY*·2HCl-labeled callosal neurons in rat sensorimotor cortex 6 days after TB injections contralaterally at C2 and *DY*·2HCl injections contralaterally in sensorimotor cortex. Note absence of *DY*·2HCl-labeled glial nuclei, and absence of *DY*·2HCl-labeled nuclei in *TB*-labeled corticospinal neurons

addition, after 21 days, neurons in the cell nests of the dorsal column nuclei showed some yellow fluorescence of nucleus and cytoplasm. This fluorescence probably represented transneuronal labeling.

In the two rats with DY·2HCl injections at L1–L2 followed by 14 days survival also many brain stem and cortical neurons showed DY·2HCl nuclear labeling (Fig. 1D). Moreover, the magnocellular elements of red nucleus also showed a nuclear as well as a pronounced cytoplasmic labeling. Yellow fluorescent glial nuclei were present only around the large neurons in red nucleus. No blue fluorescent glial nuclei occurred in the funiculi of the lower medulla oblongata.

DY·2HCl is not only transported from fiber termination areas (cf. group A) but also from broken axons as indicated by the following findings. In the 4 rats with DY·2HCl injections in the cervical cord, labeled neurons were present both in the dorsomedial and in the ventrolateral parts of red nucleus, which project to the cervical and the lumbosacral segments, respectively (Gwyn 1971; Flumerfelt and Gwyn 1974; Murray and Gurule 1979). However, in the 2 rats with lumbar DY·2HCl injections, labeled neurons were present only in the ventrolateral part.

In the 2 cats with DY·2HCl injections at C5–C8 and at L7–S1, respectively, followed by 3 weeks and 4 weeks survival, many labeled neurons were present in the brain stem and sensorimotor cortex. They showed mainly nuclear labeling (Fig. 1E). However, some cytoplasmic labeling occurred, especially in the magnocellular elements of red nucleus and, in the cat with the C5–C8 injections, also in the large pyramidal neurons of the sensorimotor cortex (Fig. 1E). Virtually no fluorescent glial nuclei were present around the retrogradely labeled neurons except for a few with dull fluorescence, adjoining the large labeled elements in red nucleus and sensorimotor cortex. As in the 4 rats, the cervical DY·2HCl injections in the cat produced retrograde labeling of neurons in both the dorsomedial and the ventrolateral parts of the magnocellular red nucleus, while the lumbar injections produced labeling only in the ventrolateral part.

In conclusion, DY·2HCl is transported retrogradely over long distances in rat and cat. DY·2HCl is also transported from damaged axons. In rat no DY·2HCl migration out of the retrogradely labeled brain stem and cortical neurons occurs after survival times of 7 days following upper cervical injections and of 14 days following lumbar injections. The same is true in cat 3 weeks following cervical and 4 weeks following lumbar injections.

Group C

'Anterograde' transport of NY through axons gives rise to fluorescent labeling of glial nuclei along these axons and in their termination area (cf. glial nuclei in SNR in group A). This 'anterograde' NY glial

labeling shows roughly the same time course as the retrograde neuronal labeling (Bentivoglio et al. 1980a). As a consequence, when neurons retrogradely labeled by NY receive afferents from the NY injection site, as occurs when labeling thalamic neurons from the cortex, the retrogradely labeled neurons tend to be surrounded by 'anterogradely' labeled glial nuclei. Under such circumstances migration of NY out of the retrogradely labeled neurons cannot be ruled out because the 'anterogradely' labeled glial nuclei cannot be distinguished from those labeled by migration of NY out of the retrogradely labeled neurons. This difficulty may be overcome by using DY·2HCl because the DY·2HCl 'anterograde' glial labeling has a much longer time course than the retrograde neuronal labeling (cf. group B).

The above has been confirmed by comparing the findings in 4 rats following 1% NY injections in sensorimotor cortex with those in 4 others following 2% DY·2HCl injections (see Table 1). In both groups of experiments the rats survived for 2, 4 (2 rats) and 6 days. In all 8 rats many retrogradely labeled neurons were present in the ventral thalamic nuclei. In the NY-injected rats many fluorescent glial nuclei were scattered between the labeled neurons. They were present after 2 days survival and became increasingly more numerous and more brilliantly fluorescent after 4 and 6 days survival (Fig. 1F). In contrast, in the DY·2HCl injected rats no fluorescent glial nuclei were present after 2 days survival, but after 4 days some dull blueish fluorescent glial nuclei occurred in a few localized patches, but were absent in other places (Fig. 1G). After 6 days they were present throughout the ventral thalamic nuclei, but they were still dull blueish fluorescent. The blue fluorescent labeling of the glial nuclei, which appeared later than the retrograde labeling of the neurons, probably reflected 'anterograde' glial labeling as indicated by the fact that after 6 days they were also present in the cerebral peduncle.

Group D

The better retention of DY·2HCl in retrogradely labeled neurons compared with NY was expected to prevent false labeling of adjoining neurons. This was confirmed in 3 rats with 2% DY·2HCl injections in sensorimotor cortex on one side and 2% TB injections ipsilaterally at C2 followed by 6 days survival (see Table 1).

The cortex of the non-injected hemisphere contained many DY·2HCl-labeled 'callosal' neurons, which were distributed over all cortical layers except

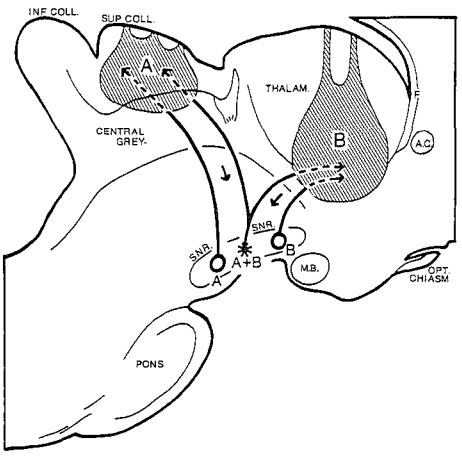

Fig. 3. Diagram of the retrograde transport of two tracers (*A* and *B*) from ventral thalamus and superior colliculus through nigral axons and collaterals to neuronal cell bodies in pars reticulata (*SNR*)

layer I, but were most numerous in layers III and V (cf. Jacobson and Trojanowsky 1974; Wise and Jones 1976). Many of the callosal neurons showed a brilliantly fluorescent nucleus which seemed somewhat pyknotic (Fig. 2B) and in addition showed some cytoplasmic fluorescence. Moreover, many dull fluorescent glial nuclei were present. Further, many fluorescent TB-labeled corticospinal neurons were present in layer V (Fig. 2B), some of which were surrounded by DY·2HCl-labeled callosal neurons. However, none of the TB-labeled corticospinal neurons showed any DY·2HCl labeling of the nucleus (Fig. 2B).

Group E

The preferential DY·2HCl labeling of the neuronal nucleus suggested that DY·2HCl could be combined with TB or FB in experiments using retrograde double labeling to demonstrate the existence of divergent axon collaterals, i.e. in the same way as has been done with NY (Kuypers et al. 1980; Huisman et al. 1981; Catsman-Berrevoets and Kuypers 1981; Bharos et al. 1981). The relatively long survival time required for the DY·2HCl-labeling and the relatively slow migration of DY·2HCl out of the retrogradely labeled neurons would make it possible to inject DY·2HCl and TB or FB at the same time. On the

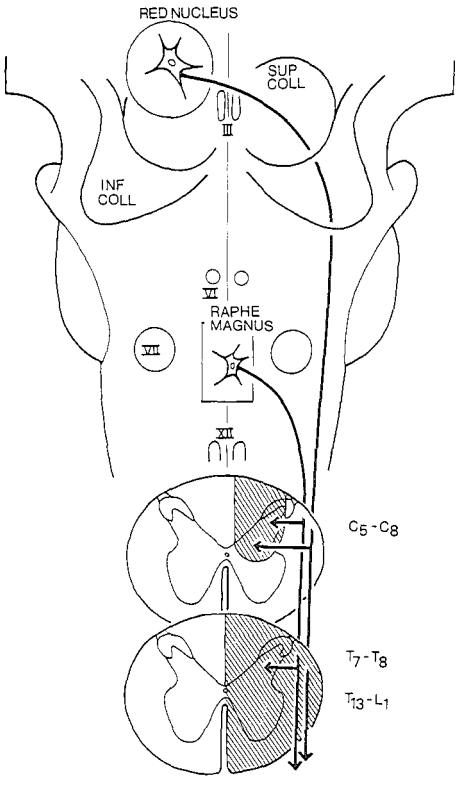

Fig. 4. Diagram of the distribution of fibers and collaterals from neurons in red nucleus and raphe magnus to the low cervical grey matter and more caudal spinal levels

other hand the relatively pronounced DY·2HCl cytoplasmic labeling could possibly mask the blue TB or FB cytoplasmic fluorescence in double-labeled neurons, thus making them difficult to recognize as double labeled. Yet, in the rats and cats of group E (see below), DY·2HCl-TB and DY·2HCl-FB double-labeled neurons could be easily distinguished from single DY·2HCl-labeled ones, both in rat and cat.

The DY·2HCl-TB double-labeled neurons in the rats when viewed with filter mirror system A (360 nm excitation wavelength) showed a blue TB-labeled cytoplasm with a yellow to white DY·2HCl-labeled nucleus. In some cases, in the blue cytoplasm, relatively large yellow fluorescent granules were present, which sometimes also showed a slight admixture of yellow DY·2HCl fluorescence. The double-labeled neurons in the cortex of the cats showed a blue fluorescent cytoplasm, which frequently contained an accumulation of orange autofluorescent granules, and a yellow to green fluorescent nucleus. This DY·2HCl nuclear labeling closely resembled NY labeling (Bentivoglio et al. 1980a; Kuypers et al. 1980).

Double Labeling of Nigral Neurons from Tectum and Thalamus. In 4 rats with DY·2HCl injections in superior colliculus and TB injections in ventral thalamus, or vice versa (Fig. 3) followed by 4 days survival, many DY·2HCl-TB double-labeled neurons were present in the SNR (Niijima and Yoshida 1982). In two of the rats the labeled neurons in the rostral two-thirds of the SNR were counted in 4 transverse sections. The percentages of the neurons labeled from the superior colliculus which were double labeled from thalamus (see Table 2) were comparable to the 30% to 50% obtained earlier (Bentivoglio et al. 1979b).

Double Labeling of Rubral and Raphe Neurons from Different Spinal Levels. In the two rats with 2% TB injections in C5–C8 dorsal grey and 2% DY·2HCl injections in white and grey matter at T7–T8 and T13–L1, respectively (Fig. 4), followed by 7 days survival, several double-labeled neurons (Fig. 5) were present in the contralateral red nucleus and many were present in nucleus raphe magnus (cf. Huisman et al. 1981, 1982; Hayes and Rustioni 1981; Martin et al. 1981). The number of TB-labeled rubral neurons and their percentages which were TB-DY·2HCl double-labeled were obtained in 9–12 transverse sections. They were comparable to those obtained in the two other rats in which NY instead of DY·2HCl was injected (see Table 2). In four transverse sections through the medulla oblongata the labeled neurons in the nucleus raphe magnus and the adjoining ventral part of the medullary medial reticular formation were counted. The average numbers of TB-labeled raphe-cervical neurons per section ranged from 33 to 57 (see Table 2). The percentages of TB-DY·2HCl double-labeled neurons in the first two rats were slightly higher than the percentages of NY-TB double-labeled neurons in the other two rats (see Table 2).

Double Labeling of Rubral Neurons from Spinal Cord and Cerebellum. Many rubral neurons in rat may be double labeled (Fig. 6) from spinal cord and cerebellum (Huisman et al. 1982, 1983). This was confirmed in the 4 rats with 2% TB injections in the cerebellar interpositus nucleus and 2% DY·2HCl (2

Table 2. Group E: Numerical comparisons of NY and DY single- and double-labeling of neurons

E1	Tracer	Site	Tracer	Site	Labeled neurons in subst. nigra pars. retic.					Animals = Rats
	TB	Sup. coll.	DY	Ventr. thal.	156 TB	425 DY	100 TB-DY	65%TB = TB-DY	24%DY = DY-TB	
	TB	Ventr. thal.	DY	Sup. coll.	387 TB	253 DY	127 TB-DY	30%TB = TB-DY	50%DY = DY-TB	
E2	Tracer	Site	Tracer	Site	Labeled neurons red nucleus			Labeled neurons raphe magnus		Animals
		Dors. grey		White+grey						
	TB	C5–C8	DY	T7 –T8	1684 TB	33%TB = TB-DY		45 TB/section	75%TB = TB-DY	Rats
	TB	C5–C8	NY	T7 –T8	2079 TB	35%TB = TB-NY		48 TB/section	42%TB = TB-NY	
	TB	C5–C8	DY	T13–L1	1638 TB	18%TB = TB-DY		57 TB/section	67%TB = TB-DY	
	TB	C5–C8	NY	T13–L1	1377 TB	25%TB = TB-NY		33 TB/section	38%TB = TB-NY	
E3	Tracer	Site	Tracer	Site	Labeled neurons in red nucleus					Animals
				White+grey						
	TB	Cerebellar Interpositus	DY	C5–C8	2824 DY	28.6%DY = DY-TB				Rats
	TB	Cerebellar Interpositus	DY	C5–C8	2464 DY	42%DY = DY-TB				
	TB	Cerebellar Interpositus	NY	C5–C8	2380 NY	20.4%NY = NY-TB				
	TB	Cerebellar Interpositus	NY	C5–C8	2168 NY	26.9%NY = NY-TB				
E4	Tracer	Site	Tracer	Site	Cortical neurons in 9 parasag. sections					Animals
	FB	White+grey C2	DY	Med. R.F. brainstem	1556 DY	12% DY = DY-FB				Cats
	FB	White+grey C2	NY	Med. R.F. brainstem	2806 NY	12.7% NY = NY-FB				

rats) or 1% NY injections (2 rats) ipsilaterally in C5–C8. The DY·2HCl-labeled rubral neurons were slightly more numerous than the NY-labeled ones (see Table 2). Moreover, the percentages of the DY·2HCl-labeled rubral neurons which were DY·2HCl-TB double-labeled in the first two rats were slightly higher than the percentages of the NY-TB double-labeled ones in the other two rats. In all 4 rats, virtually all rubro-cerebellar neurons were double labeled from spinal cord (cf. Huisman et al. 1982, 1983).

Double Labeling of Cat Corticobulbar Neurons from Spinal Cord. In cat several neurons in the rostral part of the pericruciate cortex may be double labeled from the lower brain stem and the spinal cord (Keizer and Kuypers 1982). In two cats with 3% FB injections at C2 and 2% DY·2HCl and 1% NY injections in the pontomedullary medial reticular formation, double-labeled neurons were present especially in the cortex covering the intermediate portion of the anterior sigmoid gyrus, the rostrally adjoining part of the presylvian gyrus and the lateral bank of the presylvian sulcus (Fig. 7). The single DY·2HCl- and NY-labeled neurons and the DY·2HCl-FB and NY-FB double-labeled ones were counted in 9 parasagittal sections. Almost twice as many NY-labeled neurons were present in the first cat as compared to the DY·2HCl-labeled neurons in the other (see Table 2). This probably reflected the relatively large extent of the NY injection area (cf. group A). However, in both cats virtually the same percentages of the neurons labeled from the brain stem either by NY or DY·2HCl, were FB double labeled from spinal cord (see Table 2).

In conclusion, DY·2HCl in combination with TB or FB produces a fluorescent retrograde double labeling by way of divergent axon collaterals which is as effective as that obtained with NY in combination with TB or FB.

Discussion

In frozen section material fluorescent retrograde double labeling of neurons by way of divergent axon collaterals in the brain may be demonstrated by means of two fluorescent retrograde tracers, i.e. NY

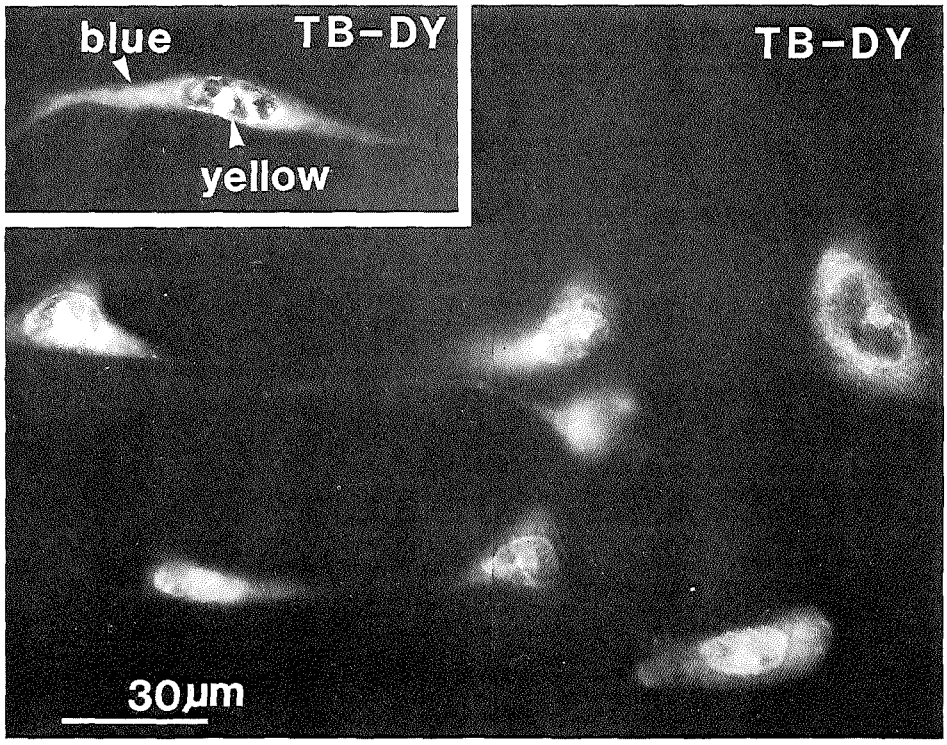

Fig. 5. Photomicrographs of *TB-DY*·2HCl double-labeled neurons in rat nucleus raphe magnus taken with filter-mirror system A (360 nm excitation wavelength) 7 days after *TB* injections in C5–C8 dorsal grey and *DY*·2HCl injections in T7–T8 white and grey matter. All neurons are *TB-DY*·2HCl double-labeled with blue *TB* fluorescent cytoplasm and yellow *DY*·2HCl fluorescent nucleus

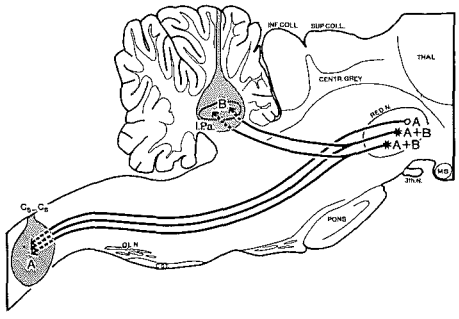

Fig. 6. Diagram of the retrograde transport of two tracers (*A* and *B*) from spinal cord and cerebellum, through rubrospinal axons and collaterals to neuronal cell bodies in red nucleus. (*I.P.a.*: Nucleus Interpositus anterior)

and TB or FB, which fluoresce with different colors at the same excitation wavelength and preferentially label different features of the cell (Kuypers et al. 1980; Catsman-Berrevoets and Kuypers 1981). The blue tracers TB and FB, give a blue fluorescence of the cytoplasm, while NY, after short survival times gives a yellow fluorescence of the nucleus at 360 nm excitation wavelength (Bentivoglio et al. 1980a). It should be cautioned, however, that some differences in the characteristics of the labeling have been observed when these various tracers were used to label motoneurons from peripheral nerves (Illert et al. 1982). Moreover, in freeze-dried material TB is not present diffusely throughout the cytoplasm, but appears as blue fluorescent granules (Björklund and Skagerberg 1979a, b).

On the basis of the findings obtained in frozen

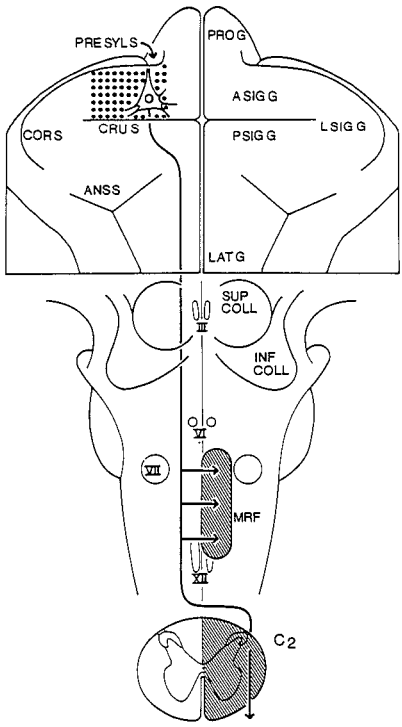

Fig. 7. Diagram of the distribution of fibers and collaterals from anterior sigmoid gyrus to lower brain stem medial reticular formation and spinal cord

section material, NY has the disadvantage that, both in vivo and in vitro, it migrates relatively rapidly out of the retrogradely labeled neurons as indicated by the fluorescence of surrounding glial nuclei. Therefore, after long survival times NY might produce false labeling of adjoining neurons (Kuzuhara et al. 1980; Bentivoglio et al. 1980a). This may be prevented by using short NY survival times and by rapid processing of the material (Bentivoglio et al. 1980a). Consequently in double-labeling experiments in which NY is combined with TB or FB the two tracers have to be injected at quite different times before sacrificing the animal. The findings in the present study indicate that this difficulty may be overcome by using DY·2HCl instead of NY. DY·2HCl, is similar to NY in that it labels mainly the neuronal nucleus, but DY·2HCl also gives some cytoplasmic labeling (experiments of groups A, B, etc.). DY·2HCl is transported retrogradely over long distance in rat and cat (experiments of group B). Effective DY·2HCl retrograde labeling requires relatively long survival times as compared to NY labeling (cf. experiments of groups A, B and Bentivoglio et al. 1980a, b), and these DY·2HCl survival times are comparable to those required for TB and FB (Bentivoglio et al. 1979a, 1980b). DY·2HCl migrates much more slowly out of the retrogradely labeled neurons than NY (cf. experiments groups A, B and Bentivoglio et al. 1980a). Even after relatively long survival times DY·2HCl does not give rise to false labeling of neighbouring neurons (experiments of group D). Moreover, the double labeling of neurons obtained with DY·2HCl in combination with TB or FB in various neuronal systems in the brain is at least as effective numerically as that obtained with NY in combination with TB or FB (experiments of group E).

NY is transported anterogradely through axons, which results in NY labeling of glial nuclei along these axons and in their termination area. This 'anterograde' NY labeling of glial nuclei has roughly the same time course as the retrograde NY neuronal labeling (experiments of group A and Bentivoglio et al. 1980a). DY·2HCl as compared to NY carries the advantage that the DY·2HCl 'anterograde' labeling of glial nuclei is much less pronounced and requires a much longer survival time than the retrograde DY·2HCl neuronal labeling (experiments of group B). Thus, when injecting DY·2HCl in the cortex instead of NY, followed by survival times sufficient to produce retrograde DY·2HCl neuronal labeling in the thalamus, no anterograde DY·2HCl-labeling of glial nuclei occurs around the DY·2HCl retrogradely labeled thalamic neurons (experiments of group C). Moreover, glial nuclei 'anterogradely' labeled by DY·2HCl tend to be dull blue fluorescent (experiments of groups A, B, C).

In kittens (Bentivoglio et al. 1980a) some faint 'anterograde' transneuronal NY labeling of pontine neurons was observed after NY injections in the cerebral cortex followed by 3 days survival. Such transneuronal labeling may also occur in rat dorsal column nuclei after DY·2HCl injections in the upper cervical segments followed by 3 weeks survival (group B). However, after 6 days survival, which suffices for effective retrograde neuronal DY·2HCl labeling in brain stem and cortex no such transneuronal DY·2HCl labeling was observed.

The above observations, therefore, lead to the conclusion that DY·2HCl may be used with advantage instead of NY in double-labeling experiments in which DY·2HCl is injected in combination with TB

or FB, because in such experiments DY·2HCl and TB or FB may be injected at the same time.

DY·2HCl shares with TB the tendency to produce a much more pronounced necrosis at the injection site than NY. However, this necrosis does not seem to interfere with the retrograde axonal transport of DY·2HCl and good retrograde neuronal labeling occurs after injecting DY·2HCl in a fiber termination area or in fiber bundles (cf. red nucleus in experiment of group B). Actually, (group E) the DY·2HCl retrograde labeling was slightly more effective than the NY labeling; the retrogradely DY·2HCl-labeled neurons were slightly more numerous than the NY-labeled ones.

DY·2HCl also has some disadvantages because the local necrosis at the injection site may cause a neurological deficit. Since TB and FB also produce necrosis at the injection sites, the simultaneous injection of DY·2HCl and TB or FB result in two areas of necrosis in the brain. In the rat experiments this has not produced any difficulty, because all the rats one day after the operation moved about in their cage in a normal way and ate and drank normally. However, the cat with FB injections in the spinal cord and simultaneous DY·2HCl injections in the brain stem remained comatose for a long time. Therefore, when injections have to be made into vital structures such as the lower brain stem, NY is preferred above DY·2HCl, or FB can be injected first and DY·2HCl one week later.

Addendum

In addition to DY·2HCl also DY 2aceturic acid (DY·2aa) has been prepared. This salt, which produces retrograde neuronal labeling similar to that obtained with DY·2HCl, is easier to prepare. However, these two compounds behave differently in the following two respects. First, DY·2aa is much more soluble in water than DY·2HCl such that 2% DY·2aa in water produces a clear, but highly viscous, yellow solution. The high viscosity makes it, according to our experiences so far, less suitable for injections in the brain because (a) it tends to clog the injection needle or glass pipette, and (b) during withdrawal of the needle or pipette from the brain, part of the DY·2aa tends to be pulled up into the needle track and sometimes on to the surface of the brain. This latter phenomenon represents a serious disadvantage, because the presence of a large amount of DY·2aa in the needle track gives rise to retrograde transport through the many axons damaged by the needle track. Secondly, DY·2aa migrates more rapidly out of the retrogradely labeled neurons than DY·2HCl. This is concluded from the observation that when 0.6 µl 2% DY·2aa was injected in rat caudate-putamen followed by 3 days survival, the heavily labeled neurons in the substantia nigra pars compacta (SNC) were surrounded by fluorescent glial nuclei.

In order to avoid the presence of a substantial amount of DY·2aa in the needle track, DY·2aa has been injected in the form of a suspension. For this purpose 2% DY·2aa in 0.2 M phosphate buffer (pH 7.2) and 2% DY·2aa in 0.15 M phosphatecitrate buffer (pH 4.0) were prepared. In these buffers DY·2aa forms a precipitate, which was homogenized in an ultrasonic water bath and than injected in a 2% concentration. Recently DY·2HCl has also been suspended in a 0.2 M phosphate buffer (pH 7.2), see Material and Methods.

When 0.6 µl injections of the two DY·2aa suspensions were injected in caudate-putamen in rats the deposits of fluorescent material in the needle track did not occur. However, the fluorescent tracers apparently still migrated relatively rapidly out of the retrogradely labeled neurons; after 3 days survival the heavily labeled neurons in the SNC were surrounded by fluorescent glial nuclei. On the other hand, when the two DY·2aa suspensions were injected in the spinal white and grey matter at T7–T8 in rats followed by 7 days survival time, the cells of origin of the descending cortical and brain stem pathways, were brilliantly labeled, but were not surrounded by fluorescent glial nuclei. Moreover, in two rats injections of the two DY·2aa suspensions were made in the spinal white and grey matter at T7–T8 in combination with TB injections in the grey matter at C5–C8, i.e. in the same way as in the experiments of group E2. In these two rats roughly the same percentages of TB-labeled neurons in the red nucleus, the raphe magnus and the adjoining reticular formation were double labeled as in the experiments of group E2.

In conclusion, DY·2HCl should be preferred above DY·2aa but in case of long distance retrograde transport a 2% DY·2aa suspension in 0.2 M phosphate buffer (pH 7.2) might also be used.

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Chapter III

Distribution of Corticospinal Neurons With Collaterals to Lower Brain Stem Reticular Formation in Cat

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Summary. The fluorescent retrograde double-labeling technique has been used to determine whether corticospinal neurons in the cat sensorimotor cortex distribute collaterals to the lower brain stem reticular formation. In this study the fluorescent tracers Nuclear Yellow and Diamidino Yellow 2HCl were used in combination with Fast Blue. One tracer was injected unilaterally in the spinal cord and the other was injected ipsilaterally in the bulbar medial reticular formation. The distribution of the retrogradely labeled neurons was studied in the contralateral hemisphere. In the sensorimotor cortex a large population of neurons was found which were labeled from the spinal cord and were double-labeled from the brain stem. These branching neurons were concentrated in the rostromedial part of area 4 and the adjoining lateral part of area 6. In this region the percentages of corticospinal neurons which were double-labeled from the brain stem ranged from 5% laterally to 30% medially. In two cats it was demonstrated by means of the anterograde transport of HRP that the corticobulbar fibers from this region which must include the corticospinal collaterals are distributed to the reticular formation of the lower brain stem. In view of the fact that the double-labeled neurons are concentrated in the anterior part of the motor cortex, those branching neurons are in all likelihood involved in the control of neck, back and shoulder movements. This control is probably exerted by way of two routes i.e. by way of the direct corticospinal connections to spinal interneurons, and by way of the indirect cortico-reticulospinal connections established by the cortical fibers to the bulbar reticular formation. The present findings suggest that this dual control may be exerted by one and the same cell.

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Introduction

The pericruciate cortex in cat projects to spinal cord as well as to mesencephalon and lower brainstem. In the lower brainstem the pericruciate fibers are distributed to the dorsal column nuclei, the spinal trigeminal complex, the lateral reticular formation, the lateral reticular nucleus and the medial reticular formation (MRF) (cf. Kuypers 1958). In a retrograde double-labeling study using HRP and tritiated apo-HRP (Rustioni and Hayes 1981) it has been demonstrated that in cat some of the cortical fibers to the dorsal column nuclei are collaterals of corticospinal axons. Moreover, in a fluorescent double-labeling study from our laboratory (Catsman-Berrevoets and Kuypers 1981) it has been shown that in rat and cat some of the cortical fibers to the mesencephalon are also collaterals of corticospinal axons. Therefore it would be of interest to know whether in cat the same is true for the pericruciate fibers to the medial reticular formation of the lower brainstem. In the present study using the retrograde fluorescent double-labeling technique (Kuypers et al. 1980; Bentivoglio et al. 1980a, b; Keizer et al. 1983), it has been demonstrated that a portion of these pericruciate fibers are collaterals of corticospinal neurons, concentrated in the anterior part of area 4 and in the adjoining part of area 6 (Hassler and Mühs-Clement 1964).

Material and Methods

In group A (two cats), the distributions of the cortical neurons projecting to the spinal cord and to the bulbar medial reticular

Table 1

Group A Retrograde HRP experiments

Case	% Tracer	µl	Injection site	% Tracer	µl	Injection site	Approach	Survival time
A1	30% HRP	28.0	C2					3 days
A2				30% HRP	1.5	MRF	dorsal	3 days

Group B Fluorescent double-labeling experiments

Case	% Tracer	µl	Injection site	% Tracer	µl	Injection site	Approach	Survival time	Survival time
B1	3% FB (DMSO)	13.0	C2	1% NY	1.8	MRF	dorsal	FB 30 days	NY 35 h
B2	3% FB (DMSO)	14.0	C2	1% NY	1.8	MRF	dorsal	FB 30 days	NY 35.5 h
B3	3% FB (DMSO)	6.4	C2	1% NY	1.8	MRF	dorsal	FB 30 days	NY 35.5 h
B4	3% FB (DMSO)	8.2	C2	2% DY·2HCl	1.8	MRF	dorsal	FB 28 days	DY·2HCl 28 d
B5	3% FB (DMSO)	8.6	C5–C6	1% NY	1.8	MRF	dorsal	FB 30 days	NY 35.5 h
B6	3% FB (DMSO)	8.2	C5–C6	1% NY	1.8	MRF	dorsal	FB 30 days	NY 35.5 h
B7	3% FB (DMSO)	6.3	T1	1% NY	1.8	MRF	dorsal	FB 30 days	NY 35.5 h
B8	3% FB (DMSO)	8.0	C2	1% NY	1.8	MRF	ventral	FB 30 days	NY 35.5 h
B9	2% DY·2HCl	7.1	C4–C5	3% FB	1.8	MRF	ventral	DY 30 days	FB 21 days
B10	2% DY·2HCl	6.4	L1	3% FB	1.8	MRF	ventral	DY 29 days	FB 17 days
B11	3% FB (DMSO)	12.8	C4–C5	1% NY	1.8	dorsal MRF	dorsal	FB 28 days	NY 35.5 h

Group C Anterograde HRP experiments

Case	% Tracer	µl	Injection site	Survival time
C1	30% HRP	0.4	Ant.Sigmoid.G.	3 days
C2	30% HRP	0.4	Ant.Sigmoid.G.	3 days

formation (MRF) were studied by means of the retrograde transport of HRP. In group B (11 cats), fluorescent retrograde tracers were used to study the distribution of the cortical neurons in the pericruciate cortex which project either to the spinal cord, or to the lower brainstem reticular formation or to both. In group C (two cats) the corticobulbar projections from the anterior part of area 4 and the adjoining part of area 6 were traced with anterogradely transported HRP, following small HRP injections in these parts of the pericruciate cortex. In all cats the injections were made under Nembutal anaesthesia. The cats which were injected in the spinal cord were administered morphine for the first one or two days after the operation.

Group A

In cat A1 a laminectomy was made at C2 and approximately 30 HRP injections (28 µl 30% HRP in water) were made unilaterally in the spinal white and grey matter by means of a glass micropipette (tip diameter 60–80 µm).

In cat A2, 1.5 µl of 30% HRP dissolved in 5% polyvinyl pyrolidon (PVP) in water was injected in the MRF of the medulla oblongata and lower pons, divided over a rostrocaudal row of six injections between the obex and the abducens nucleus, 1.5 mm from the midline. The needle was introduced into the brainstem from above after exposing the floor of the IV ventricle through a midline split of the cerebellum. After 3 days the cats were deeply anaesthetised with Nembutal and transcardially perfused with 6% Dextran in 0.9% saline (1 l), followed by cacodylate buffered (pH 7.2) paraformaldehyde (0.5%)-glutaraldehyde (2.5%) mixture (3 l) at room temperature which was followed by cacodylate buffered (pH 7.2) 8% sucrose solution (2 l) at 4° C. Subsequently the brain and spinal cord were dissected and immediately cut in sections on a freezing microtome. The frontal lobe contralateral to the injections was cut sagitally in 30 µm sections. Every other section was mounted, air dried and incubated with Tetramethylbenzidine (TMB), (Mesulam 1978). The injection areas in spinal cord and lower brainstem were cut transversely in frozen sections and every fourth section was incubated with Diaminobenzidine (DAB) (Graham and Karnovsky 1966). Every other incubated section of cortex, brain stem and spinal cord was counterstained with cresyl violet. The sections were studied microscopically under darkfield illumination. One out of every four sections through the cortex (250 µm apart) and the injection areas was charted with the aid of an X-Y plotter connected by means of transducers to the microscope stage. In the frontal lobe sections through the area extending from the medial aspect of the hemisphere to the coronal sulcus the labeled cortical neurons were counted.

Group B

Eleven cats were each injected with two different fluorescent retrograde tracers i.e. either with Fast Blue (FB) and Nuclear Yellow (NY) or with FB and Diamidino Yellow Dihydrochloride (DY·2HCl). NY is transported retrogradely much faster than FB and DY·2HCl (cf. Bentivoglio et al. 1980a, b; Kuypers et al. 1980; Keizer et al. 1983). It soon migrates out of the retrogradely labeled neurons and may produce fluorescence of surrounding glial and neuronal nuclei. This NY migration may be prevented by using a short NY survival time (Bentivoglio et al. 1980a). Therefore in the cases in which NY was used FB was injected first and NY approximately four weeks later; a short time before the animal was sacrificed (see Table 1). The rate of transport of DY·2HCl equals that of FB, and DY·2HCl migrates only very slowly out of the retrogradely labeled neurons (Keizer et al. 1983). Therefore these

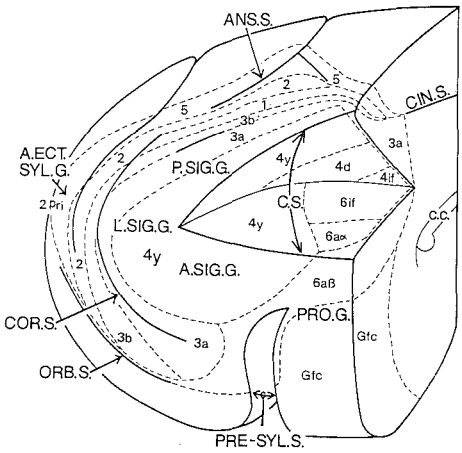

Fig. 1. Rostral part of the right hemisphere of cat with cytoarchitectonic subdivisions of the pericruciate cortex (Hassler and Mühs-Clement 1964). The cruciate sulcus and the pre-sylvian sulcus are shown opened. Abbreviations: A.ECT.SYL.G, anterior ecto-sylvian gyrus; ANS.S., ansate sulcus; A.SIG.G., anterior sigmoid gyrus; c.c., corpus callosum; CIN.S., cingulate sulcus; COR.S., coronal sulcus; C.S., cruciate sulcus; Gfc, granular frontal cortex; L.SIG.G., lateral sigmoid gyrus; ORB.S., orbital sulcus; PRE-SYL.S., pre-sylvian sulcus; P.SIG.G., posterior sigmoid gyrus; PRO.G., proreate gyrus

two tracers can be injected at the same time. However, after such double injections, the cats remained comatose for a long time. Therefore in later experiments the spinal (DY·2HCl) injections were made first and the brainstem (FB) injections 1 week later; after the cat had recovered and was eating by itself and walking around in its cage.

In the spinal cord 15–30 glass micropipette penetrations were made through which 6–14 µl of either 3% FB dissolved in a 2% solution of dimethylsulfoxide (DMSO) in water or of 2% DY·2HCl dissolved in a 0.2 M phosphate buffer pH 7.2 were deposited in the spinal white and grey matter. Multiple penetrations were made in order to interrupt as many corticospinal fibers as possible because FB and DY·2HCl are transported effectively from broken axons (Kuypers et al. 1980; Bentivoglio et al. 1980b; Keizer et al. 1983) Moreover DMSO has been shown to enhance

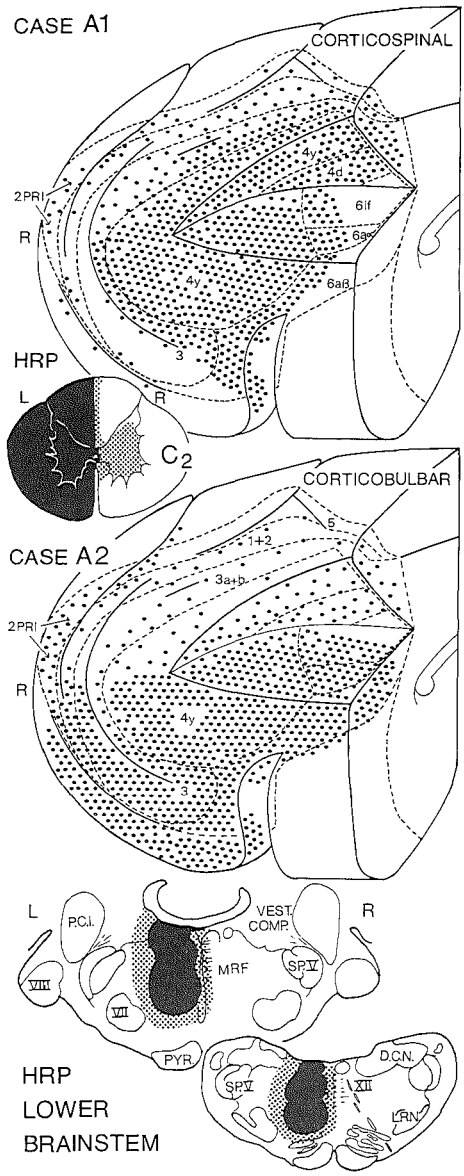

Fig. 2. Diagrammatic representations of the distributions of the retrogradely HRP-labeled neurons in the peri-cruciate cortex in cat after contralateral HRP injections at C2 (case A1, upper part) and after contralateral HRP injections in the bulbar medial reticular formation (case A2, lower part). Dotted areas represent HRP diffusion around the injection areas. The brainstem injection was made by introducing the injection needle from above. Abbreviations: D.C.N., dorsal column nuclei; L.R.N., lateral reticular nucleus; MRF, medial reticular formation; P.C.I., pedunculus cerebelli inferior; PYR, pyramid; SP.V, spinal trigeminal complex; VEST.COMP., vestibular complex; VII, facial nucleus; VIII, cochlear nucleus; XII, hypoglossal nucleus

the uptake of the tracers when injected in fiber bundles (Huisman et al. 1982).

In nine of the 11 cats FB was injected in the spinal cord; in five cases (B1–B4, B8) at C2, in one case (B11) at C4–C5, in two cases (B5–B6) at C5–C6 and in one case (B7) at T1. In eight of the nine cases (B1–B3, B5–B8, B11) NY was injected in the lower brainstem MRF while in one case (B4) DY·2HCl was injected. In the two remaining cases (B9, B10) the tracer injections were reversed i.e. DY·2HCl was injected in spinal cord (in case B9 at C4–C5 and in case B10 at L1) and FB was injected in the lower brainstem MRF.

In eight (B1–B7, B11) of the 11 cases the brainstem injections were made from dorsal (cf case A2). In one (B11) of these eight cases the injection area was restricted to the dorsal part of the MRF (see Table 1). In the remaining 3 cases (B8, B9, B10) the injections were made from ventral through the pyramidal tract ipsilateral to the spinal injection. The concentrations, the amounts of the tracers and the survival times are given in Table 1. After the appropriate survival times the cats were deeply anaesthetized with Nembutal and transcardially perfused with 2.7% saline (1 l), followed by cacodylate or citrate buffered (pH 7.2) 30% formalin (3 l, 60 min) at room temperature, which was followed by cacodylate or citrate buffered (pH 7.2) 8% sucrose (2 l, 30 min) at 4.0° C. Brain and spinal cord were then immediately dissected and deeply frozen in dry ice until the sections were cut. The frontal lobe contralateral to the injections was cut sagitally in 30 μm sections on a freezing microtome and every other section was immediately mounted and air dried, but not coverslipped. The injection areas in brain stem and spinal cord were cut transversally and every fourth section was mounted.

The sections were studied with a Leitz Ploemopack fluorescence microscope, equipped with filter-mirror system A providing excitation light of 360 nm wavelength. The immersion oil was applied directly to the sections. In every fourth or sixth of the mounted sections through the frontal lobe (250 μm and 360 μm apart) the distribution of the retrogradely labeled cortical neurons was charted with the aid of an X-Y plotter connected by means of transducers to the microscope stage. In the frontal lobe sections through the area extending from the medial aspect of the hemisphere to the coronal sulcus the single- and double-labeled corticospinal neurons were counted. The injection areas in the brainstem were also plotted. After plotting, one out of every two sections through brainstem and frontal lobe were counterstained with cresyl violet, for indentification of the cytoarchitectonic cortical areas and their laminae and for delineation of the injection areas in the brainstem.

Group C

In two cats (C1, C2) the fibers descending from the anterior sigmoid gyrus were visualized by means of anterograde HRP-labeling. For this purpose 0.4 μl 30% HRP dissolved in water was injected in this cortical area by means of a glass micropipette (four injections, depth 2–3 mm). After 3 days the cats were anaesthetised with Nembutal and perfused in the same way as the cats of group A. The cortical injection areas were cut sagitally in 30 μm frozen sections. In case C1 they were reacted with DAB and in case C2 with TMB. The lower brainstem of cat C1 was cut sagitally but that of cat 2 was cut transversally. The sections were mounted, air-dried and reacted with TMB (Mesulam 1978). Every other section was counterstained with cresyl violet.

Results

Group A. Retrograde HRP Experiments

Labeling of Corticospinal Neurons. The HRP injection at C2 (cat A1) was largely confined to the white and grey matter of one half of the spinal cord. However, HRP reaction products were also present in the dorsal funiculus and in the grey matter around the central canal on the other side (see Fig. 2).

In the frontal lobe contralateral to the C2 injections, the retrograde HRP-labeled neurons were concentrated in the pericruciate areas. The bulk of them was found in areas 4γ, 4δ, the lateral parts of 6aα, 6aβ and 6if, and area 3a (Figs. 1 and 2). However, substantial numbers were also found in the lateral and dorsal banks of the presylvian sulcus. A smaller number of labeled neurons was found in areas 3b, 1 and 2, 5, in the banks of the orbital sulcus and in area 2-Pri (anterior ectosylvian gyrus) (Fig. 2). All labeled neurons were situated in lamina V (Fig. 3a) and in area 4 included many Betz cells. In 34 parasagittal sections (see Material and Methods), 11,380 labeled corticospinal neurons were counted (Table 2), 80% were situated in area 4, area 3a, the lateral part of area 6 and the banks of the presylvian sulcus (see Fig. 2); 20% were situated in areas 3b, 1, 2, 5 and 2-Pri.

Labeling of Corticobulbar Neurons. The HRP injection in the brainstem (cat A2) was strictly unilateral and involved the MRF between the abducens nucleus and the obex. The injection area infringed upon the medial vestibular nucleus and the rostral pole of the gracile nucleus but spared the pyramidal tracts (see Fig. 2).

In the frontal lobe contralateral to the brainstem injections the HRP-labeled corticobulbar neurons

Fig. 3A, B. Darkfield photomicrographs of HRP-labeled cortical neurons in parasagittal sections through the peri-cruciate cortex at the level of the pre-sylvian sulcus. **A** HRP-labeled corticospinal neurons after injections at C2. **B** HRP-labeled corticobulbar neurons after injections in bulbar MRF. Note the population of corticospinal neurons which extends dorsally into upper bank of cruciate sulcus (*white arrows* in **A**) and that of the corticobulbar neurons which extends anteriorly onto gyrus proreus (*white arrow* in **B**). **C–H** Photomicrographs of fluorescent retrogradely labeled neurons taken with filter-mirror system A (360 nm excitation wavelength). **C** Population of single FB-labeled corticospinal neurons in area 4γ with a blue fluorescent cytoplasm. **D** Single FB-labeled corticospinal neuron with labeling of apical dendrites. **E** Large blue FB-labeled corticospinal neuron with lipofuscine autofluorescence (Yellow-brown) in cytoplasm. **F** DY·2HCl-labeled corticospinal neuron with golden-yellow fluorescent nucleus. **G, H** FB-DY·2HCl double-labeled neurons with blue fluorescent cytoplasm and a yellow fluorescent nucleus

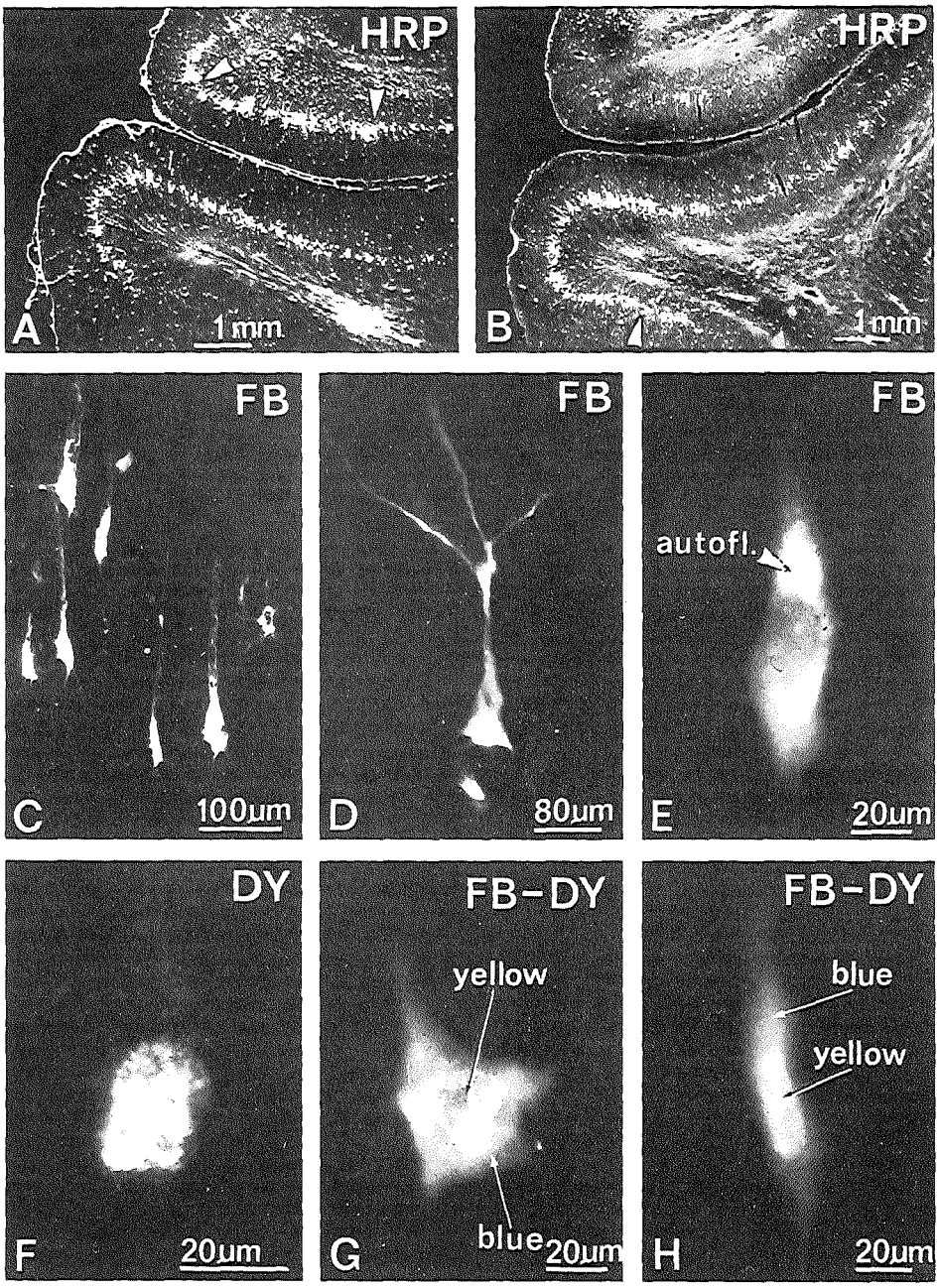
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Fig. 3A, B

were located more rostrally than the corticospinal neurons (case A1) and were situated mainly in the precruciate areas (Fig. 2). They were found in the rostral part of area 4γ, and in areas 6aα, 6aβ, 6if; i.e. the ventral bank of the cruciate sulcus and on the entire surface of the anterior sigmoid gyrus (Fig. 2). They were also found in the lateral and dorsal banks of the pre-sylvian sulcus, in the banks of the coronal and orbital sulci, and on the surface of the corresponding gyri (Figs. 1 and 2). Relatively fewer labeled neurons were found in area 2-Pri, in area 5 and in the caudal part of the dorsal bank of the cruciate sulcus. Only a few labeled neurons were found in the posterior sigmoid gyrus. All labeled neurons were situated in lamina V and were pyramidal in shape (Fig. 3b).

The distributions of corticospinal and corticobulbar neurons thus overlapped (Fig. 3a, b). The area of overlap comprised mainly the caudal part of the dorsal bank of the cruciate sulcus, its entire ventral bank, the lateral part of area 6, the anterior sigmoid gyrus and the lateral and dorsal banks of the presylvian sulcus (Fig. 2).

Group B. Fluorescent Tracer Experiments

Spinal and Medullary Fluorescent Injection Areas. The FB injection areas consisted of three concentric zones. The inner zone 1, fluoresced brightly white to blue and contained orange granules. It consisted of structureless debris and in the Nissl stained sections did not show any histological texture. Zone 2 was relatively narrow, displayed numerous blue fluorescent glial cells and neurons and also showed blue fluorescence of the ground substance. In the Nissl sections it was darkly stained, because of the many glial nuclei. Zone 3 contained only fluorescent glial cells and in its periphery blue fluorescent fibers. It faded gradually into the non-fluorescent tissue (Fig. 4).

The NY injection areas were quite different. Zone 1 was very narrow and showed little necrosis. It contained a dense accumulation of brightly yellow fluorescent glial nuclei and was darkly blue in the Nissl sections. Zone 2 was much wider and contained many yellow fluorescent glial cells and neurons and a yellow fluorescent ground substance. This zone blended gradually into zone 3. This zone contained only fluorescent glial nuclei and faded gradually into the non-fluorescent tissue.

The DY·2HCl injection areas had a relative wide yellow-brown fluorescent zone 1, which consisted of fluorescent tracer and fluorescent necrotic tissue. Zone 2 was narrow. It displayed yellow fluorescent glial and neuronal elements and a yellow to blue fluorescent ground substance. Zone 3 was also narrow and contained only yellow fluorescent glial nuclei. After injections of 0.2 µl, the FB and the NY injection areas were roughly of the same size and the inner two zones measured approximately 2 mm in diameter. However, the DY·2HCl injection areas were much smaller and the two inner zones measured approximately 1 mm in diameter.

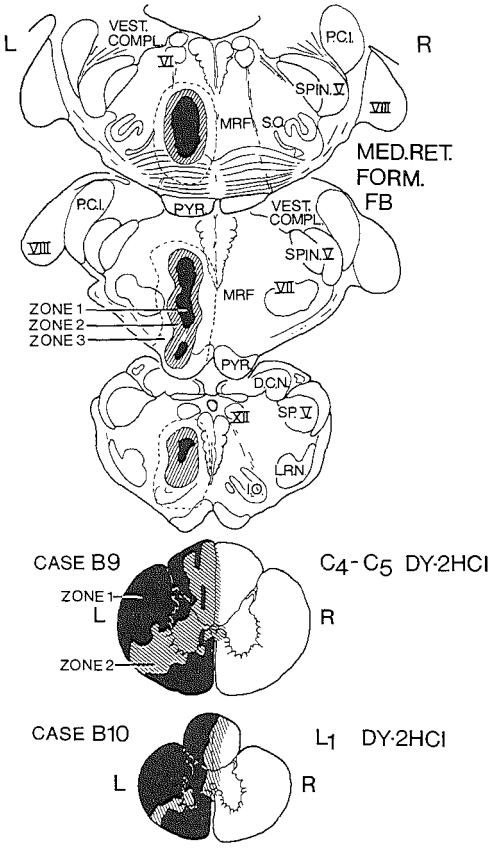

Fig. 4. Upper part: FB injection area in bulbar medial reticular formation. For description of the three concentric zones see text. Note that the injection was made from below through the pyramidal tract. Lower part: DY·2HCl injection area in spinal cord at C4–C5 (case B9) and at L1 (case B10). Abbreviations: I.O., inferior olive; S.O., superior olive; VI, abducens nucleus. For other abbrevations see Fig. 2

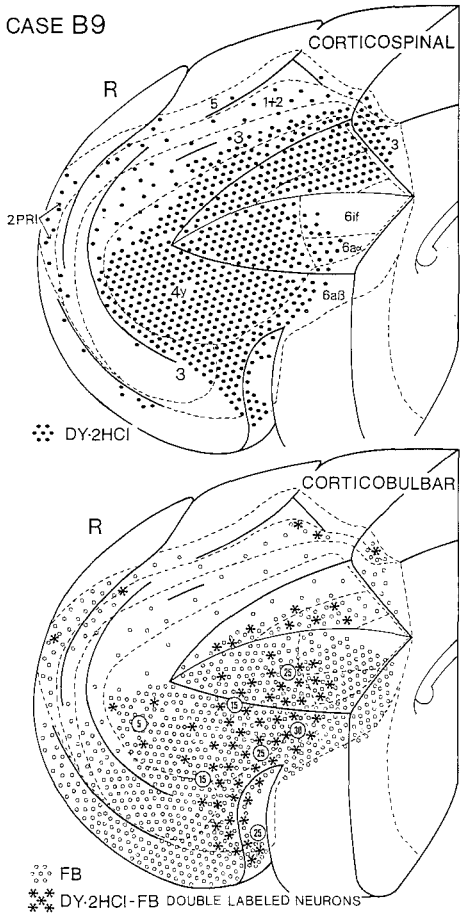

Fig. 5. Diagrammatic representation of the distributions of the single DY·2HCl-labeled corticospinal neurons, the single FB-labeled corticobulbar neurons and the FB-DY·2HCl double-labeled ones in case B9 after DY·2HCl spinal injections at C4–C5. The encircled numbers in the bottom figure refer to the percentages of labeled corticospinal neurons which were double labeled from the brain stem in different parts of the precruciate cortex. Note that the percentages decrease from medial to lateral

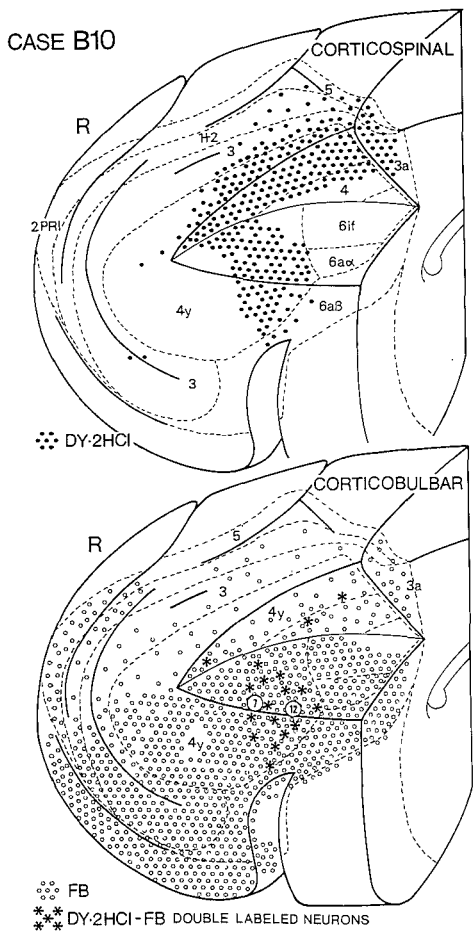

Fig. 6. Diagrammatic representation of the distributions of the single DY·2HCl-labeled corticospinal neurons, the single FB-labeled corticobulbar neurons and the FB-DY·2HCl double-labeled ones in case B10 after DY·2HCl spinal injections at L1. Note that the areal distribution of the double-labeled neurons is more restricted than after injections at C4–C5 (see Fig. 5)

The spinal FB and DY·2HCl injections were centered in the ventral and lateral funiculi (Fig. 4). They appeared as brilliantly fluorescent, structureless masses. The remaining white and grey matter of the ipsilateral side showed a brilliantly fluorescent ground substance. The contralateral side did not show any fluorescence of the ground substance, but the grey matter contained many FB- or DY·2HCl-labeled neuronal elements. The brainstem injections were made ipsilateral to the spinal injections. In cases B1–B7 and B11 they were made from dorsal and the injection areas were of roughly the same size

Table 2. Number of labeled neurons in cases A1, B3, B9 and B10

Case	Tracer	µl	Site	Tracer	µl	Site	Number of sections charted[a]	Number of labeled corticospinal neurons in these sections		Estimates of total numbers of labeled corticospinal neurons	
A1	HRP	28.0	C2				34	11,380 HRP		60,000	
								single+double	double	single+double	double
B3	FB	6.4	C2	NY	1.8	MRF	39	7,086 FB	874 FB-NY	38,000	4200
B9	DY·2HCl	7.1	C4–C5	FB	1.8	MRF	37	10,139 DY	837 DY-FB	55,000	4000
B10	DY·2HCl	6.4	L1	FB	1.8	MRF	35	3,784 DY	67 DY-FB	19,000	350

[a] In the above cases one out of eight sections was charted

as the HRP injection area (cat A2). In the remaining three animals (B8, B9, B10) the injections were made from ventral through the pyramidal tract (Fig. 4). In these cases the injection areas involved the pyramid and the dorsally adjoining medial tegmental field, but spared the contralateral pyramid and the contralateral tegmental field. They extended rostrocaudally from the middle of the trapezoid body to the middle of the inferior olive and did not involve the dorsal column nuclei (Fig. 4).

Distribution of Labeled Corticospinal Neurons. The most pronounced fluorescent retrograde labeling of corticospinal neurons was obtained in cases B3, B9 and B10 which will be described in detail. The distribution of the corticospinal neurons in case B3 (FB) and in case B9 (DY·2HCl) in the hemisphere contralateral to the C2 and C4–C5 injections was the same as in case Al (HRP). About 80% of the labeled neurons were situated in areas 4γ, 4δ, the lateral parts of areas 6aα, 6aβ, 6if, in area 3a and the lateral and dorsal banks of the pre-sylvian sulcus. The remaining 20% were situated in areas 3b, 1, 2, 5 and in area 2-Pri (Fig. 5). All labeled neurons were pyramidal in shape and were located in lamina V, (Fig. 3C, D, E, F). In area 4 they included many Betz cells. In case B3, 7086 labeled neurons were counted in 39 parasagittal sections, and in case B9 10,139 were counted in 37 parasagittal sections (Table 2). In case B10 with injections at L1 (DY·2HCl), the labeled corticospinal neurons showed a much more restricted distribution than in cases B3 and B9. They were found only in the banks of the cruciate sulcus, the medial part of the anterior sigmoid gyrus and the medial two thirds of the posterior sigmoid gyrus along the cruciate sulcus (Fig. 6). About 90% were situated in areas 4γ, 4δ, 3a and in the lateral part of area 6. The remaining 10% were situated in areas 3b, 1 and 2. No labeled neurons were present in area 5 and in area 2-Pri (Fig. 6). In 35 parasagittal sections, 3784 labeled corticospinal neurons were counted (Table 2).

In cases B1, B2, B4 and B8 with injections at C2 (FB) fewer labeled corticospinal neurons were present than in cases B3 and B9, but the distributions were approximately the same. In cases B5 and B6 with injections at C5–C6 (FB) the number of labeled neurons was smaller than in cases B1, B2, B4 and B8 and the areal distribution was slightly more restricted, because virtually no labeled neurons occurred in the lateral bank of the pre-sylvian sulcus and only few were present in the rostromedial part of area 4, and in the lateral part of area 6. In case B7 with injections at Th1 (FB) the labeled neurons occupied a slightly larger area than after injections at L1 (case B10), such that in case B7 several labeled neurons were also present on the lateral sigmoid gyrus and in area 5.

In conclusion, after progressively more caudal injections in the spinal cord the area of the labeled corticospinal neurons becomes more and more restricted to the medial parts of the peri-cruciate cortex.

Distribution of Labeled Corticobulbar Neurons. The areal distributions of the labeled corticobulbar neurons in cases B1, B2, B3, B5, B6, B7 and B8 (NY) in case B4 (DY·2HCl) and in cases B9 and B10 (FB) were similar to that in case A2 (HRP). All labeled neurons were pyramidal in shape and were situated in lamina V. The bulk of them was present in the rostral part of area 4γ, in areas 6aα, 6aβ and 6if, in the lateral and dorsal banks of the pre-sylvian sulcus, in the orbital gyrus as well as in the lower half of the coronal gyrus (Figs. 5 and 6). However, in case B11 with NY injections in the dorsal part of the MRF only a few NY-labeled neurons were present in the coronal and orbital gyri.

Distribution of Double-Labeled Cortical Neurons. In all cases (group B) the areas containing the neurons labeled from the spinal cord and those containing the

neurons labeled from the brainstem overlapped, mainly in the anterior part of the sensorimotor cortex. After C2 injections the area of overlap comprised (a) the caudal part of the upper bank of the cruciate sulcus, (b) a major part of its ventral bank, including the lateral parts of the areas 6aα, 6aβ and 6if, (c) the anterior sigmoid gyrus, and (d) the lateral and dorsal banks of the pre-sylvian sulcus (cf. HRP experiments). In this area of overlap single FB- and single NY- or DY·2HCl-labeled neurons were present as well as double-labeled ones (Fig. 3G, H). The latter showed FB labeling of the cytoplasm and NY or DY·2HCl labeling of the nucleus (Fig. 3G, H). These double-labeled neurons must distribute branching axons to the spinal cord and the lower brainstem.

In case B3 (FB at C2) and in case B9 (DY·2HCl at C4–C5) very many double-labeled neurons were present in the area of overlap. The largest number of double-labeled neurons was found in the intermediate part of the ventral bank of the cruciate sulcus and in the adjoining intermediate part of the anterior sigmoid gyrus. The percentages of labeled corticospinal neurons which were double-labeled differed in different parts of the area of overlap. Thus, at the border between gyrus proreus and anterior sigmoid gyrus 30% of the labeled corticospinal neurons were double labeled. When moving anteriorly and laterally the percentage decreased such that in the lateral bank of the pre-sylvian sulcus, in the medial portion of the anterior sigmoid gyrus and in the intermediate parts of the ventral and dorsal banks of the cruciate sulcus 25% were double-labeled. Further laterally, in the intermediate part of the anterior sigmoid gyrus and the lateral parts of the banks of the cruciate sulcus, 15% were double-labeled and in the lateral part of the anterior sigmoid gyrus and in the lateral sigmoid gyrus only 5% (Fig. 5). In case B3, 874 FB-NY double-labeled neurons were counted in 39 sagittal sections and in case B9, 837 FB-DY·2HCl double-labeled neurons were counted in 37 sections (Table 2).

In cases B1, B2, B4 and B8 with FB injections at C2 fewer corticospinal neurons were labeled than in cases B3 and B9 and also fewer double-labeled ones were found. Yet, the percentages of the double-labeled corticospinal neurons as well as their areal distribution were similar to those in cases B3 and B9. In case B11 with FB injections at C4–C5 and a relatively small NY injection dorsally in the MRF, the number of labeled corticospinal neurons was roughly the same as in cases B1, B2, B4 and B8, but the percentages of double-labeled corticospinal neurons were much smaller, with a maximum of 14%.

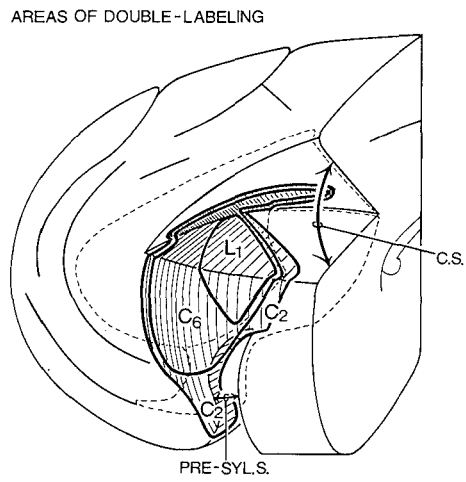

Fig. 7. Diagrammatic representation of the differences of the distribution areas of the double-labeled neurons after injections at C2, C6, and L1 (see for abbreviations Fig. 1)

Following progressively more caudal injections in the spinal cord, the area of the labeled corticospinal neurons becomes more and more restricted to the medial parts of the sensorimotor cortex. As a consequence the area of corticospinal-corticobulbar overlap, which contains the double-labeled neurons, becomes more and more restricted to the medial parts of the precruciate cortex (Fig. 7). Thus in cases B5 and B6 with C5–C6 FB injections the area of the double-labeled neurons was smaller than in cases B3 and B9 with C2 and C4–C5 injections respectively, because in case B5 and B6 no double-labeled neurons were found in the lateral bank of the pre-sylvian sulcus. In addition, fewer double-labeled neurons were present in the rostromedial part of area 4 and in the lateral part of area 6. Yet, the percentage of the double-labeled corticospinal neurons in the different parts of the area of overlap were approximately the same as in cases B3 and B9, ranging from 25–30% medially to 5% laterally. In case B7 with injections at Th1 (FB) and in case B10 with injections at L1 (DY·2HCl) the area of the double-labeled corticospinal neurons was even smaller and covered only the anteromedial part of area 4 (Figs. 6 and 7). In this area in case B10 7–12% of the corticospinal neurons were double-labeled and in 35 parasagittal sections 67 FB-DY·2HCl double-labeled neurons were counted (Table 2).

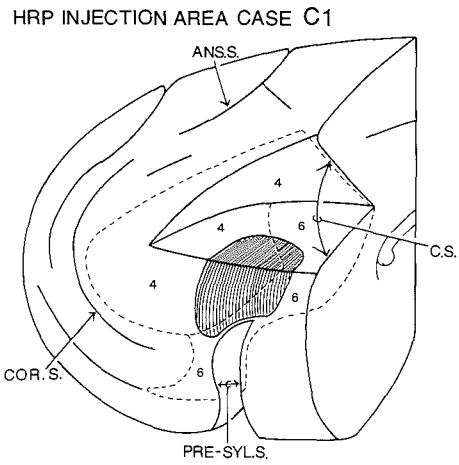

Fig. 8. HRP injection area in the rostro-medial part of area 4 and the lateral part of area 6 of case C1, as demonstrated by the DAB method (see for abbreviations Fig. 1)

Group C. Anterograde HRP Labeling of Corticobulbar Fibers

In two cases an attempt was made to delineate the brainstem distribution of the corticobulbar fibers, including the corticospinal collaterals, which originate from the cortical area containing the double-labeled corticospinal neurons. For this purpose in cases C1 and C2 small HRP injections were made in the medial part of the anterior sigmoid gyrus (Fig. 8) and the distribution of the anterogradely HRP-labeled cortical fibers in the lower brainstem was studied.

The anterograde HRP fiber-labeling in the two cases (C1 and C2) was most obvious in fiber bundles viewed at low magnification. However, at high magnification the individual HRP-labeled fibers were difficult to distinguish, although occasionally their course was suggested by a row of HRP-positive granules. In certain areas a diffuse distribution of HRP positive granules occurred which presumably represented labeling of axon terminals.

In cats C1 and C2 distinct bundles of anterograde HRP-labeled fibers could be traced through the internal capsule, cerebral peduncle, and pons into the pyramidal tract in which the labeled fibers were present exclusively ipsilaterally. Many of these fibers could be traced through the pyramidal decussation into the contralateral dorso-lateral funiculus. However some descended into the ipsilateral ventral funiculus. A large number of HRP-labeled corticobulbar fibers left the pyramidal tract between the trapezoid body and the inferior olive. They fanned out through the tegmentum on both sides in a dorsal and a lateral direction. In sagittal sections it was observed that they also fanned out in a rostro-caudal direction, thus reaching rostrally the tegmentum of the pons dorsal to the trapezoid body and caudally the tegmentum of the medulla oblongata dorsal to the inferior olive. More caudally, at the level of the inferior olive, labeled fibers left the pyramidal tract from its dorso-lateral aspect and coursed through the ipsilateral and contralateral inferior olive to the tegmentum on both sides (cf. Kuypers 1958). In the lower pons and medulla oblongata HRP-positive granules were present diffusely throughout the reticular formation on both sides, though few were present in its dorsal parts. In transverse sections the HRP-positive granulation was most dense in the center of the tegmentum and in parasagittal sections it was most pronounced between the levels of the trapezoid body and the inferior olive. These areas correspond largely with the nucleus reticularis gigantocellularis (Meessen and Olszewski 1949; Taber et al. 1960; Taber 1961), in which also some retrogradely HRP-labeled neurons were present which apparently project directly to the precruciate cortex. In the rostral medulla oblongata the diffuse anterograde HRP-labeling in the reticular formation was equally dense on both sides but more caudally it was most pronounced on the contralateral side. Anterograde HRP-labeling also occurred in the lateral reticular nucleus contralaterally. However, no HRP-labeling was present in the spinal trigeminal complex and in the dorsal column nuclei. These findings indicate that the corticobulbar fibers, including the corticospinal collaterals, from the medial part of the anterior sigmoid gyrus to the lower brainstem are distributed mainly to the reticular formation.

Discussion

The corticobulbar fibers from the sensorimotor cortex to the lower brainstem tegmentum are distributed bilaterally (Kuypers 1958). However, from the present findings conclusions regarding corticospinal collateralization can be drawn only in regard to the fibers which are distributed contralaterally, because the brainstem injections and the spinal injections were made on the same side (Fig. 9). This had the advantage that the possible involvement of the pyramidal tract by the brainstem injections would not produce false double-labeling because none of the pyramidal fibers on that side are derived from the

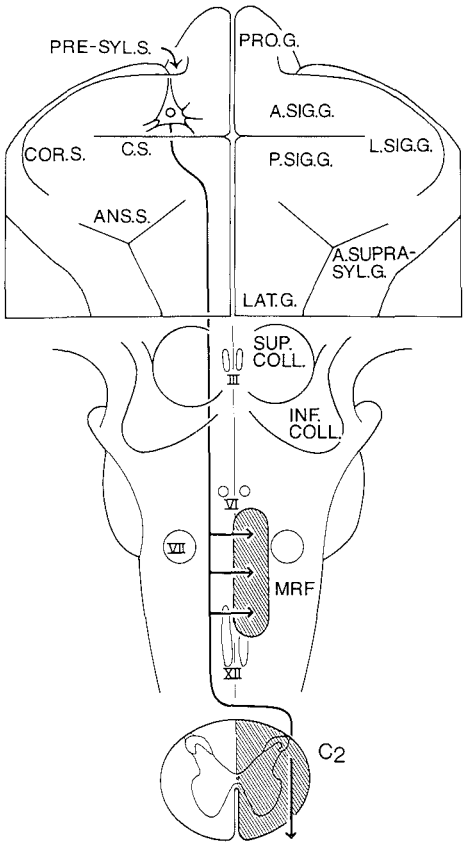

Fig. 9. Diagram of corticospinal fibers which give off collaterals to the contralateral bulbar medial reticular formation of the lower brainstem. Hatching indicates schematically the injection areas in spinal cord and medial reticular formation. Note that both injections were made on the same side, and that the distribution of the labeled neurons was studied in the contralateral hemisphere. Abbreviations: A. SUPRA-SYL.G., anterior suprasylvian gyrus; INF.COLL., inferior colliculus; SUP.COLL., superior colliculus; III, oculomotor nucleus (For other abbreviations see Figs. 1 and 2)

contralateral sensorimotor cortex (cf. Biedenbach and DeVito 1980; findings group C). Moreover, the brainstem injections could also be made from ventral through the pyramidal tract, which avoided involvement of the rostral part of the dorsal column nuclei by the injection area.

The fluorescent tracers FB, NY and DY·2HCl were used. They are transported from fiber termination areas as well as from damaged fibers (Huisman et al. 1982; Huisman 1983; Kuypers and Huisman 1983; Keizer et al. 1983) in the same way as HRP (Kuypers and Maisky 1975). Therefore when the tracers were injected in the cervical white and grey matter, labeled corticospinal neurons were present throughout the contralateral pericruciate cortex. The areal distributions of the FB- and the DY·2HCl-labeled corticospinal neurons (group B) were similar to that of the HRP-labeled corticospinal neurons (group A) and were approximately the same as obtained in earlier experiments (Armand et al. 1974; Berrevoets and Kuypers 1975; Catsman-Berrevoets and Kuypers 1981; Armand and Aurenty 1977; Groos et al. 1978; Biedenbach and DeVito 1980).

The maximum number of FB-labeled corticospinal neurons (case B3) was estimated at 38,000 and that of the DY·2HCl-labeled corticospinal neurons (case B9) was estimated at 55,000. These numbers are of a similar order of magnitude to the maximum number of HRP-labeled neurons (case A1) which was estimated at 60.000. The pyramidal tract at the level of the inferior olives contains approximately 80,000 fibers (Crevel and Verhaart 1963). If all these fibers continued into the spinal cord, 50–75% of the corticospinal neurons in the three cases would have been labeled. However, since some of the pyramidal fibers terminate in the medulla oblongata (Rossi and Brodal 1956; Kuypers 1958) probably less than 80,000 fibers reach the spinal cord which implies that a higher percentage of the corticospinal neurons were labeled.

The experiments of groups A and B show that the areal distributions of the labeled corticobulbar neurons after NY, FB and DY·2HCl injections in the MRF of the lower brain stem were similar to that after HRP injections. These neurons were present in the rostral parts of area 4, in area 6 and in the lateral and dorsal banks of the presylvian sulcus. However, labeled neurons were also present on the surfaces of the coronal and orbital gyri. These gyri carry the sensorimotor face representation (Delgado and Livingstone 1955; Woolsey 1958) and give rise to corticobulbar fibers which are distributed from the pyramidal tract through the bulbar MRF to the lateral reticular formation and the trigeminal sensory nuclei (Kuypers 1958; Mizuno et al. 1968; Dunn and Tolbert 1982). Therefore the neurons in these gyri were probably labeled from these corticobulbar fibers which passed through the injection area. This is further supported by the fact that in case B11 with dorsal injections in the MRF, which must have interrupted only a few of those corticobulbar fibers, only a few labeled neurons were present in these gyri.

After combined injections of the fluorescent tracers in the spinal cord and the MRF (group B) the contralateral pericruciate cortex contained large numbers of double-labeled neurons. After the cervical injections these neurons, which must distribute their stem axon to the spinal cord and a collateral to the lower brainstem, were concentrated in the following regions; (1) the lateral parts of area 6aα and 6aβ, (cf. Hassler and Muhs-Clement 1964), where 30% of the corticospinal neurons were double-labeled, (2) the lateral and dorsal banks of the presylvian sulcus, where 25% were double-labeled, (3) the caudal part of the dorsal bank of the cruciate sulcus, its entire ventral bank and the surface of the anterior sigmoid gyrus, where 15–25% were double-labeled and (4) the lateral part of the anterior sigmoid gyrus where 5% were double-labeled. Thus the double-labeled corticospinal neurons were present mainly in those parts of the sensorimotor cortex which carry the representations of back movements, neck movements and shoulder movements (Nieoullon and Rispal-Padel 1976), while virtually none were present in the areas, which carry the representations of wrist movements, forepaw movements and hindlimb movements. However, after spinal injections at L1 the double-labeled neurons were mainly present medially in the area of corticobulbar spinal overlap i.e. in the representation area of back movements.

The anterograde HRP transport findings in group C show that the corticobulbar fibers from the area containing the bulk of the double-labeled neurons (cf. group B) are distributed exclusively to the lower brainstem reticular formation, bilaterally. This in turn strongly suggests that the collaterals of the corticospinal neurons in this cortical area are also distributed to the lower brainstem reticular formation. The existence of such collaterals was suggested by the findings of Scheibel and Scheibel (1957) although Cajal was unable to find such collaterals (Cajal 1952). The corticobulbar fibers in question, including the corticospinal collaterals, are distributed mainly to the nucleus reticularis gigantocellularis but also to the nucleus reticularis pontis caudalis and the nucleus reticularis ventralis of the medulla oblongata (Rossi and Brodal 1956; Kuypers 1958). These nuclei contain many reticulospinal neurons (Torvik and Brodal 1957; Nyberg-Hansen 1966; Petras 1967; Kuypers and Maisky 1975; Wilson and Peterson 1981). This suggests that the corticobulbar fibers, including the corticospinal collaterals, establish indirect cortico-reticulo-spinal connections. This is supported by the electrophysiological findings, that reticulospinal neurons in these brainstem areas receive monosynaptic inputs from the contralateral sensorimotor cortex (Magni and Willis 1964; Peterson et al. 1974) and that disynaptic pyramidal connections to motoneurons of certain neck muscles are established by pyramidal collaterals to the lower brainstem reticular formation (Alstermark et al. 1983a, b).

The location of the double-labeled neurons in the sensorimotor cortex suggests that the corticospinal neurons with collaterals to the lower brainstem reticular formation are mainly involved in the control of neck and back movements and of shoulder movements. This would be in accordance with the earlier notion that the brainstem reticulospinal system mainly controls axial, body and integrated limb-body movements (Lawrence and Kuypers 1968), which is supported by the fact that the monosynaptic reticulospinal connections to motoneurons are preferentially established with motoneurons of neck and back muscles (cf. Wilson and Peterson 1981). The control of the activity of neck and back muscles from the motor cortex may therefore take place along two routes; (a) by way of the direct corticospinal fibers to spinal interneurons and (b) by way of indirect cortico-reticulo-spinal connections. The direct corticospinal connections may be more focussed than the indirect ones, since a relatively small percentage of the cortical fibers distribute collaterals to both the cervical and the lumbar cord (Shinoda et al. 1976) while a relatively large percentage of the reticulospinal fibers gives rise to such collaterals (Peterson et al. 1975). Because of the relatively large diameter of the reticulospinal fibers (cf. Busch 1964) the indirect cortico-reticulo-spinal activity probably reaches the motoneurons earlier than the direct corticospinal activity, as suggested also by electrophysiological findings (Alstermark et al. 1983a, b). The relatively fast indirect cortico-reticulospinal connections may produce a synergistic activation of neck and back muscles as exemplified by the types of movements elicited by electrical stimulation of the motor cortex after transection of the pyramidal tract (Tower 1944; Woolsey et al. 1972). The slower direct corticospinal connections may then superimpose a finer regulation upon this brainstem control. The fact that the rostral part of the motor cortex contains a considerable number of neurons with collaterals to the lower brainstem reticular formation and the spinal cord suggests that in certain cases this dual control may be exerted by the same neurons.

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Note added in proof. When using DY·2HCl it was dissolved in a 0.2 M phosphate buffer (pH 7.2) in which it forms a precipitate. This precipitate was homogenized in an ultrasonic waterbath prior to filling the syringe or pipette. However, recently several investigators have encountered difficulties with this procedure, because DY·2HCl in this form tends to clog the pipette. These difficulties can be overcome by dissolving DY·2HCl in a mixture of ethyleneglycol and 0.2 M phosphate buffer (pH 7.2) varying from equal volumes of both to one volume ethyleneglycol and four volumes phosphate buffer. The DY·2HCl suspensions, thus obtained are less viscous after ultrasonic homogenization, than the original one and the pipette is therefore easier to fill and to empty. However, the addition of ethyleneglycol seems to slightly enhance the migration of DY·HCl out of the retrogradely-labeled neurons as indicated by fluorescent labeling of surrounding glial nuclei. On the other hand, this glial labeling was observed only after retrograde transport of DY·2HCl over short distances in combination with a relatively long survival time i.e. from caudate-putamen to substantia nigra in rat with 3 days survival. However, it could be prevented by using a low percentage of ethyleneglycol in phosphate buffer i.e. 20% and restricting the survival time to 2 days.

Chapter IV

Distribution of Corticospinal Neurons With Collaterals to the Lower Brain Stem Reticular Formation in Monkey (Macaca Fascicularis)

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Summary. An earlier retrograde double-labeling study in cat showed that up to 30% of the corticospinal neurons in the medial and anterior parts of the precruciate motor area represent branching neurons which project to both the spinal cord and the reticular formation of the lower brain stem. These neurons were found to be concentrated in the rostral portion of the motor cortex, from where axial and proximal limb movements can be elicited. In the present study the findings in the macaque monkey are reported. The fluorescent retrograde tracer DY was injected unilaterally in the spinal cord at C2 and the fluorescent tracer FB was injected ipsilaterally in the medial tegmentum of the medulla oblongata. In the contralateral hemisphere large numbers of single DY-labeled corticospinal neurons and single FB-labeled corticobulbar neurons were present. A substantial number of DY-FB double-labeled corticospinal neurons were also found, which must represent branching neurons projecting to both the spinal cord and the bulbar reticular formation. These neurons were present in: 1. The anterior portion of the "cingulate corticospinal area" in the lower bank of the cingulate sulcus; 2. The supplementary motor area (SMA); 3. The rostral part of precentral corticospinal area; 4. The upper portion of the precentral face representation area; 5. The caudal bank of the inferior limb of the arcuate sulcus; 6. The posterior part of the insula. In these areas 10% to 30% of the labeled neurons were double-labeled. The functional implications of the presence of branching corticospinal neurons in these areas is discussed.

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Key words: Corticospinal collaterals – Premotor cortex – Motor cortex – Supplementary motor cortex – Fluorescent tracers – Monkey

Abbreviations

A = nucleus ambiguus, AS = arcuate sulcus, C = cuneate nucleus, Cing. S. = cingulate sulcus, corp. call. = corpus callosum, CS = central sulcus, Cx = external cuneate nucleus, DCN = dorsal column nuclei, dl = dorsolateral intermediate zone, IO = inferior olive, IP = intraparietal sulcus, Lat. Fis. = lateral fissure, LR = lateral reticular nucleus, LS = lunate sulcus, ML = medial lemniscus, MLF = medial longitudinal fascicle, mn = motoneuronal pool, MRF = medial reticular formation, Occ. = occipital pole, P = pyramid, PG = pontine grey, PS = principle sulcus, RB = restiforme body, RF = reticular formation, S = solitary nucleus, SPV = spinal trigeminal complex, STS = superior temporal sulcus, Sup. Col. = superior colliculus, TB = trapezoid body, VC = vestibular complex, vm = ventromedial intermediate zone, III = nucleus oculomotorius, VI = nucleus abducens, VII = nucleus, n. facialis, X = motor nucleus n. vagus, XII = nucleus hypoglossus.

Introduction

In an earlier fluorescent double-labeling study (Keizer and Kuypers 1984) it was demonstrated that in cat up to 30% of the corticospinal neurons in the medial and anterior parts of the precruciate motor area represent branching neurons which distribute collaterals to the contralateral medial reticular formation of the lower brain stem. Thus, these branching neurons are concentrated in those areas in which head, neck, back and shoulder movements are represented (Nieoullon and Rispal-Padel 1976). In the present fluorescent double-labeling study an attempt has been made to determine whether such branching neurons also exist in the macaque monkey. This was of special interest because the macaque monkey

possesses a highly differentiated motor cortex (Woolsey et al. 1958; Asanuma and Rosen 1972; Murphy et al. 1978; Kwan et al. 1978; McGuinness et al. 1980; Lemon 1981) and a wealth of anatomical and electrophysiological data are available regarding its efferent connections (Kuypers 1958; 1960; Kuypers and Lawrence 1967; Kuypers and Brinkman 1970; Jankowska et al. 1975; Catsman-Berrevoets and Kuypers 1976; Jones and Wise 1977; Biber et al. 1978; Murray and Coulter 1981; Sessle and Wiesendanger 1982; Toyoshima and Sakai 1982; Lawrence et al. 1985; Buys et al. 1986).

In three macaque monkeys the tracers Diamidino Yellow (DY.2HCl, Keizer et al. 1983) and Fast Blue (FB, Bentivoglio et al. 1980) were injected in the medullary medial reticular formation and the upper cervical dorsolateral funiculus respectively. The double-labeling findings in the macaques were similar to those in the cats (Keizer et al. 1984) and demonstrated that the rostral part of the precentral corticospinal area (Catsman-Berrevoets and Kuypers 1976; Jones and Wise 1977; Toyoshima and Sakai 1982) contains branching corticospinal neurons which distribute collaterals to the reticular formation of the lower brain stem. However, in contrast to the findings in the cat, branching corticospinal neurons in the macaque were also present in several other areas, e.g. the lower bank of the cingulate sulcus, the supplementary motor area, the precentral face representation area, the posterior bank of the inferior limb of the arcuate sulcus and the insula.

Material and methods

Three monkeys (Macaca fascicularis) were each injected with Fast Blue (FB) and Diamidino Yellow 2HCl (DY.2HCl), under Nembutal anaesthesia. In all these monkeys (5.0–7.4 µl) 2% DY.2HCl dissolved in a 0.2 M phosphate buffer (pH 7.2) was injected *unilaterally* into the *dorsal part of the lateral funiculus of the spinal cord at C2* by means of a glass micropipette. Multiple penetrations were made in order to interrupt as many corticospinal fibers as possible, because DY.2HCl is transported effectively from broken axons (Keizer et al. 1983). One week later, 7% FB in water (1.2–2.8 µl) was injected *ipsilaterally* into the *medial tegmental field of the lower brain stem*, by way of a ventral approach through a hole drilled through the basal part of the occipital bone. The pipette penetrations into the brain stem were made through the pyramidal tract ipsilateral to the spinal injections. This procedure made it possible to make injections in the brain stem which were restricted to the medial tegmental field and did not involve more dorsally located structures such as the dorsal column nuclei. In addition this procedure guaranteed that only corticobulbar neurons were FB labeled in the contralateral hemisphere and no corticospinal neurons, because the latter neurons do not distribute their fibers through the contralateral pyramidal tract through which the injections were made.

In the first two monkeys (A1, A2) the needle penetrations were made from the caudal edge of the pons to midolivary levels (2.0–2.8 µl), while in the third monkey (A3) the injections were restricted to the pontomedullary junction (1.2 µl). Four weeks after the DY injections the monkeys were sacrificed with an overdose of Nembutal and transcardially perfused with saline (1 l) followed by citrate buffered (pH 7.2) 20% formaline (3 l) at room temperature, which was followed by citrate buffered 10% sucrose (2 l) at 4° C. The brains were immediately dissected and the hemispheres contralateral to the injections were photographed, and subsequently cut coronally in 30 µm sections on a freezing microtome. The injection areas in brainstem and spinal cord were cut transversally. Every fourth section was mounted, air dried but not coverslipped. The sections were studied with an Olympus BH-2 fluorescence microscope, equipped with filter-mirror system U providing excitation light of 360 nm wavelength. In one out of every sixteen sections through the cortex (500 µm apart, total number of sections studied 62 (A1) and 58 (A2)), the distributions of the retrogradely single- and double-labeled neurons were charted with the aid of an x-y plotter. All sections that were plotted were subsequently counterstained with cresyl violet. This provided a general idea about the cytoarchitectonic characteristics of the cortical areas containing the retrogradely labeled neurons. However, the different areas and subareas could not be delineated precisely. Therefore, their boundaries were largely inferred from the gross surface anatomy (see Wise 1985).

Results

In the three monkeys, the DY injection areas at C2 were entirely restricted to one half of the spinal cord. They involved the dorsal two thirds of the lateral funiculus and the adjoining parts of the spinal grey matter (Fig. 1B). The FB injection areas in the brain stem were restricted to the side ipsilateral to the spinal injections. In the first two cases (A1, A2), these FB injection areas extended dorsally through the pyramid into the medial reticular formation (Fig. 1B). In both cases the injection areas extended rostrocaudally from a level between the motor V nucleus and the VI nucleus to levels through the middle of the inferior olive. The dorsal column nuclei and the pyramidal decussation were not involved (see Material and methods). In the third monkey (A3), the pipette penetrations were orientated differently and extended from the surface of the pontomedullary junction into the basal pons. The injection area involved the pyramidal tract, a very small portion of the pontine nuclei and several of the pyramidal bundles in the basal pons all on the side ipsilateral to the spinal injections. The tegmentum of the medulla oblongata and the pons were not involved.

In all three cases the hemispheres contralateral to the injections contained large numbers of DY-labeled corticospinal neurons in the upper two thirds of the precentral gyrus, in the upper portion of its lower one third (face representation area, Woolsey et al. 1952) and in the adjoining parts of the anterior bank of the central sulcus (Figs. 1A, C, 2A). This population of DY-labeled corticospinal neurons

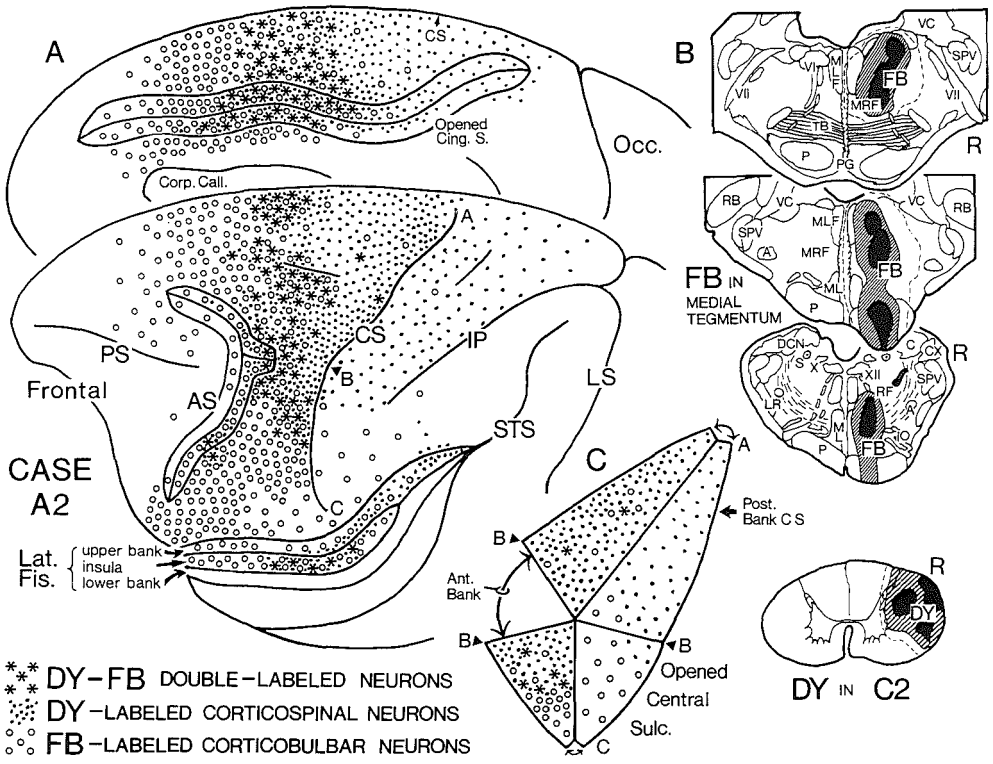

Fig. 1A–C. Diagrammatic representation of the distributions of the single DY-labeled corticospinal neurons (dots), the single FB-labeled corticobulbar neurons (circles) and the DY-FB double-labeled neurons (asterisks) (**A**, **C**), after DY injections at C2 (**B** lower part) and FB injections in the lower brain stem (**B** upper part). Note: double-labeled neurons are present in the lower bank of the cingulate sulcus, in the SMA, in the rostral part of the precentral gyrus, in the arcuate spur region and in a region within the face representation of the primary motor cortex

extended anteriorly in diminishing densities into the area medial to the upper limb of the arcuate sulcus. Labeled corticospinal neurons were present also in the posterior bank of the inferior limb of this sulcus as well as in the cortex lining the arcuate spur. The population of labeled corticospinal neurons on the convexity of the hemisphere extended onto the medial surface of the hemisphere, into the area of the motor cortex and the rostrally adjoining supplementary motor area (SMA, Fig. 1A) including the upper bank of the cingulate sulcus. This population of DY-labeled neurons on the medial aspect of the hemisphere extended anteriorly up to the level of the posterior extent of the arcuate sulcus. A separate population of DY-labeled corticospinal neurons was present in the lower bank of the cingulate sulcus. This cingulate population extended slightly more rostrally than that in the SMA and continued caudally up to the level of the central sulcus. On the convexity of the hemisphere, DY-labeled corticospinal neurons were also present in the upper two thirds of the postcentral gyrus, in the anterior part of the superior parietal lobule, as well as in the parietal operculum and in the caudal half of the insula (Fig. 1A). All DY-labeled neurons were located in cortical layer V. In the hemispheres contralateral to the injections also many FB-labeled corticobulbar neurons were present (Figs. 1A, 2B). A limited number of such neurons was located in the lower one third of the postcentral gyrus. This sparsely popu-

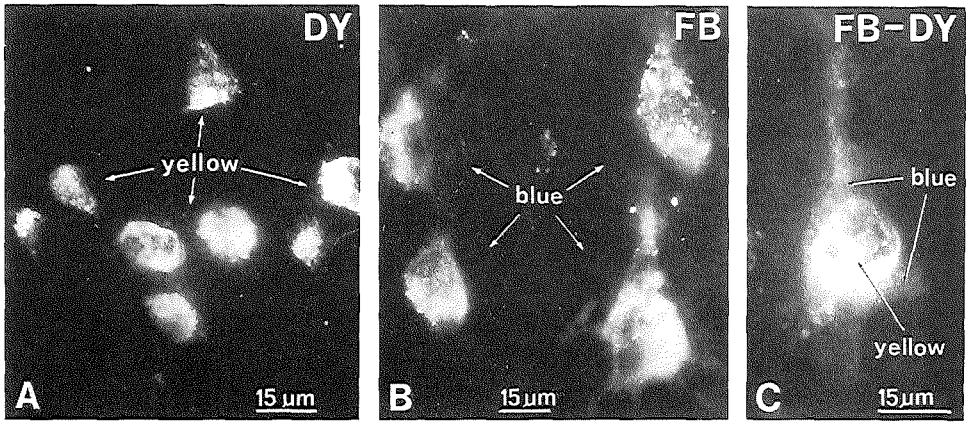

Fig. 2A–C. Photomicrographs of fluorescent retrogradely labeled neurons taken with filter-mirror system U (360 nm excitation wavelength). **A** Population of single DY-labeled corticospinal neurons with a yellow fluorescent nucleus in the SMA. **B** Population of single FB-labeled corticobulbar neurons with a blue fluorescent cytoplasm in area 6. **C** Double-labeled neuron with a yellow fluorescent nucleus and a blue fluorescent cytoplasm situated lateral to the superior precentral dimple

Table 1. Percentages of the corticospinal and corticobulbar neurons which were double-labeled (DL) in the different cortical areas of Case A2 (see Fig. 1)

Cortical area	number of sections	number of double-labeled (DL) neurons counted	% corticospinal neurons DL	% corticobulbar neurons DL
SMA, including upper bank cingulate s.	20	287	20% DL	26% DL
Lower bank cingulate s.	18	69	19% DL	17% DL
Anterior precentral corticospinal area	15	107	19% DL	30% DL
Face area	11	62	18% DL	21% DL
Lower limb arcuate s.	3	7	13% DL	7% DL
Insula	14	32	10% DL	8% DL

lated field of FB-labeled neurons extended laterally into the parietal operculum and the caudal part of the insula. It extended also anteriorly into the most lateral portion of the precentral gyrus. (Fig. 1A, C). A field of more densely-packed FB-labeled corticobulbar neurons was present in the lateral one third of the precentral gyrus. This field extended anteriorly into the caudal bank of the inferior limb of the arcuate sulcus, and into the banks of the arcuate spur; it extended laterally into the rostral part of the insula and the adjoining part of the orbitofrontal cortex (Fig. 1A). The field of FB-labeled neurons extended also into the area medial to the upper limb of the arcuate sulcus, from where it continued posteriorly in diminishing densities into the anterior part of the precentral corticospinal area. FB-labeled neurons were present also in the banks of the upper limb of the arcuate sulcus and more laterally in the cortex medial to the caudal half of the principal sulcus.

The population of the FB-labeled corticobulbar neurons extended from the lateral surface of the hemisphere onto its medial surface. Here the FB-labeled neurons were present in the region of the SMA, overlapping with the population of DY-labeled corticospinal neurons in this area (Fig. 1A). FB-labeled neurons were present also in the superior frontal gyrus anterior to the SMA as well as in the upper and lower banks of the cingulate sulcus, and the adjoining upper part of the cingulate gyrus at these levels. This distribution of the FB-labeled corticobulbar neurons is in keeping with earlier HRP findings (Catsman-Berrevoets and Kuypers 1976).

According to the present findings the distribution of the FB-labeled corticobulbar neurons extended more anteriorly than that of the DY-labeled cortico-

spinal neurons. However, the two populations showed an extensive overlap. The most striking feature of the area of overlap was the presence of many DY-FB double-labeled neurons (Figs. 1A, 2C). They must represent branching corticospinal neurons which also distribute collaterals to the lower brain stem (Fig. 3). Proceeding from medial to lateral, these double-labeled neurons were concentrated in the following regions. 1. The anterior portion of the cingulate corticospinal area in the lower bank of the cingulate sulcus, 2. The region of the SMA and the anterior portion of the posteriorly adjoining precentral motor cortex, on the medial surface of the hemisphere, 3. The rostral part of the precentral corticospinal area on the convexity of the hemisphere. In this region several concentrations of double-labeled neurons could be distinguished, i.e. along the upper margin of the hemisphere, at the level of the superior precentral dimple, as well as in the banks of the spur of the arcuate sulcus and the surrounding cortex, 4. The upper portion of the face representation area where a band of double-labeled neurons extended from the spur of the arcuate sulcus postero-laterally, into the anterior bank of the lateral one third of the central sulcus, 5. The caudal bank of the inferior limb of the arcuate sulcus, 6. The posterior part of the insula. In the second case a slighty larger number of double-labeled neurons was present than in the first case (A2). In several sets of sections of the second case the single- and double-labeled neurons in the various areas were counted. In these areas 10% to 30% of the labeled neurons were found to be double-labeled (see Table 1).

In the third monkey, in which the FB injections were small and largely restricted to the pyramidal tract and the basal pons (see M and M), only a very few scattered FB-labeled neurons were present in the contralateral hemisphere, while the distribution of the DY-labeled corticospinal neurons was roughly the same as in the other two monkeys. However, none of these neurons were double-labeled.

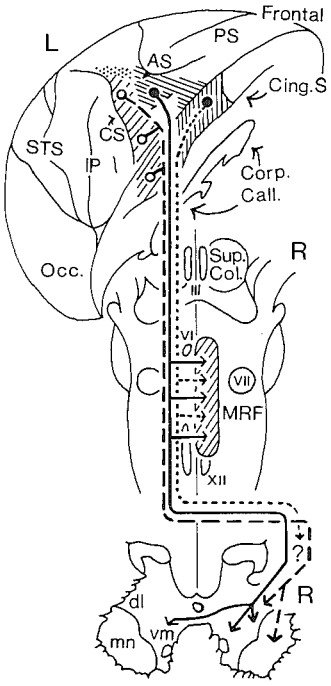

Fig. 3. Diagram showing the differences in spinal projections from the posterior and the anterior parts of the precentral corticospinal area. Corticospinal fibers from major portions of the posterior part project to the dorsal and lateral parts of the intermediate zone and to the motoneuronal pool contralaterally; many of the corticospinal fibers from the anterior part typically project to the ventromedial parts of the intermediate zone bilaterally. Several of the corticospinal fibers from the anterior part give off collaterals to the medial subdivision of the bulbar reticular formation

Discussion

In the three monkeys the distributions of the labeled neurons were studied only in the left hemisphere. The spinal DY injections aimed at labeling the corticospinal neurons were made contralaterally in the right C2 dorsolateral funiculus (Fig. 3). Corticobulbar neurons in this hemisphere could be labeled from either half of the brain stem since the corticobulbar fibers are distributed bilaterally (Kuypers 1958; Kuypers and Lawrence 1967). The almost complete absence of FB-labeled neurons in the left hemisphere of the third monkey indicated that in the macaque as in cat (Biedenbach and DeVito 1980; Keizer and Kuypers 1984) the fibers in each pyramidal tract are derived almost exclusively from the ipsilateral hemisphere. Therefore the FB injections in the brain stem tegmentum aimed at labeling corticobulbar neurons in the left hemisphere could be made through the right pyramidal tract because these injections would not cause labeling of pyramidal tract neurons in the left hemisphere. However, these injections labeled only those corticobulbar neurons which distribute fibers contralaterally.

The distributions of the DY-labeled corticospinal neurons and the FB-labeled corticobulbar neurons in the left hemisphere were in keeping with earlier findings (Kuypers 1958, 1960; Kuypers and Lawrence 1967; Catsman-Berrevoets and Kuypers 1976; Jones

and Wise 1977; Biber et al. 1978; Künzle 1978; Murray and Coulter 1981; Toyoshima and Sakai 1982; Sessle and Wiesendanger 1982; Martino and Strick 1987; Hutchins et al. (in press)). The present experiments demonstrated that these two distributions overlap extensively, which so far had only been inferred from comparing the findings in different single tracer experiments. The presence of double-labeled neurons in the areas of overlap indicated that these areas contain branching neurons which distribute divergent collaterals to the spinal cord and the lower brain stem (Fig. 3).

The lateral one third of the precentral gyrus, which contained many double-labeled neurons in its upper part (Fig. 1A), distributes fibers bilaterally to the bulbar motor nuclei and lateral reticular formation (Kuypers 1958, 1960; Kuypers and Lawrence 1967), which contains the bulk of the interneurons projecting to the bulbar motor nuclei (Holstege et al. 1977). The lateral one third of the precentral gyrus and probably in particular its upper part, also distributes fibers to the grey matter of the upper cervical segments (Kuypers 1958). The FB injections into the brain stem probably damaged the corticobulbar fibers which pass from the left pyramidal tract, cross the midline to the right lateral reticular formation. The DY injections in the C2 dorsolateral funiculus probably involved the crossed corticospinal fibers to the upper cervical grey. Therefore the double-labeled neurons in question probably represented branching neurons which distribute one set of crossed collaterals to the bulbar lateral reticular formation and another set to the upper cervical grey. These cortico-bulbo-spinal collaterals may project via interneurons to the bulbar motoneurons innervating the muscles of the pharynx and larynx and to upper cervical motoneurons innervating the extrinsic laryngeal muscles.

In view of the present findings, it is of interest that the corticospinal fibers in monkey also distribute collaterals to the dorsal column nuclei (DCN). The cells of origin of the cortico-DCN-spinal collaterals are situated mainly in the postcentral, somatosensory areas 1 and 2 (Bentivoglio and Rustioni 1986).

The double-labeled neurons in the anterior part of the precentral corticospinal area, (i.e. around the superior precentral dimple and close to the spur of the arcuate sulcus) in all likelihood represent branching corticospinal neurons which distribute collaterals to the lower brain stem medial reticular formation (Kuypers 1958, 1960; Kuypers and Lawrence 1967). The stem-fibers of these neurons probably terminate especially in the ventro-medial part of the spinal intermediate zone (Kuypers and Brinkman 1970). These branching corticospinal neurons are located in areas in which movements of the head and neck, shoulder, and body axis are represented (Woolsey et al. 1958; Kwan et al. 1978; Wong et al. 1978; cf. Freund and Hummelstein 1984, 1985). This is in keeping with the observation that the reticulospinal neurons in the lower brain stem are preferentially involved in steering axial movements, proximal limb movements and integrated limb-body movements (Lawrence and Kuypers 1968; cf. Wilson and Peterson 1981; Alstermark et al. 1983a, b). The location of this group of branching corticospinal neurons in the monkey has much in common with their location in the cat (Keizer and Kuypers 1985), where they are situated in the anterior and medial parts of the precruciate corticospinal motor area. These parts carry the representations of head, neck and shoulder movements (Nieoullon and Rispal-Padel 1976) and project preferentially to the ventromedial parts of the spinal intermediate zone on both sides (Armand et al. 1985). The few double-labeled neurons in the posterior bank of the inferior limb of the arcuate sulcus indicate that of this separate set of corticospinal neurons (cf. Martino and Strick 1987), some also distribute collaterals to the lower brain stem tegmentum.

The bulk of the double-labeled neurons on the medial aspect of the hemisphere, above the cingulate sulcus were located in the SMA (Woolsey et al. 1958; Mitz and Wise 1987). The concentration of double-labeled neurons on the convexity of the hemisphere close to its medial margin (see Fig. 1A), probably represented a lateral extension of the SMA population. However, this concentration may also have been part of the concentration of double-labeled neurons in the anterior portion of the precentral corticospinal area, since in the second animal these two concentrations were continuous.

The presence of a mixture of corticobulbar and corticospinal neurons throughout the SMA, combined with branching corticospinal-bulbar neurons might explain that electrical stimulation of the SMA (Woolsey et al. 1958; Mitz and Wise 1987) in particular after ablation of the motor cortex (Wiesendanger et al. 1973) elicits mainly proximal limb movements. The diffuse distribution of corticobulbar and branching corticospinal neurons throughout the SMA may be related to the observation that in the SMA the representations of proximal and distal movements are intermixed (Mitz and Wise 1987).

The anterior parts of the SMA carry the representation of oro-facial movements (Woolsey et al. 1952; Mitz and Wise 1987). Therefore some of the double-labeled neurons in this region may distribute collaterals to the bulbar lateral reticular formation rather than to the medial reticular formation.

The population of labeled corticospinal neurons in the lower bank of the cingulate sulcus (cingulate corticospinal area) was distinct from that in the SMA and extended more anteriorly (see also Hutchins et al. (in press)). This cingulate corticospinal area receives cortico-cortical fibers from the post-arcuate cortex (Barbas and Pandya 1987) and projects, together with the postarcuate cortex and the SMA, to the precentral motor cortex (Muakassa and Strick 1979; Godschalk et al. 1984). A relatively large proportion of the corticospinal neurons in the cingulate corticospinal area were found to be branching neurons. Hutchins et al. (in press) recognised in this area a rostral and a caudal subdivisions. The present findings suggest that the branching corticospinal neurons may be concentrated in the rostral subdivision.

The premotor cortex of Fulton and his collaborators (Fulton 1935; Jacobson 1934) corresponds roughly to the upper part of area 6 of the Vogts (Fulton 1935). Destruction of this premotor cortex interferes with the performance of skilled movements of the contralateral limb without producing gross weakness (Jacobson 1934). Unilateral destruction of the postarcuate cortex (cf. Matsumara and Kubota 1979; Muakassa and Strick 1979) in combination with the premotor area (Fulton 1935) and the SMA (Woolsey et al. 1955; Mitz and Wise 1987) interferes with visually guided movements (Moll and Kuypers 1975, 1977; Passingham 1985). These areas differ from the precentral motor cortex such that the activity of many neurons in these non-primary motor areas is especially related to motor preparation (Tanji et al. 1980; Rizzolatti et al. 1981a, b; Weinrich and Wise 1982; Weinrich et al. 1984; Godschalk et al. 1985; Wise 1985), while the activity of many neurons in the precentral motor cortex is related directly to the execution of movements (Godschalk et al. 1981; Tanji 1985). However, Woolsey et al. (1952) pointed out that the premotor cortex of Fulton and his collaborators (1935) overlaps the rostral part of the precentral motor area (MI) in particular those regions carrying the representations of axial and proximal movements (Woolsey et al. 1952; cf. Freund and Hummelstein 1985). Correspondingly branching corticospinal neurons in the anterior part of the precentral corticospinal area are located in the same region as e.g. the "premotor" neurons related to movements of the hand and especially of the foot described by Kurata and his collaborators (1985). It is not clear whether the corticospinal and corticobulbar projections from the rostral part of the precentral corticospinal area, emanating either from different neurons or from branching neurons, are of critical importance in subserving the above "premotor" functions. Yet, the differences in the projections of corticospinal neurons in the anterior versus the posterior parts of the precentral corticospinal area may be of further assistance in clarifying the anatomical subdivisions of this region.

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Chapter V

Branching Cortical Neurons in Cat, Which Project to the Colliculi and to the Pons: A Retrograde Fluorescent Double-labeling Study

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Summary. The fluorescent double-labeling technique has been used to determine whether the corticopontine and the corticotectal fibers in the cat are derived from two different sets of neurons or whether they are derived from branching neurons which distribute collaterals to the pontine grey and the colliculi. After unilateral DY.2HCl injections in the pontine grey and FB injections in the ipsilateral colliculi, large numbers of FB-DY.2HCl double-labeled neurons were present in the cortex of the ipsilateral hemisphere. However, the labeled neurons in its rostral part may have represented pyramidal tract neurons which were labeled retrogradely because their fibers descended through the DY.2HCl injection area. Therefore, also DY.2HCl injections were made in the pyramid (i.e. caudal to the pons) and the cortical pyramidal tract area, containing the retrograde DY.2HCl-labeled neurons, was delineated. In the rest of the experiments only the DY.2HCl-labeled neurons in the caudal two thirds of the hemisphere (outside the pyramidal tract area) were taken into account because only these neurons could, with confidence, be regarded as corticopontine neurons. In some anterograde HRP transport experiments the trajectories of the corticotectal and the corticopontine fibers were visualized. On the basis of the findings the DY.2HCl injections in the pontine grey were placed such that they could not involve any of the corticotectal fibers passing from the cerebral peduncle to the colliculi. Thus artifactual double-labeling of cortical neurons was avoided. However, also under these circumstances many double-labeled neurons were present in the caudal two thirds of the hemisphere. This led to the conclusion that in the cat a large proportion of the corticopontine neurons in the caudal two thirds of the hemisphere represent branching neurons which also distribute collaterals to the colliculi. The parietal (anterior part of the lateral gyrus, middle and posterior suprasylvian gyri) and the cingulate areas together contained three quarters of all labeled corticopontine neurons outside the pyramidal tract area. In the parietal areas roughly 25% of them were double-labeled and in the cingulate area 14%. However, in the visual areas 18 and 19 a much larger percentage (30–60%) was double-labeled. In a recent study from our laboratory it was found that in the cat the pyramidal tract fibers distribute an abundance of collaterals to the pontine grey. Therefore, a large proportion of all corticopontine connections in this species appear to be established by branching neurons which also distribute fibers to other cell groups in the brain stem and the spinal cord.

Key words: Corticopontine collaterals – Corticotectal neurons – Cortex – Cat – Fluorescent tracers

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Introduction

The corticopontine neurons which represent the first link in the main cerebro-cerebellar pathway are distributed throughout a major portion of the neocortex (Kawamura and Chiba 1979; Albus et al. 1981; Brodal 1982). The cell bodies of these neurons are situated in cortical layer V, which also contains the cell bodies of the neurons which distribute their fibers to the brain stem and the spinal cord. Some of these projecting neurons represent corticotectal neurons which distribute their fibers to the colliculi (Berrevoets and Kuypers 1975; Gilbert and Kelly 1975; Kawamura and Konno 1979; Catsman-Berrevoets and Kuypers 1981). The corticotectal

Table 1

Group A Retrograde fluorescent labeling of pyramidal tract neurons

Case	%	Tracer	µl	Injection site	%	Tracer	µl	Injection site	Survival time	Survival time
A1	7%	FB	8.2	sup. + inf. colliculi	2%	DY	4.6	ipsil. pyramid	FB 17 days	DY 11 days
A2	7%	FB	9.6	sup. + inf. colliculi	2%	DY	5.0	ipsil. pyramid	FB 17 days	DY 11 days
A3	7%	FB	9.0	sup. + inf. colliculi	2%	DY	5.2	ipsil. pyramid	FB 18 days	DY 11 days

Group B Anterograde HRP fiber labeling

Case	%	Tracer	µl	Cortical injection site	Survival time
B1	30%	HRP	24.6	Lat. Gyrus, S. Sylvian Gyri (see Fig. 3)	3 days
B2	30%	HRP	8.3	E. Sylvian Gyri, P. Sylvian Gyrus (see Fig. 3)	3 days

Group C Fluorescent double-labeling experiments

Case	%	Tracer	µl	Injection site	%	Tracer	µl	Injection site	Survival time	Survival time
C1	7%	FB	4.2	sup. + inf. colliculi	2%	DY	4.8	ipsil. caudal 1/2 pons	FB 20 days	DY 13 days
C2	7%	FB	7.6	sup. + inf. colliculi	2%	DY	8.0	ipsil. caudal 2/3 pons	FB 18 days	DY 12 days
C3	7%	FB	7.8	sup. + inf. colliculi	2%	DY	5.0	ipsil. caudal 2/3 pons	FB 19 days	DY 13 days
C4	7%	FB	4.0	inferior colliculus	2%	DY	2.6	ipsil. rostro-caudal pons	FB 19 days	DY 13 days
C5	7%	FB	7.8	sup. + inf. colliculi	2%	DY	6.4	ipsil. rostro-caudal pons	FB 21 days	DY 16 days

neurons, as the corticopontine neurons, are present in almost all neocortical areas (Kawamura and Konno 1979). Therefore using a single tracer technique it is very difficult to determine whether the corticopontine and the corticotectal fibers are derived from two different sets of neurons or whether they are derived from branching neurons which distribute divergent collaterals to the pons and the colliculi.

In the present study the fluorescent tracers Fast Blue (FB) (Bentivoglio et al. 1980) and Diamidino Yellow dihydrochloride (DY.2HCl) (Keizer et al. 1983) were injected to clarify this point. FB and DY were injected ipsilaterally in the colliculi and the pontine grey respectively. After these injections many double-labeled neurons were present in various cortical areas, in both the rostral and caudal parts of the hemisphere. This suggested that many of the corticopontine neurons represented branching neurons which distributed collaterals to the colliculi. However, in order to ascertain the existence of such branching neurons the following two points had to be demonstrated. a) The cortical neurons, DY.2HCl-labeled from the pontine injection, truely represented corticopontine neurons and did not represent pyramidal tract neurons which were labeled because the DY.2HCl injections damaged the pyramidal tract fibers passing through the pons. b) The double-labeled cortical neurons did not merely represent corticotectal neurons which were double-labeled because the two fluorescent tracer injections involved the same corticotectal fibers in two different places along their trajectory. In two sets of control experiments these two points could be demonstrated convincingly. Therefore it was concluded from the double-labeling findings that a large portion of the corticopontine fibers were derived from branching neurons which also distributed fibers to the colliculi.

Material and methods

In all the animals of the three groups described below the surgery, the injections and the perfusions were made under deep Nembutal anaesthesia.

Group A

In cases A1–A3 the cortical areas containing labeled neurons after DY.2HCl injections in the pyramidal tract were delineated. 2% DY.2HCl in 0.2 M phosphate buffer (pH 7.2) was injected in the pyramid and the adjoining bulbar medial reticular formation immediately caudal to the pons by means of several glass micropipette penetrations, which were made from ventral through a hole drilled in the base of the skull (Table 1). 6 to 7 days earlier 7% FB in distilled water had been injected in the ipsilateral colliculi by means of several glass micropipette penetrations. They were made from above after gently lifting the occipital lobe and removing part of the bony tentorium cerebelli. These additional injections of FB in the colliculi made it possible to clarify a side issue, i.e. the existence of branching pyramidal tract neurons which distribute collaterals to the colliculi. One day after either of the two injections the animals were eating and walked around in the cages. Seventeen to eighteen days after the tectal injection the animals were sacrificed.

Group B

In cases B1 and B2, the trajectories of the fibers from "visual" and "auditory" cortical areas to the colliculi and the pons were visualized. HRP injections were made in "visual" (B1) and "auditory" (B2) cortical areas (Table 1). In case B1, 30% HRP in distilled water was injected (123 glass micropipette penetrations, depth 2–3 mm) in the lateral gyrus and in the middle and posterior suprasylvian gyri (24.6 µl). In case B2, 30% HRP was injected (83 penetrations, depth 2–3 mm) in the middle and posterior ectosylvian gyri, the posterior sylvian gyrus, and the banks of the posterior tips of the sylvian and ectosylvian sulci (8.3 µl). The animals were allowed to survive for three days.

Group C

In cases C1–C5 the distributions of the double-labeled cortical neurons were studied after FB injections in the colliculi combined with DY.2HCl injections in the ipsilateral pontine grey (Table 1). In cases C1–C3 and C5, 7% FB was injected in the superior and inferior colliculi. In case C4 7% FB was injected in the inferior colliculus only. In all cases (C1–C5) five to seven days later 2% DY.2HCl was injected in the caudal one third of the pontine grey and in the median, paramedian and ventral regions of its middle one third. In cases C4–C5 larger 2% DY.2HCl injections were made which also involved the paramedian and ventral regions of the rostral one third of the pontine grey. The animals were allowed to survive for seventeen to twenty-one days after the tectal injections.

The cats injected with fluorescent tracers (groups A and C), were transcardially perfused with 1.5% saline (1 l), followed by citrate buffered (pH 7.2) 30% formalin (3 l) at room temperature, which was followed by citrate buffered (pH 7.2) 10% sucrose (2 l) at 4° C. The brains were immediately dissected and the hemispheres were photographed. The hemispheres of cases A2, A3, C1 and C3–C5 were cut coronally (stereotaxic plane: Snider and Niemer 1961) in 30 µm sections on a freezing microtome. Every fourth section was mounted and air dried but not coverslipped. In cases A1 and C2 the hemispheres were divided in two parts by a coronal cut through the ansate sulcus. The rostral part was cut sagitally, the caudal part was cut coronally. In all these cases the injection areas in the brainstem were cut transversally in 30 µm sections and every fourth section was mounted. All the sections were studied with a Leitz Ploemopack fluorescence microscope equipped with filter-mirror system A (excitation light: 360 nm wavelength). Immersion oil was applied directly to the sections. In every eight section (1 mm apart) the distributions of the retrogradely single- and double-labeled cortical neurons were charted with the aid of an X–Y plotter. In the charts the numbers of DY single-labeled and DY-FB double-labeled corticopontine neurons in the different cortical areas were counted and the percentages of the DY-labeled neurons which were double-labeled were calculated. The borders of the cytoarchitectonic cortical areas were first approximated on the basis of the various descriptions available in the literature (Woolsey 1961; Otsuka and Hassler 1962; Hassler and Muhs-Clement 1964; Heath and Jones 1971; Tusa et al. 1979; Tusa and Palmer 1980 (Fig. 1)). Subsequently an attempt was made to recognize the actual borders of these areas in the Nissl stained frozen sections. The injection areas in the colliculi and the pons were also plotted and the sections were then counterstained with cresylviolet. The cats injected with HRP (group B), were transcardially perfused according to Mesulam (Mesulam 1982). The hemispheres were photographed and brainstems and hemispheres were cut transversally in 30 µm frozen sections. Every fourth section was mounted and air dried. The brainstem sections were incubated with Tetramethylbenzidine (TMB) (Mesulam 1982), while the cortical injection areas were incubated with Diamidinobenzidine (DAB). The sections were studied under darkfield illumination.

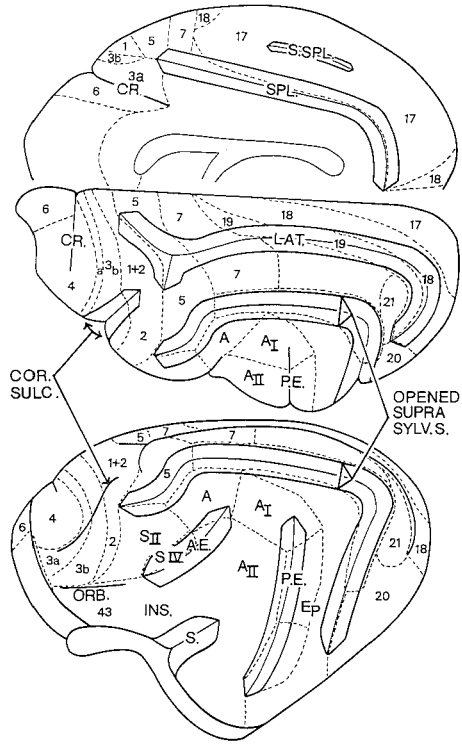

Fig. 1. Medial, dorsal and lateral views of the left hemisphere of cat with cytoarchitectonic subdivisions of the cerebral cortex, according to the descriptions of Woolsey 1961; Hassler and Muhs-Clement 1964; Heath and Jones 1971; Tusa et al. 1979; Tusa and Palmer 1980. Note that some sulci are shown opened

Results

Group A. Retrograde fluorescent labeling of pyramidal tract neurons

In cases A1–A3 the DY.2HCl injection areas (Keizer et al. 1983; Keizer and Kuypers 1984; Kuypers and Huisman 1984) involved one pyramid, the dorsally adjoining tegmentum and the caudal part of the trapezoid body. The injection areas extended rostrocaudally from the caudal one third of the trapezoid

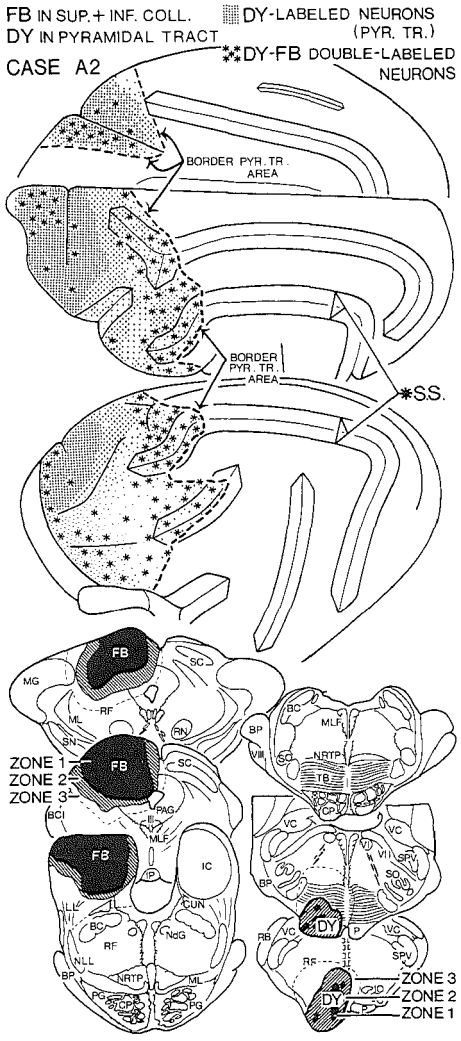

Fig. 2. Diagrammatic representation of the retrogradely DY.2HCl single-labeled and DY.2HCl-FB double-labeled pyramidal tract neurons (upper part) after DY.2HCl injections in the pyramidal tract caudal to the pons and FB injections in the dorsal mesencephalon (lower part). For descriptions of the three concentric zones see Keizer and Kuypers 1984. The border of the cortical pyramidal tract area is indicated by a dotted line. For cytoarchitectonic areas see Fig. 1

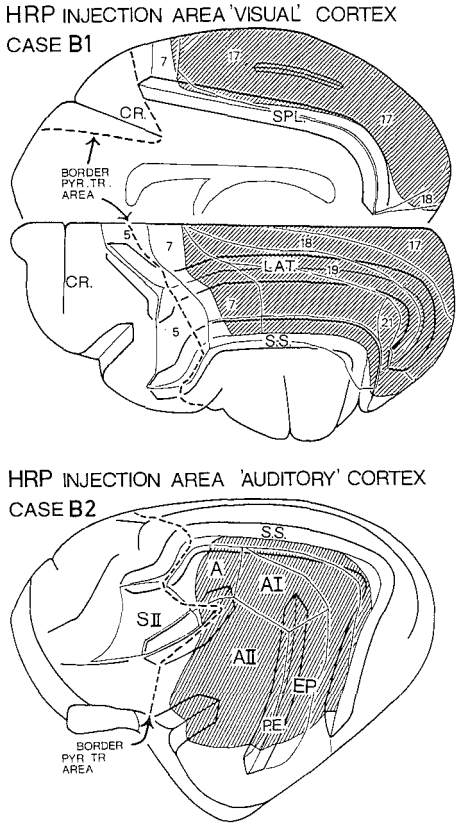

Fig. 3. Upper part: HRP injection area in the visual and parietal cortical regions (case B1). Note that the injection area does not involve the pyramidal tract area. Lower part: HRP injection area in the auditory cortical regions (case B2). Note that the injection area involves the caudal portions of the banks of the anterior ectosylvian sulcus

body to the middle of the inferior olive and spared the basal pons (Fig. 2). The FB injection areas in the mesencephalon involved the ipsilateral superior and inferior colliculi, the pretectal nuclei, the adjoining dorsal part of the mesencephalic reticular formation and the dorsolateral part of the periaqueductal grey. The injection areas extended rostro-caudally from the caudal tip of the pulvinar to the caudal surface of the inferior colliculus (Fig. 2).

In all three cases many DY.2HCl single-labeled, FB single-labeled and DY.2HCl-FB double-labeled

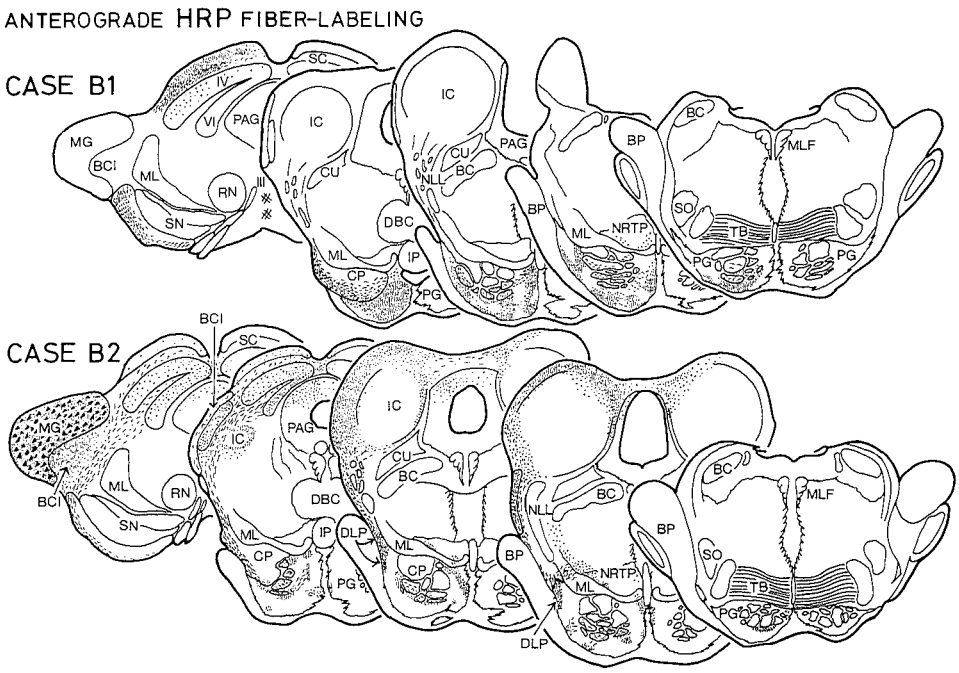

Fig. 4. Upper part: Distribution of anterogradely HRP-labeled fibers and terminals in the superior colliculus and the pontine grey after ipsilateral HRP injections in the visual and parietal cortical regions (case B1; see Fig. 3). Note absence of HRP-labeled fibers in the mesencephalic tegmentum and the preferential distribution of terminal labeling in the rostral pons. Lower part: Distribution of anterogradely HRP-labeled fibers and terminals in the mesencephalon and the pontine grey after ipsilateral HRP injections in auditory cortical regions (case B2; see Fig. 3). Note heavy labeling of the dorsolateral pontine nucleus (DLP, third and fourth section), and the trajectory of labeled fibers from the cerebral peduncle to the inferior colliculus (second section)

neurons were present in the frontal one third of the ipsilateral hemisphere, while only FB single-labeled neurons were present in its caudal two thirds (Figs. 2, 8). The present description will only deal with the areal distributions of the DY.2HCl single-labeled neurons and of the DY.2HCl-FB double-labeled ones, which were DY.2HCl labeled from the injections immediately caudal to the pons. The FB single-labeled neurons in the caudal two thirds of the hemisphere were distributed in roughly the same way as in the cases of group C where their distribution will be described in detail. The DY.2HCl single-labeled (pyramidal tract) neurons in the frontal part of the hemisphere were present in areas 4 and 6, in areas 3, 1 and 2, in area 5, on the surface of the anterior ectosylvian gyrus (SII), and in the dorsal bank of the anterior ectosylvian sulcus (SIV, Fig. 2). They were also present in the orbital gyrus and in area 43 (Hassler and Muhs-Clement 1964) (Figs. 1, 2). DY.2HCl-FB double-labeled neurons were found in area 6 and in the adjoining rostro-medial part of area 4. In case A1 in 9 parasagittal sections, through the rostro-medial portion of the motorcortex, 1665 DY.2HCl-labeled pyramidal tract neurons were present of which 19.2% were double-labeled. Double-labeled neurons were also present on the surface of the posterior two thirds of the orbital gyrus where 15–20% of the DY.2HCl-labeled neurons were double-labeled (Fig. 2), and in areas 5, SII and SIV (cf. Catsman-Berrevoets and Kuypers 1981). Therefore in the double-labeling experiments of group C (see below), only the single- and the double-labeled cortical neurons in the caudal two thirds of the hemisphere i.e. outside the cortical areas described above could with confidence be regarded to represent corticopontine neurons.

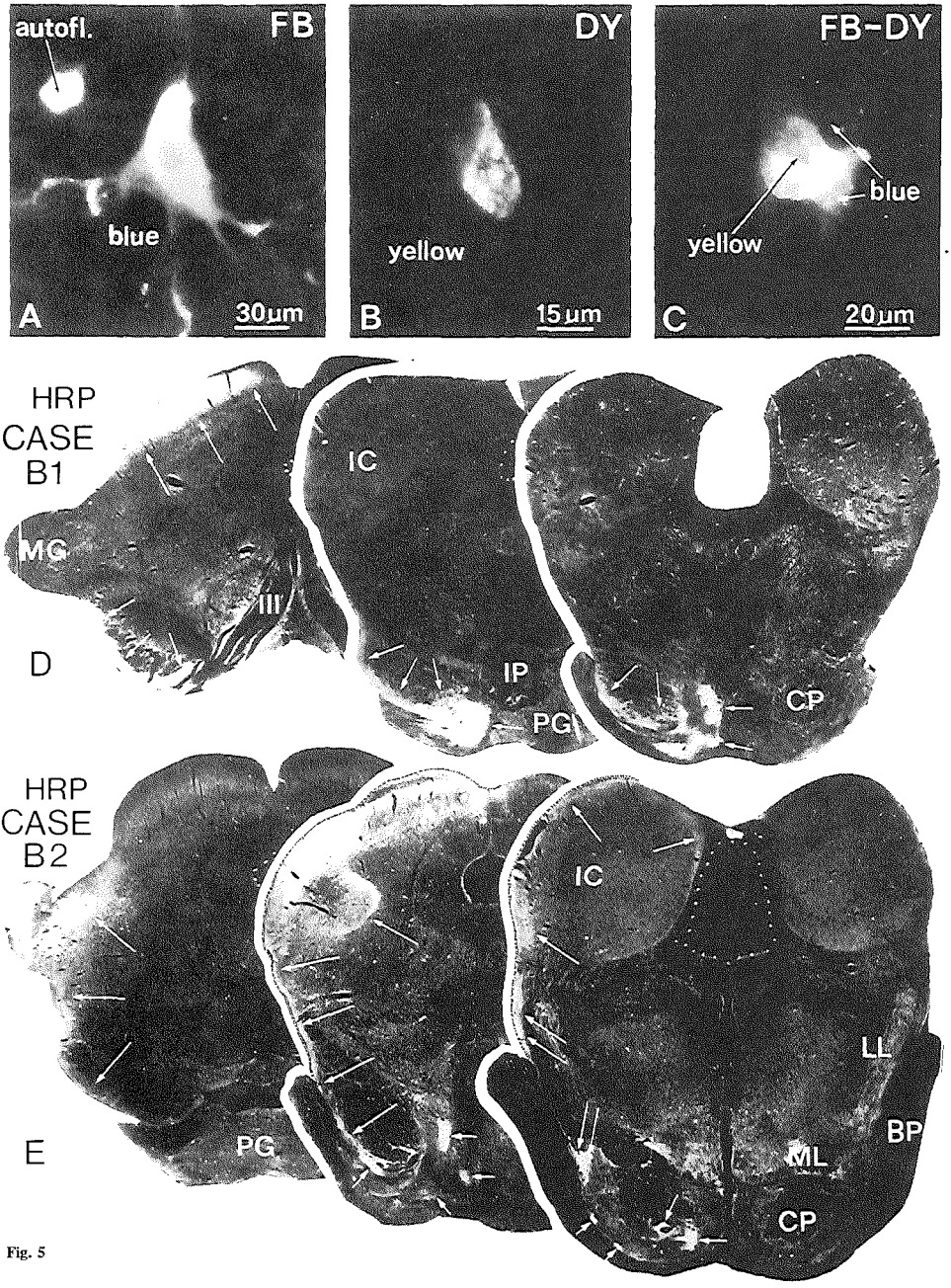
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Fig. 5

Group B. Anterograde HRP fiber labeling

In case B1 the HRP injection area involved the lateral gyrus including its medial aspect as well as the middle and posterior suprasylvian gyri including the adjoining medial bank of the suprasylvian sulcus (Fig. 3). Rostrally, the injection area extended into the caudal part of area 7 and caudally it involved the occipital pole except for its ventrolateral surface (Fig. 3). A distinct bundle of HRP-labeled fibers proceeded from the injected cortical areas into the internal capsule where it split up into two separate fiber groups. One group descended through the cerebral peduncle to the pons, while the other proceeded through the dorsal thalamus to the superior colliculus (Figs. 4B1, 5D and 6). At the mesodiencephalic junction the first group of labeled fibers was located laterally in the cerebral peduncle. More caudally these fibers gradually spread out along the ventral aspect of the peduncle (Figs. 4B1 and 5D). The fibers of the second group were distributed primarily to the superficial layers of the superior colliculus, though a few were distributed to the intermediate layers. No HRP-labeled fibers could be traced from the cerebral peduncle to the superior colliculus (Figs. 4B1, 5D). In the pons HRP-positive granules were present in the paramedian, peripeduncular and ventral nuclei with the largest quantity being present in the rostral half of the pons. A few granules were present in the nucleus reticularis tegmenti pontis (Figs. 4B1, 5D) and no HRP-labeled fibers were observed either in the ponto-medullary tegmentum or the pyramid.

In case B2 the HRP injection area involved the middle and posterior ectosylvian gyri, the sylvian gyrus and the lateral bank of the suprasylvian sulcus. Rostrally it extended into the banks of the most posterior parts of the anterior ectosylvian sulcus and of the sylvian sulcus as well as into the posterior insular region (Fig. 3). HRP-labeled fibers could be traced from the injected cortex through the cerebral peduncle into the pons. In the rostral mesencephalon labeled fibers coursed from the peduncle dorsally along the lateral aspect of the mesencephalon to the superior colliculus, where many HRP-positive granules were present in its intermediate and deep layers (Figs. 4B2, 5E, 6). In the caudal mesencephalon, the dorsally coursing fibers formed a distinct bundle passing to the inferior colliculus, where dense accumulations of HRP-positive granules were present in its pericentral and external layers (Figs. 4B2, 5E, 6). Many granules were also present in the medial geniculate body, together with HRP-labeled neurons. In the pons HRP-positive granules were present mainly in its rostro-caudal middle one third. Dense accumulations were present in the dorsolateral nucleus and moderate amounts were present in the paramedian, lateral and ventral nuclei (Figs. 4B2, 5E). The HRP-labeled fibers which coursed from the cerebral peduncle to the inferior colliculus were also present at the level of the rostral

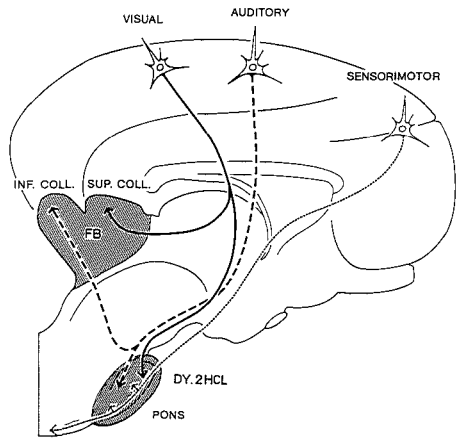

Fig. 6. Diagram showing the trajectory of divergent axon collaterals from branching neurons in visual and auditory cortical areas to the colliculi and the pons, and of pyramidal tract fibers which give off collaterals to the pons. Hatching indicates schematically the fluorescent injection areas in the colliculi and the pons. Note that the auditory fibers to the inferior colliculus (dotted line) pass in close proximity to the pontine injection area, and that the pyramidal fibers descend through the pons

Fig. 5A–C. Photomicrographs of fluorescent retrogradely labeled neurons taken with filter-mirror system A. **A** FB single-labeled corticotectal neuron with a blue fluorescent cytoplasm. Note lipofuscine autofluorescence (yellow-brown) of an unlabeled neighbouring neuron. **B** DY.2HCl single-labeled corticopontine neuron with a yellow fluorescent nucleus. **C** DY.2HCl-FB double-labeled neuron with a blue fluorescent cytoplasm and a yellow fluorescent nucleus. **D, E** Darkfield photomicrographs of anterogradely HRP-labeled fibers and terminals in transverse sections through the upper brain stem. **D** Labeled fibers in the cerebral peduncle and terminal labeling in the superficial layers of the superior colliculus and patches of labeling in the rostral pontine grey, after injections in the visual and parietal cortex. **E** Labeled fibers in the cerebral peduncle and terminal labeling in the colliculi and the pontine grey, after injections in the auditory cortex. Note labeled fibers running from the cerebral peduncle to the inferior colliculus (middle section, four arrows) and right section (upper pair of arrows), and in close proximity the heavily labeled dorsolateral pontine nucleus (right section, lower pair of arrows)

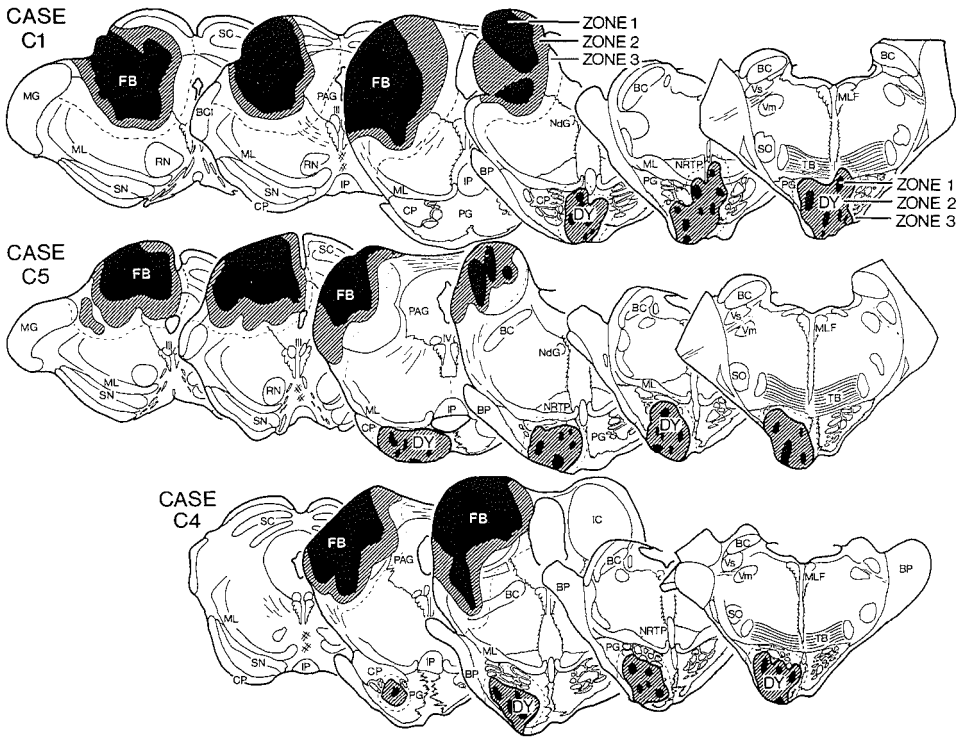

Fig. 7. Fluorescent injection areas in the dorsal mesencephalon and the pons. For description of the three concentric zones see Keizer and Kuypers 1984. Two upper rows: FB injection area in the colliculi and the dorsal mesencephalic reticular formation, and DY.2HCl injection area in the pontine grey. Note that in both cases (C1 and C5) the lateral portions of the pons are spared by the DY.2HCl injection area, and that in case C5 the DY.2HCl injection area extends into the most rostral portion of the pons. Bottom row: FB injection area in the inferior colliculus and the adjoining mesencephalic reticular formation, and DY.2HCl injection area in the pons including its rostral part (case C4)

pons where they passed alongside the dorsolateral pontine nucleus (Figs. 4B2, 6). Therefore the fluorescent tracer injections in the pons in the cases of group C were restricted to its medial and ventral parts, avoiding the area of the dorsolateral nucleus. However, this implied that the corticopontine neurons in the central part of the cortical auditory area, which project to the dorsolateral nucleus (Brodal 1972), would probably not be retrogradely labeled (see group C).

Group C. Double-labeling experiments

The FB injection areas in the mesencephalon of the cases C1–C3 and of case C5 were of roughly the same size as those in the cases of group A (Figs. 2, 7). They were strictly unilateral and extended rostrocaudally from the most caudal parts of the pulvinar to the caudal surface of the inferior colliculus (Fig. 7). They involved the superior and inferior colliculi as well as varying portions of the adjoining pretectal nuclei, the dorsal part of the mesencephalic reticular formation and of the dorsolateral quadrant of the periaqueductal grey (PAG). In case C4 the injection area was restricted to the inferior colliculus, and the adjoining portions of the mesencephalic tegmental field and of the PAG (Fig. 7).

Distribution of labeled corticotectal neurons. The retrogradely FB-labeled cortical neurons, labeled from the tectum (corticotectal neurons), showed a

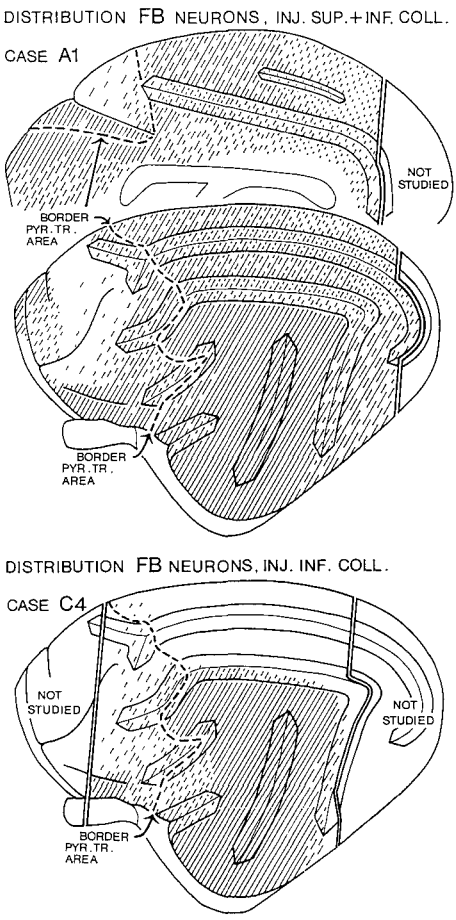

Fig. 8. Diagrammatic representation of the distributions of the FB-labeled corticotectal neurons in case A1, after ipsilateral FB injections in both colliculi and in case C4, after ipsilateral FB injections in the inferior colliculus. Note in case A1 the sparsity of labeled neurons in the dorsolateral part of area 4 and in the primary somatosensory area. For cytoarchitectonic subdivisions see Fig. 1

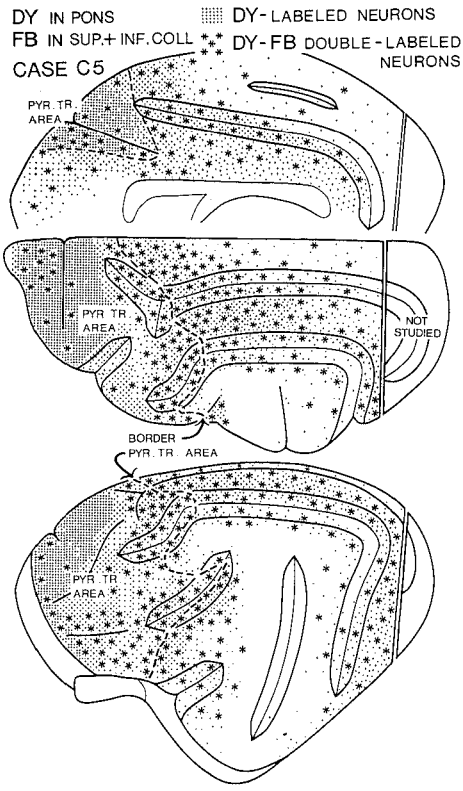

Fig. 9. Diagrammatic representation of the distributions of the DY.2HCl single-labeled and the DY.2HCl-FB double-labeled neurons in case C5. Labeled neurons outside the pyramidal tract area represent single- and double-labeled corticopontine neurons, while within the pyramidal area they represent corticopontine neurons as well as pyramidal tract neurons. For cytoarchitectonic subdivisions see Fig. 1

blue fluorescence of cytoplasm and proximal dendrites, but showed no labeling of the nucleus (Fig. 5A). In all the cases of this group the FB-labeled neurons were situated in cortical layer V. The distributions of the FB-labeled corticotectal neurons including the DY.2HCl-FB double-labeled ones were plotted in detail in the cases A1 (see group A), C1 and C2 with FB injections in both colliculi. They were most numerous in case C1, but their distributions were the same in all three cases (cf. Fig. 8, upper part). The findings in case C1 will be described in detail.

Large numbers of FB-labeled neurons including DY.2HCl-FB double-labeled ones were present in the frontal one third of the hemisphere (pyramidal tract area) i.e. in area 6, in the rostromedial part of area 4, in the ventral bank of the cruciate sulcus, in the caudal part of its dorsal bank, and in the frontal granular cortex (Figs. 1, 8). Only a few scattered FB-

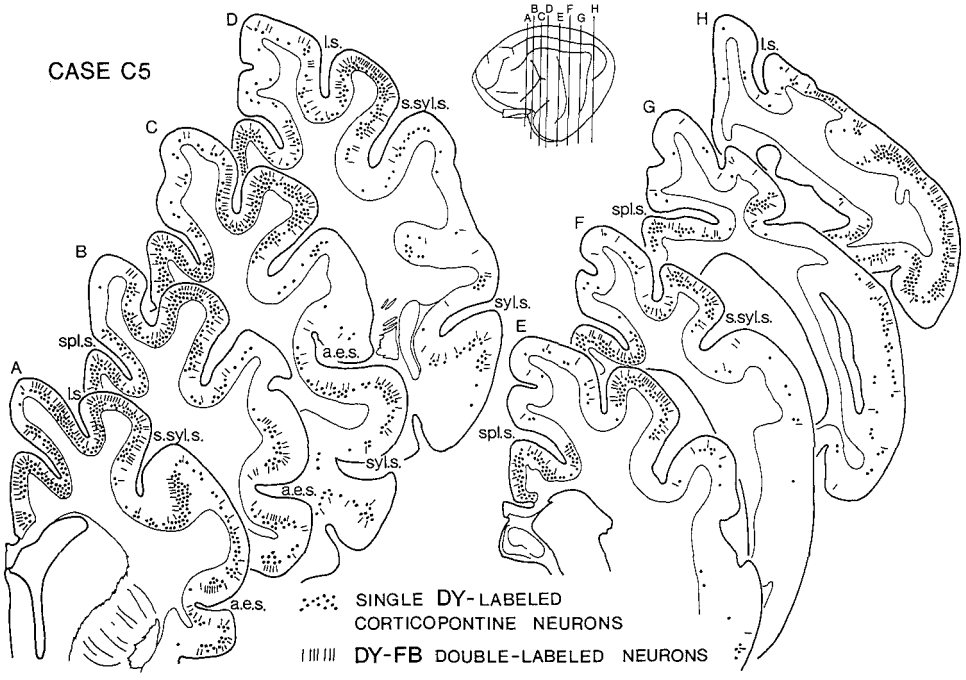

Fig. 10. Diagrammatic representation of coronal sections (**A–H** 30 μm thickness) through the left hemisphere ipsilateral to the brain stem injections in case C5, showing the DY.2HCl single-labeled (dots) and the DY.2HCl-FB double-labeled corticopontine neurons (rods). Each symbol represents one labeled neuron. All labeled neurons were present in cortical layer V. Because of lack of space the total cortical depth has been filled with symbols. Note abundance of labeled neurons in the suprasylvian and cingulate gyri. For cytoarchitectonic subdivisions see Fig. 1

labeled neurons were present in the posterior and lateral sigmoid gyri and the banks of the coronal sulcus, i.e. in the caudal and caudolateral part of area 4 and the primary somatosensory cortex (Fig. 8). In the caudal two thirds of the hemisphere (i.e. caudal to the pyramidal tract area) the FB-labeled neurons were distributed evenly throughout the cortex (Fig. 8). Many, densely packed, FB-labeled neurons were present in the orbital gyrus (including area 43) and in the auditory regions. Moderate densities were found in areas 18 and 19, areas 5 and 7, areas 20 and 21 and in the frontal half of the cingulate gyrus. Low densities were found in the caudal half of the cingulate gyrus and in SII. The density of labeled corticotectal neurons was lower in area 17 than in areas 18 and 19 (Fig. 8). In case C4 with FB injections in the inferior colliculus only, the FB-labeled neurons, including the DY.2HCl-FB double-labeled ones, were largely restricted to the auditory cortex (Fig. 8, lower part). Large numbers were present in areas A, AI, AII and Ep and on the surface of the orbital gyrus (area 43) (Figs. 1, 8). In addition labeled neurons were present in the insular cortex, in SII and in area 5.

The DY.2HCl injection areas in the pons were different in size but were always restricted to the pontine grey and always involved the pyramidal bundles. In cases C1–C3 the injection areas were restricted to the caudal two thirds of the pons (Fig. 7C1). In case C1, the injection area involved mainly the median, paramedian and ventral pontine nuclei and the nucleus reticularis pontis, the latter however mainly contralaterally (Fig. 7C1). In cases C2 and C3 they involved the entire caudal one third of the pontine grey and the paramedian, ventral and lateral nuclei of its middle one third. However, in all three cases they spared the dorsolateral nucleus. In

Table 2. Labeled corticopontine neurons in cases of Group C

Case	Tracer	Site	Tracer	Site	Number of corticopontine neurons counted	% double-labeled (DL)
C1	FB	sup. + inf. colliculi	DY	caudal 1/2 pons	4508 DY	25.3% DL
C3	FB	sup. + inf. colliculi	DY	caudal 2/3 pons	3447 DY	25.6% DL
C4	FB	inferior colliculus	DY	rostro-caudal pons	4336 DY	4.6% DL
C5	FB	inf. + sup. colliculi	DY	rostro-caudal pons	6913 DY	22.0% DL

case C4, but especially in case C5 the injection areas extended further rostrally than in the other cases and involved also the paramedian, peripeduncular and ventral nuclei in the rostral one third of the pontine grey, sparing however its lateral parts (Figs. 7C5, C4).

Distribution of labeled corticopontine neurons. The retrogradely DY.2HCl single-labeled neurons, labeled from the injections in the pons (corticopontine neurons), showed a yellow fluorescent nucleus, while the DY.2HCl-FB double-labeled ones also showed a blue fluorescent cytoplasm (Figs. 5B and C). Large numbers of single- and double-labeled corticopontine neurons were present in all five cases (C1–C5; Figs. 9 and 10). In four cases (C1, C3–C5) the labeled corticopontine cells were counted in coronal sections taken from the area between the ansate sulcus and the occipital pole of the hemisphere (see Table 2).

The widest distribution of the DY.2HCl-labeled cortical neurons was observed in cases C4 and C5 with large pontine injections involving the entire rostro-caudal extent of the basal pons (Fig. 7). Case C5 will be dealt with in detail but only the distributions outside the pyramidal tract area (see group A) will be described. The largest number of DY.2HCl-labeled corticopontine neurons including the DY.2HCl-FB double-labeled ones were present in the parietal areas (caudal part of area 5 and area 7), and in areas 20 and 21. These regions together contained nearly one half of the total number of labeled corticopontine neurons, counted outside the pyramidal tract area (see Table 3). The cingulate gyrus contained one fifth of the total population of counted neurons. In areas 18 and 19 the labeled neurons were relatively less densely packed but a relatively higher percentage was double-labeled (see Table 3). DY.2HCl-labeled corticopontine neurons including double-labeled ones were also present in the periphery of the auditory cortex, i.e. in the rostral part of area AII (caudal to the line connecting the posterior tips of the anterior ectosylvian and the sylvian sulci), in the banks of the sylvian sulcus and in the suprasylvian fringe area. However, only a few scattered ones were present in area Ep, and none were present in the auditory areas A and AI (Figs. 9, 10, see Table 3).

Table 3. Areal distributions of labeled corticopontine neurons Case C5 (see text)

Cortical area	Labeled neurons counted	% Double-labeled (DL)
Front. Gran.	65 DY	20% DL
cingulate	1290 DY	14% DL
caudal 5, 7, 20, 21	3357 DY	22% DL
17	435 DY	12% DL
18, 19	307 DY	40% DL
Clare-Bisshop	220 DY	24% DL
SII	569 DY	22% DL
lower bank A.E.S.	180 DY	29% DL
Insular	207 DY	28% DL
auditory (periphery)	345 DY	24% DL

The FB-labeled corticotectal neurons were counted in only a limited number of sections. Therefore no solid data can be provided regarding the percentages of the corticotectal neurons that were double-labeled. However, the impression was gained that the percentages in the various cortical areas ranged from 5% to 30%.

In case C4 in which the FB injections were restricted to the inferior colliculus, 143 DY.2HCl-labeled corticopontine neurons were counted in the auditory-regions and 22% of them were double-labeled (Fig. 11). In the lower bank of the anterior ectosylvian sulcus 171 labeled neurons were counted of which 12% were double-labeled (Fig. 11). The lack of labeled corticopontine neurons in the central

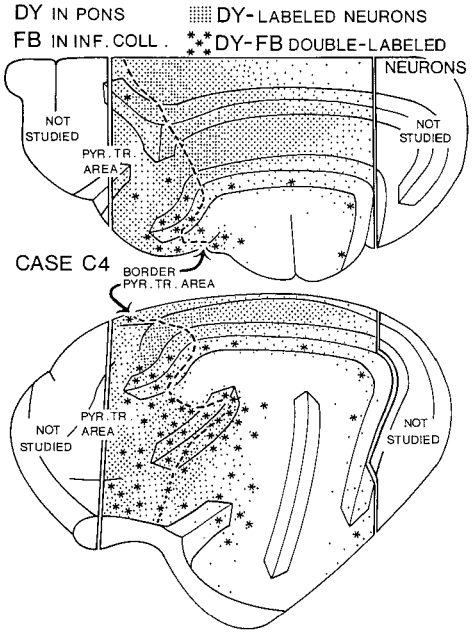

Fig. 11. Diagrammatic representation of the distribution of DY.2HCl single-labeled and DY.2HCl-FB double-labeled neurons after DY.2HCl injections in the pons and FB injections in the inferior colliculus (case C4, see Fig. 7). Note presence of double-labeled corticopontine neurons in the periphery of the auditory regions and the lack of labeled neurons in its centre. For cytoarchitectonic subdivisions see Fig. 1

part of the auditory cortex confirms the expectations based on the fact that the DY.2HCl injections spared the dorsolateral part of the rostral pons, which receives the bulk of the corticopontine projections from the auditory areas (Brodal 1972).

Discussion

In the cases of Group C with FB injections in the colliculi and DY.2HCl injections in the ipsilateral pontine grey many FB-labeled neurons and many DY.2HCl-labeled neurons were present in the neocortex of the ipsilateral hemisphere. Moreover, many of the DY.2HCl-labeled neurons were DY.2HCl-FB double-labeled. This suggested that many corticopontine neurons represented branching neurons which also distributed collaterals to the mesencephalon, especially the colliculi. In the cases of Group A with DY.2HCl injections in the pyramidal tract and the medullary tegmentum immediately caudal to the pons, the DY.2HCl-labeled pyramidal tract neurons were restricted to the rostral one third of the hemisphere where they were restricted to the pyramidal tract area as outlined by earlier retrograde neuronal labeling studies (cf. Biedenbach and DeVito 1980; Keizer and Kuypers 1984). This implied that in the cases of Group C only the DY.2HCl-labeled neurons outside this pyramidal tract area, i.e. mainly in the caudal two thirds of the hemisphere, could with confidence be regarded to represent corticopontine neurons. On the basis of the findings in Group B the double labeling in Group C could not have resulted from the fact that the two tracer injections involved the same corticotectal fibers at different places along their trajectory. Therefore the double labeling of the DY.2HCl-labeled corticopontine neurons in the caudal two thirds of the hemisphere in the cases of Group C may be interpreted to indicate that many of these corticopontine neurons represented branching neurons which distributed collaterals to the colliculi. This is in keeping with electrophysiological findings (Baker et al. 1983).

The labeled corticopontine neurons in the caudal two thirds of the hemisphere were most densely packed in the anterior part of the lateral gyrus, and in the middle and posterior suprasylvian gyri (caudal part of area 5, area 7 and areas 20 and 21). This region contained one half of all labeled corticopontine neurons of which roughly 25% were double-labeled. In the cingulate gyrus one fifth of all labeled corticopontine neurons were present and 14% was double-labeled. The parietal and cingulate areas together therefore contained the bulk of the labeled corticopontine neurons outside the pyramidal tract region. In areas 18 and 19 the labeled corticopontine neurons were less densely packed than in the parietal regions and represented less than one tenth of the total population. However, the percentages of the double-labeled neurons in these visually related areas were considerably higher than in the parietal areas and ranged from 30% to 60%. These relatively high percentages are in keeping with electrophysiological findings (Baker et al. 1983) but the reason for their occurrence remains obscure.

In case C4 in which the FB injections involved only the inferior colliculus the FB-labeled neurons in the caudal two thirds of the hemisphere were restricted to the auditory cortex and the immediately adjoining areas (Figs. 1, 8). However, the DY.2HCl-FB double-labeled corticopontine neurons as the DY.2HCl single-labeled ones were only present in the periphery of the FB-labeled cortical area, i.e. in

the suprasylvian fringe area and in the area caudal to the connecting line between the posterior tips of the anterior ectosylvian and the sylvian sulci. These two areas are commonly regarded to subserve auditory functions (Woolsey 1961). However, in these areas the percentages of the DY.2HCl-labeled corticopontine neurons which were double-labeled were lower than in the cases with FB injections in both colliculi. This strongly suggests that the peripheral parts of the auditory areas also contain corticopontine neurons which distribute collaterals to the superior colliculus. This is supported by earlier findings (cf. Kawamura et al. 1978; Stein et al. 1983) and is in keeping with the distribution of the anterogradely labeled fibers to the superior colliculus in case B2.

Some of the DY.2HCl-labeled corticopontine neurons in the cases of Group C may have been DY.2HCl-FB double-labeled from the pretectal nuclei, the pulvinar, the mesencephalic reticular formation and the PAG rather than from the colliculi, since the FB injections involved also these structures. The pulvinar receives mainly fibers from cortical cells situated in layer VI of the neocortex. In none of the cases labeled cells were present in layer VI. Therefore, labeling of the pulvinar could not have contributed considerably to the results. The PAG in monkey and rat receive cortical connections from the frontal granular cortex (Kuypers and Lawrence 1967; Leichnetz et al. 1981) and the cingulate gyrus (Wyss and Sripanidkulchai 1984). Some of the double-labeled neurons in these cortical areas may therefore represented branching corticopontine neurons which distributed collaterals to the PAG.

The double-labeled neurons in area 6 and the rostro-medial part of area 4 of Group A, which were also present in group C (see Figs. 2, 9), were located in the same areas as some of the branching corticospinal neurons which distribute collaterals to the colliculi and the mesencephalic reticular formation (Catsman-Berrevoets and Kuypers 1981), and in the same area as some of the corticospinal neurons which distribute collaterals to the medulla oblongata (Keizer and Kuypers 1984). Because of their identical location, some of these double-labeled neurons in the various experiments may therefore have been identical. If this is true it would imply that the rostral part of the motor cortex contains highly branching neurons which project to the spinal cord and in addition distribute collaterals to the colliculi and the reticular formation at different brain stem levels.

The widespread distribution of corticotectal neurons throughout the cerebral cortex in Groups A and C confirmed earlier observations (Kuypers and Lawrence 1967; Garey et al. 1968). However, the present findings showed that these corticotectal neurons are not concentrated in the banks of sulci as reported (Holländer 1974), but are evenly distributed throughout the various cortical regions. In the present cases as well as in earlier HRP studies (Kawamura and Konno 1979; Stein et al. 1983) only very few labeled corticotectal neurons were present in the caudal and lateral parts of area 4 and in the primary somatosensory area while they were quite numerous in the rostral part of the motor cortex. This would be in keeping with the concept that the superior colliculus and the rostromedial part of the motor cortex differ from the caudal part of the motor cortex such that the former probably steer mainly eye and head movements as well as general orientating movements of the body while the latter plays a crucial role in steering highly fractionated distal extremity movements (Kuypers 1981).

The central issue of the present study is the double-labeling of corticopontine neurons after tracer injections in the pontine grey and the colliculi (Group C). This choice was later supported by the fact that not all branching corticopontine neurons of the present study may have distributed collaterals to the colliculi but some may have projected to the dorsal mesencephalic reticular formation and others to the PAG. None the less the vast majority of the double-labeled corticopontine neurons must also have represented corticotectal neurons. In earlier single tracer and electrophysiological studies these branching neurons were not distinguished from the pure corticopontine neurons or the pure corticotectal neurons. In order to clarify the physiological importance of the branching it would be of interest to explore in what sense these branching neurons differ from the other cortical neurons which project either only to the colliculi or only to the pontine grey. Related findings were obtained in regard to the corticopontine connections from the pyramidal tract area in the rostral one third of the hemisphere. Thus in cat and lower animals very many of the pyramidal tract neurons, including the corticospinal ones are branching neurons which distribute collaterals to the pontine grey (Cajal 1952; Ugolini and Kuypers 1986). These data combined with those of the present studies suggest the existence of a general organisational principle to the effect that in many species at least a proportion of the connections from the cortical mantle to the pontine grey are established by branching neurons which also distribute collaterals to either the brain stem or the spinal cord or both. It therefore may be assumed that these neurons when transmitting instructions to these subcortical and spinal cell groups transmit simultaneously a copy of these instructions via the pons to the cerebellum. In respect to the copy transmitted from the motor

cortex it seemed reasonable to assume that in the cerebellum this copy, after comparison with information arising from the periphery could be used to adjust the motor command.

Abbreviations

A.E.	anterior ectosylvian sulcus
a.e.s.	anterior ectosylvian sulcus
BC	brachium conjunctivum
BCI	brachium colliculus inferior
BP	brachium pontis
cor. sulc.	coronal sulcus
CP	cerebral peduncle
CR.	cruciate sulcus
CUN	cuneiform nucleus
DBC	decussation brachium conjunctivum
DLP	dorsolateral pontine nucleus
IC	inferior colliculus
inf. coll.	inferior colliculus
INS.	insula cortex
IO	inferior olive
IP	interpeduncular nucleus
LAT.	lateral sulcus
l.s.	lateral sulcus
MG	medial geniculate body
LL	lateral lemniscus
ML	medial lemniscus
MLF	medial longitudinal fascicle
NdG	dorsal nucleus of Gudden
NLL	nucleus lateral lemniscus
NRTP	reticular tegmental pontine nucleus
ORB.	orbital sulcus
P	pyramid
PAG	periaqueductal grey
P.E.	posterior ectosylvian sulcus
RF	reticular formation
PG	pontine grey
RB	restiform body
RN	red nucleus
S.	sylvian sulcus
SC	superior colliculus
SN	substantia nigra
SO	superior olive
SPV	spinal trigeminal complex
S.S.	suprasylvian sulcus
s.syl.s.	suprasylvian sulcus
S.SPL.	suprasplenial sulcus
SPL.	splenial sulcus
spl.s.	splenial sulcus
sup. coll.	superior colliculus
syl.s.	sylvian sulcus
TB	trapezoid body
VC	vestibular complex
Vm	trigeminal motor nucleus
Vs	trigeminal principle nucleus
III	oculomotor nucleus
IV	trochlear nucleus
VI	abducens nucleus
VII	facial nerve
VIII	vestibulo-trochlear nerve

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Chapter VI

Large Layer VI Cells in Macaque Striate Cortex (Meynert Cells) Project to Both Superior Colliculus and Prestriate Visual Area V5

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Summary. Layer VI of macaque striate cortex contains a number of large solitary neurones called Meynert cells. It has been shown earlier that these Meynert cells project to the posterior bank of the superior temporal sulcus (area V5), but it has also been shown that they project to the superior colliculus. In retrograde fluorescent double-labelling experiments, it was found that Meynert cells represent a class of neurones which distribute divergent axon collaterals to the posterior bank of the superior temporal sulcus and to the superior colliculus, i.e. to a distant cortical and a subcortical structure. This feature appears to be unique among projecting neurones in monkey visual cortex.

Key words: Meynert cells – Monkey visual cortex – Superior colliculus – Axon collateralization – Double labelling

Introduction

The solitary cells of Meynert (Meynert 1867) form a class of conspicuously large neurones in primate striate cortex that are located, in the macaque, in layer VI (Le Gros Clark 1942; Lund 1973; but see Chan-Palay et al. 1974). They are distributed evenly at a relatively low density (about 25 per square mm), the mean distance to the next neighbour being about 0.13 mm (see Winfield et al. 1981). In retrograde tracer studies, they have been found to send their axons to the posterior bank of the superior temporal sulcus (Lund et al. 1975; Maunsell and Van Essen 1983; Fries and Zeki 1983) which contains a visual area concerned with the analysis of visual movement that was originally termed the "motion area of the STS" and later V5 (Zeki 1974; Zeki 1978). In New World monkeys, Meynert cells have been found to project to cortical area MT (Spatz 1975). Similarities in function, connections and location have led to the assumption that area MT in New World monkey is homologous to V5 so that the term MT has been used also in the macaque (see Van Essen et al. 1981). Further, Meynert cells have been found in New World monkeys to project to the pulvinar (Spatz et al. 1981).

The unexpected finding that Meynert cells also project to the superior colliculus (Fries and Distel 1983; Fries 1984) suggested the possibility that they might send axon branches to both superior colliculus and prestriate visual area V5. This notion was supported by the fact that, after injections of horseradish peroxidase in either structure, locally every single Meynert cell was found to be labelled. We have attempted to answer this question by means of the retrograde fluorescent double-labelling technique (see Kuypers et al. 1980), using the fluorescent substances Fast Blue (FB; Bentivoglio et al. 1980) and Diamidino Yellow·dihydrochloride (DY·2HCl; Keizer et al. 1983).

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Material and methods

In two monkeys (macaca fascicularis) Fast Blue (FB) injections were made in the superior colliculus and Diamidino Yellow (DY·2HCl) injections were made in the posterior bank of the superior temporal sulcus, under barbiturate anaesthesia. First, the splenium of the corpus callosum was cut in the midline and 7% FB dissolved in distilled water was injected unilaterally into the superior colliculus by means of a glass pipette (14–19 penetrations, 5.0–7.6 µl in total). Subsequently, the anterior bank of the superior temperal sulcus ipsilateral to the collicular injection was removed by subpial aspiration and 2% DY·2HCl dissolved in a 0.2 M phosphate buffer (pH 7.2) was injected into the medial two thirds of its posterior bank by means of a large number of pipette penetrations (96–114 penetrations, 19.2–22.8 µl in total). After 14

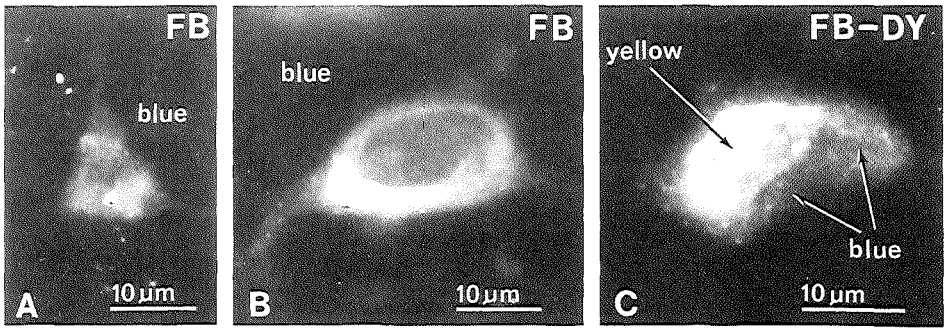

Fig. 1A–C. Photomicrographs of single FB- and FB-DY·2HCl double-labelled neurones in striate cortex, taken with filter-mirror system U (360 nm excitation wavelength). **A** Small FB-labelled corticotectal neurone in layer V. Note the pyramidal shape and lightly labelled apical dendrite. **B** Large single FB-labelled Meynert cell in layer VI. Note the ovoid shape of its cell body and labelling of basal dendrites. **C** Large FB-DY·2HCl double-labelled Meynert cell in layer VI with a blue fluorescent cytoplasm and a yellow fluorescent nucleus. Note the eccentric position of the nucleus

to 19 days, the monkeys were sacrificed with an overdose of barbiturate and transcardially perfused with 0.9% salaine (11), followed by citrate buffered (pH 7.2) 20% Formaline (31) at room temperature, which was followed by citrate buffered (pH 7.2) 10% sucrose (21) at 4° C. The brains were immediately dissected and the hemispheres ipsilateral to the injections were photographed. The hemispheres were cut horizontally in 30 µm sections on a freezing microtome and the upper brain stems were cut transversally. Every fourth section was mounted and air dried but not coverslipped. The material was studied with an Olympus BH–2 fluorescence microscope, equipped with filter-mirror system U providing excitation light of 360 nm wavelength. In every fourth mounted section of the hemisphere, the distributions of the retrogradely single FB-labelled neurones (blue fluorescent cytoplasm), the single DY·2HCl-labelled neurones (yellow fluorescent nucleus) and the double-labelled neurones (both features) were charted with the aid of a X–Y plotter.

Results

The FB injections in the superior colliculus included all of its layers and extended into dorsal parts of the tegmentum and the central grey. Anteriorly, some label spread into pretectal nuclei and, marginally, into the posterior pulvinar complex (Fig. 2D). In striate and prestriate visual cortical areas, large numbers of FB-labelled pyramidal neurones were present in layer V, forming a dense row (Fig. 1A and Fig. 2A and B). In addition, large FB-labelled neurones were found in striate cortex below the layer of the FB-labelled corticotectal cells, i.e. in layer VI. These neurones often showed an ovoid cell-body with eccentric positioned nucleus and large far reaching basal dendrites but only a small apical dendrite. By their laminar position, their size of 25–35 µm and their particular morphology (see Lund 1973; Stürmer et al. 1981; Fries and Distel 1983; Winfield et al. 1983), they were identified as Meynert cells (Fig. 1B and Fig. 2C).

The DY·2HCl injections were confined to the cortex of the posterior bank of the superior temporal sulcus with only minor diffusion into underlying white matter (Fig. 2A and B). DY·2HCl-labelled cells were scattered in striate cortex in layer IVB which is known to contain cells projecting to the posterior bank of the superior temporal sulcus (Lund et al. 1975; Maunsell and Van Essen 1983; Fries and Zeki 1983). Occasionally, single DY·2HCl-labelled Meynert cells were found.

The most striking feature of the present findings was that a large proportion (40–50%) of the FB-labelled Meynert cells appeared to be double labelled with FB *and* DY·2HCl (Fig. 1C and Fig. 2C). This finding indicates that Meynert cells represent branching neurones which distribute axon collaterals to the superior colliculus and to the posterior bank of the superior temporal sulcus. However, none of the scattered DY·2HCl-labelled cells in layer IVB (originally also called "Meynert cells" (Meynert 1867)) was double-labelled, and also none of the layer V corticotectal cells (Fig. 1A). Further, no double-labelled cells were found in prestriate visual cortex. The FB-labelled corticotectal cells in these areas were located strictly in layer V throughout (Fig. 2A and B), whereas DY·2HCl labelled cells occurred only in upper layers in V2, V3 and V4 and in upper and lower (i.e. layer IV) layers in V3A (see Maunsell and Van Essen 1983; Fries and Zeki 1983). Hence, double-labelling was confined solely to Meynert cells and did not occur in any other cells, thus rendering a technical artifact highly unlikely.

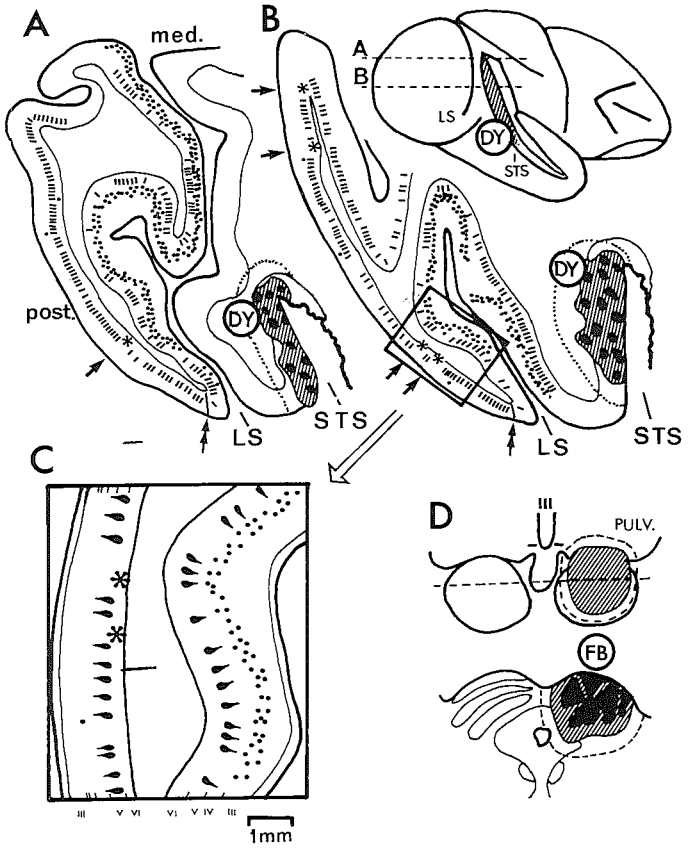

Fig. 2A–D. Distribution of retrogradely labelled cells after DY·2HCl injections in the posterior bank of the superior temporal sulcus and FB injections in the ipsilateral superior colliculus. **A and B** Drawings of posterior parts of horizontal sections taken at levels indicated on the sideview of the hemisphere. The DY·2HCl injection site is shaded; the dotted line marks the zone of diffusion. In striate and prestriate visual cortex, rods indicate FB single-labelled corticotectal neurones, dots indicate DY·2HCl single-labelled corticocortical neurones and asterisks indicate FB-DY·2HCl double-labelled neurones. The boundary between areas 17 and 18 is indicated by the double arrow. **C** shows the laminar distribution of labelled cells in striate (left cortical strip) and prestriate (right cortical strip) cortex in higher magnification. **D** Dorsal view (above) and cross-section across the ipsilateral collicular FB injection site (below). LS: Lunate sulcus; STS: superior temporal sulcus

Discussion

In previous HRP studies it was found that Meynert cells could be labelled retrogradely from the superior colliculus and the posterior bank of the superior temporal sulcus (Lund et al. 1975; Maunsell and Van Essen 1983; Fries and Distel 1983; Fries 1984). In light of these observations, the present findings indicate unequivocally that Meynert cells have branching axons projecting to both these structures. Technical artifacts inherent of the method of double-labelling with fluorescent tracers (for detailed discussion see Keizer at al. 1983) can be safely excluded. Both injection sites were quite distant from striate cortex and only the large cells of Meynert in layer VI were double-labelled. This double-labelling cannot be due to label uptake of corticotectal fibers in white matter damaged in the course of the cortical DY·2HCl injection, since none of the layer V corticotectal cells were double-labelled.

Thus, Meynert cells are distinct from other projection neurones in striate cortex. Neither are they located in the layers of origin of corticocortical projections (i.e. layers II and III) nor in the layer of corticotectal projections (i.e. layer V; see Lund et al. 1975). Further, the pattern of divergent projections by branching axons to a cortical and subcortical structure has, to our knowledge, not been described for any other cortical projection neuron in monkey. In cat, some corticotectal cells in the Clare-Bishop area have been found to send axon collaterals through the corpus callosum to homotopical regions in the contralateral hemisphere (Weber et al. 1983).

The particularity of the pattern of axon collateralization in Meynert cells is emphasized by the

fact that other corticotectal cells in monkey seem to have few cortical axon collaterals either to the contralateral hemisphere or locally (Keizer and Fries, in preparation). Although large layer V pyramids have been shown to send recurrent axon collaterals back to the cortex (see Cajal 1964; Lund 1973; Gilbert and Wiesel 1983), our own results using fluorescent double-labelling techniques revealed a surprisingly low number of corticotectal cells in layer V that were double-labelled after collicular FB injections and small local injections of DY·2HCl into striate cortex.

Apart from the interesting question, whether this pattern of divergent axon collaterals is genetically determined or whether the collaterals are simply not eliminated in early postnatal development (see Innocenti 1981), Meynert cells deserve interest with respect to the functional role of this double projection. Both target structures, i.e. the superior colliculus and prestriate visual area V5 are related to movement in the field of view. However, whereas collicular cells possess no directional specificity, V5 cells are highly direction specific (see Schiller and Stryker 1972; Zeki 1974). Meynert cells have far reaching basal dendrites but only thin and short apical dendrites (Lund 1973; Winfield et al. 1983; Fries 1983). They may therefore sample information mainly from layers V and VI where they could integrate information from large areas of visual space. Hence, they might be specialized elements to detect visual movement. The question of why they distribute their information to such different structures deserves further study.

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Denken ist inneres Handeln.

Immanuel Kant,
1724-1804

Chapter VII

General Discussion

VII.1 Fluorescent neuronal tracers

At the turn of the century the existence of axon collaterals was demonstrated by Ramon Y Cajal using the Golgi silver impregnation technique (Cajal 1911, see Cajal 1952). In his drawings of Golgi material of the brain stem he showed, for example, that pyramidal tract fibers emit axon collaterals projecting to the tegmentum of the lower brain stem. However, in many instances he could not determine the precise cortical origin and the target areas of these divergent axoncollaterals.

The retrograde HRP technique, developed in the seventies, (Kristensson and Olsson 1971, Lavail and Lavail 1972) demonstrated that the cells of origin of the cortical pathways descending to the brain stem and the spinal cord were situated exclusively in cortical lamina V, and that their areal distributions overlapped considerably (Armand et al.1974, Berrevoets and Kuypers 1975, Groos et al. 1977, Weisberg and Rustioni 1976, 1977, 1979, Gilbert 1975, Catsman-Berrevoets et al. 1979). These findings, the drawings of Cajal and the electrophysiological findings (Tsukahara et al. 1968, McComas and Wilson 1968, Endo et al. 1973, Atkinson et al. 1974, Allen et al. 1975) demonstrated that the pyramidal fibers emit collaterals to the red nucleus, to the pons and to the dorsal column nuclei. This brought about the need for the development of a "quantitative" anatomical technique demonstrating divergent axon collaterals.

In the seventies three anatomical methods became available, employing two different retrograde tracers, which after being retrogradely transported through each collateral could be demonstrated independently in the same neuronal cell body. These methods are:

A. The HRP-tritiated apo-HRP technique, developed by Hayes and Rustioni (1979, 1981), which makes use of the retrograde axonal transport of HRP and tritiated, enzymatically inactive apo-HRP (Kristensson and Olsson 1971, Lavail and Lavail 1972).

B. The HRP-Iron dextran technique (Cesaro et al. 1979, Olsson and Kristensson (1978), in which the two tracers are demonstrated histochemically by processing the section for HRP and subsequently demonstrating the presence of ferric iron by means of the Perl's reaction.

C. The fluorescent double-labeling technique, developed by Kuypers and co-workers (Kuypers et al. 1977, Vanderkooy et al. 1978, Vanderkooy and Kuypers 1979, Bentivoglio et al. 1979a, b, Bentivoglio et al. 1980a, b, Catsman-Berrevoets et al. 1980, for review see Kuypers et al. 1980), which makes use of several fluorescent substances which after being transported retrogradely through each axon collateral and can be demonstrated independently in the neuronal cell body by means of fluorescence microscopy (Kuypers et al. 1977, 1980).

In 1981 when the present study was commenced, the retrograde fluorescent double-labeling technique made use of the combination of Nuclear Yellow (NY,

Bentivoglio et al. 1980a, b) and True Blue (TB) in rat (Bentivoglio et al 1979a, b, Kuypers et al. 1980, Huisman et al 1981), and of NY in combination with Fast Blue (FB) in cat and monkey (Bentivoglio et al. 1980a, Kuypers et al. 1980, Catsman-Berrevoets and Kuypers 1981, Bharos et al. 1981, Huisman et al. 1982). NY produces a yellow fluorescent labeling of the nucleus at 360 nm excitation wavelength, while TB and FB produce a blue fluorescence of the cytoplasm at this same wavelength. After long survival times, however, NY migrates out of the retrogradely labeled neurons into neighbouring neurons, producing false neuronal labeling (Kuzuhara et al. 1980, Bentivoglio et al. 1980b). This could be prevented by restricting the NY survival time (Bentivoglio et al. 1980b, Catsman-Berrevoets et al. 1980). This complicates double-labeling experiments since TB and FB must be injected first and NY later, i.e. a short period before the animal is sacrificed (Kuypers et al. 1980). The NY survival period has to be choosen carefully in order to prevent false neuronal labeling. In some experiments, this may also cause an organizational problem as exemplified by the experiments described in chapter III, in which the NY survival period was 35.5 hours. This implied that either the injections in the brain or the sacrifice of the animal had to be made at night. This problem could be circumvented when NY was replaced by a retrograde fluorescent tracer which like NY would produce a yellow fluorescence of the nucleus, but would require survival times comparable to those of TB and FB, without leaking out of the retrogradely labeled neurons. Diamidino Yellow Dihydrochloride (DY.2HCl), which was found after testing a large number of chemical substances provided by Dr. O. Dann and which appeared to meet these requirements (Chapter II).

The features of DY as a retrograde fluorescent neuronal tracer were obtained in a number of experiments in several neuronal systems in the brain of rat and cat. It may be concluded that: a. DY-labeled neurons show a yellow fluorescent nucleus and some dull yellow fluorescence of the cytoplasm of the cell body and dendrites at 360 nm excitation wavelength, which resembles NY-labeled neurons, but DY migrates much more slowly out of the retrogradely labeled neurons than NY (Chapter II, group A).

b. DY is transported retrogradely over long distances in rat as well as in cat. Moreover, it was demonstrated that DY is effectively taken up and transported from broken axons. Since after DY injections in the cervical cord, labeled neurons were present in the dorsomedial and ventrolateral parts of the red nucleus, which project to the cervical and lumbar segments respectively (Gwyn 1971, Flumerfelt and Gwyn 1974). Effective retrograde DY labeling requires a survival time comparable to that of TB and FB (Chapter II, Group B).

c. DY is also transported anterogradely through axons, which results in blue fluorescence of glial nuclei along these axons and in their termination area. However, this anterograde labeling of glial nuclei has a much longer time course than required for sufficient retrograde neuronal labeling (Chapter II, group C).

d. In rat sensorimotor cortex the callosal and the corticospinal neurons form two separate populations (Catsman-Berrevoets et al. 1980). No false DY retrograde labeling occurs from DY-labeled callosal neurons to adjoining TB-labeled corticospinal neurons, even after long survival times (Chapter II, group D).

e. On the basis of these findings it was expected that DY could be combined with TB and FB in retrograde double-labeling experiments and that DY could be injected at the same time as TB and FB. In a number of different neuronal systems in the brain of rat and cat, it was confirmed that the double labeling of neurons by way of axoncollaterals with DY in combination with TB and FB was as effective as that obtained with NY in combination with TB and FB (Chapter II, group E).

DY, TB and FB have the tendency to produce a much more pronounced necrosis at the injection site than NY. However, with respect to DY this necrosis does not seem to interfere with the uptake and retrograde axonal transport of DY, since it produces effective labeling of neurons after being injected in a fiber termination area or in fiber bundles (Chapter II, group B).

After injecting 0.4 μl DY, the injection area contained in its center a mass of brown-yellow fluorescent material consisting of DY and necrotic tissue, surrounded by a zone displaying blue tissue fluorescence and containing a dense accumulation of fluorescent glial and neuronal nuclei. This area measured about 1 mm in diameter. This is considerably smaller than the injection areas resulting from the same amount of NY and FB (2 mm). In a methodological study, the effective uptake zone of the DY injection area seemed to be even smaller, measuring 100 μm in diameter and was restricted to the DY deposit and its immediate surrounding tissue (Conde 1987). This implies, especially in retrograde double-labeling experi-

ments in which the percentage of branching neurons has to be estimated, that a large number of needle penetrations have to be made in order to label as many neurons as possible.

A comparison of the number of labeled neurons obtained with DY and HRP in the cat indicates that DY is at least as sensitive as HRP. The maximum number of DY-labeled corticospinal neurons after spinal DY injections at C2 was estimated of 55,000 and that of HRP-labeled corticospinal neurons of 60,000. In contrast, comparing the efficacies of FB and HRP, the findings indicate that FB is half as sensitive as HRP, since the maximum number of FB-labeled corticospinal neurons after injections at C2 was estimated of 38,000. However, after FB injections in the fiber termination areas, i.e. in the spinal cervical grey at C5-C6, instead of in the dorsolateral funiculus, in which the bulk of the corticospinal fibers descend (Armand and Kuypers 1982, Armand et al. 1985), approximately the same number of FB-labeled neurons was present in the forelimb representation of the motor cortex in cat as obtained with HRP (Chapter III, groups A and B). After FB injections in the dorsolateral funiculus (i.e. hemi-infiltration at C2) much less FB-labeled neurons were obtained than after HRP and DY injections, even if FB was dissolved in dimethylsulfoxide (Huisman et al. 1982).

When a comparison is made between the retrograde fluorescent double-labeling technique and the HRP-tritiated apo-HRP technique (Hayes and Rustioni 1979, 1981), the earlier results suggested that the retrograde fluorescent tracers produce a larger percentage of double-labeled neurons (Hayes and Rustioni 1981, c.f. Huisman et al. 1981, 1982). However, a comparison between both methods in demonstrating the existence of corticospinal neurons with branching axons to the cuneate nucleus in cat revealed a similar percentage of branching corticocuneate neurons. Thus, about 20% of the labeled corticocuneate neurons in area 3a were double-labeled, using the HRP-tritiated apo-HRP technique, while 14% to 16% double-labeled corticocuneate neurons were obtained throughout the pericruciate area with the retrograde fluorescent double-labeling technique (Rustioni and Hayes 1981, Bentivoglio and Rustioni 1986). For a precise comparison of these results it is of importance to know that area 3a contains the bulk of double-labeled corticocuneate neurons within the pericruciate area. However, the percentages of double-labeled corticospinal neurons cannot be determined with this method since their number will be underestimated (see chapter I.3.).

The local necrosis produced by DY, TB and FB at the injection site has the disadvantage that the simultaneous injection of two tracers results in two areas of necrosis in the brain, which may cause serious neurological deficits. Thus when injections with these tracers have to be made in vital structures such as in the lower brain stem or the spinal cord, NY may be preferred above DY, or FB can be injected first and DY one week later.

After DY became commercially available, it has been used succesfully in combination with TB in rat and FB in cat and monkey in a number of retrograde double-labeling studies (Cavada et al. 1984, Godschalk et al. 1984, Huisman et al. 1983, 1984, Keizer and Kuypers 1984, Ilinski et al. 1985, Fries et al. 1985, Yanagihara and Niimi 1985, Bentivoglio and Rustioni 1986, Lawes and Payne 1986, Innocenti et al. 1986, Bentivoglio and Molinari 1986, Gonzalo-Ruiz and Leichnetz 1987, Keizer et al. 1987, Verburgh 1987, Audinat et al. 1988, Keizer and Kuypers 1989).

VII.2. The corticospinal and -bulbar fibers in cat and monkey

In cat the fluorescent tracers NY, DY and FB were used, while in monkey FB was used only in combination with DY. The spinal injections were made at high cervical levels (C2 or C4-C5), aimed at labeling the corticospinal neurons in the contralateral hemisphere. Corticobulbar neurons in this hemisphere could be labeled from either half of the brain stem since the corticobulbar fibers are distributed bilaterally in cat and monkey (Kuypers 1958, Kuypers and Lawrence 1967). In the present study the bulbar injections and the spinal injections were made on the same side. This had the advantage that the involvement of the pyramidal tract by the brain stem injections would not produce false double labeling in the contralateral hemisphere, because the pyramidal fibers on the side of injection are derived from the ipsilateral hemisphere only (Biedenbach and DeVito 1980; findings chapter III, group C). Moreover, the injections could also be made from ventral through the pyramidal tract, thus avoiding involvement of the dorsal column nuclei by the injection area. However, conclusions regarding corticospinal collateralization can be drawn only with regard to the fibers which are distributed contralaterally.

In cat the areal distributions of labeled cortical neurons in the pericruciate cortex, obtained after injections of FB, NY and DY in the contralateral half of the spinal cord at C2 (Chapter III, group B) were similar to that of the HRP-labeled corticospinal neurons (Chapter III, group A). Moreover, the present findings were approximately the same as obtained in earlier experiments (Armand et al. 1974, Berrevoets and Kuypers 1975, Armand and Aurenty 1977, Groos et al. 1978, Biedenbach and DeVito 1980, Catsman-Berrevoets and Kuypers 1981).

The experiments of Chapter III, groups A and B show that the areal distributions of the labeled corticobulbar neurons after FB, NY and DY injections in the medial reticular formation of the lower brain stem were similar to those after HRP injections.

The retrograde HRP findings demonstrated, indirectly, that the distributions of corticospinal and corticobulbar neurons overlapped (Chapter III, Figs. 2, 3a, b). The area of overlap comprised mainly the caudal part of the dorsal bank of the cruciate sulcus, its entire ventral bank, the lateral part of area 6, the anterior sigmoid gyrus and the lateral and dorsal banks of the presylvian sulcus. Moreover, all HRP-labeled neurons were present in cortical lamina V and were pyramidal in shape.

In monkey, the distributions of the DY-labeled corticospinal neurons and the FB-labeled corticobulbar neurons in the hemisphere contralateral to the spinal and brain stem injections, were in keeping with earlier findings (Kuypers 1958, 1960, Kuypers and Lawrence 1967, Catsman-Berrevoets and Kuypers 1976, Jones and Wise 1977, Biber et al. 1978, Kunzle 1978, Murray and Coulter 1981, Toyoshima and Sakai 1982, Sessle and Wiesendanger 1982, Martino and Strick 1987). According to the present findings the distribution of the FB-labeled corticobulbar neurons extended more anteriorly than that of the DY-labeled corticospinal neurons. However, the two populations showed an extensive overlap, which so far had only been inferred from comparing the findings in different single tracer experiments.

VII.3. Collateralization in the corticospinal tract in cat and monkey

In cat, in the area of overlap of the labeled corticospinal and the labeled corticobulbar neurons many double-labeled neurons were present. These double-labeled neurons, which must represent branching corticospinal neurons projecting to the spinal cord as well as to the bulbar reticular formation, were concentrated in the lateral parts of area 6 and the rostral part of area 4, i.e. in the rostro- (medial) part of the motor cortex (Fig. 8B). According to a electrophysiological mapping study (Nieoullon and Rispal-Padel 1976) the branching corticospinal neurons were present mainly in those parts of the motor cortex which carry the representations of movements of back, neck and shoulder, while virtually none were present in the areas which carry the representations of wrist, forepaw and hindlimb respectively. The area containing the branching corticospinal neurons also corresponds largely with Armands common zone (Armand and Aurenty 1977). This zone projects bilaterally to the ventromedial part of the intermediate grey matter of both spinal enlargements (Armand and Kuypers 1980, Armand et al. 1985). According to anterograde HRP-findings (see Chapter III, group B) and anterograde degeneration experiments (Rossi and Brodal 1956, Kuypers 1958), the corticobulbar fibers from the area containing the bulk of the branching corticospinal neurons are distributed bilaterally, mainly to the central tegmental area of the lower pons and the medulla oblongata. This region, i.e. the paramedian reticular formation comprises the nucleus reticularis gigantocellularis and the nucleus reticularis pontis caudalis. These nuclei contain many reticulospinal neurons, which like the rostral motor cortex projects bilaterally to the ventromedial part of the spinal intermediate zone (Torvik and Brodal 1957, Nyberg-Hansen 1966, Petras 1967, Kuypers and Maisky 1975, Peterson et al. 1975, Basbaum et al. 1978, Holstege et al. 1979, Holstege and Kuypers 1982, Wilson and Peterson 1981, Huerta and Harting 1982). This suggest that the corticobulbar fibers, including the corticospinal collaterals, establish indirect cortico-reticulo-spinal connections). This is in keeping with electrophysiological findings, which showed that the reticulospinal neurons in these regions receive monosynaptic inputs from the contralateral sensorimotor cortex (Magni and Willis 1964, Peterson et al. 1975). In addition it has been shown that disynaptic pyramidal connections to motoneurons of certain neck muscles are established by pyramidal collaterals to the reticular formation of the lower brain stem (Alstermark et al. 1983a, b).

In light of these findings it is of interest, that after injections of DY in the pyramidal tract immediately

caudal to the pons and FB injections in the ipsilateral superior colliculus and the adjoining mesencephalic reticular formation (Chapter V, groups A and C), double-labeled neurons were present in area 6 and the rostro-medial part of area 4 (Fig. 8A). It is therefore most likely that a considerable number of the branching neurons, which appeared to distribute axon collaterals both to the bulbar as well as to the mesencephalic reticular formation and the superior colliculus were located in the same area as the branching corticospinal neurons which distribute collaterals to the bulbar reticular formation. Further, it was demonstrated that some of the cortico-mesencephalic fibers in the rostral part of the motor cortex are derived from branching corticospinal neurons (Catsman-Berrevoets and Kuypers 1981). Since in all these studies double-labeled neurons were present in the rostro-medial part of the motor cortex, it might be assumed that some of these double-labeled neurons are identical. If this assumption is correct, it would imply that the rostral part of the motor cortex in cat contains highly branching neurons which project to the spinal cord and in addition distribute collaterals to the colliculi and the mesencephalic and bulbar medial reticular formation (Fig. 8C).

It is of interest to note that the corticospinal fibers in cat also distribute collaterals to the dorsal column nuclei. These branching corticospinal neurons are mainly situated in area 3a, but are also present in areas 4, 1 and 2, and some in areas 3b and 5 (Fig. 8E, Rustioni and Hayes 1981).

In a recent anatomical study it was demonstrated in cat, that very many of the pyramidal tract fibers derived from the sensorimotor cortex distribute collaterals to the pontine grey (Ugolini and Kuypers 1986). Thus, the populations of branching corticospinal neurons which distribute collaterals to the mesencephalic and bulbar reticular formation may also distribute collaterals to the pontine nuclei (Figs. 8A, B, C, E). However, these pontine collaterals are by no means specific to the above mentioned branching corticospinal neurons, since the cortico-spinal neurons situated in the representation areas of the fore- and hindlimb (Armands specific zone) also distribute collaterals to the pontine nuclei (Fig. 8D, Ugolini and Kuypers 1986).

In monkey, it was demonstrated that the anterior part of the precentral corticospinal area (Catsman-Berrevoets and Kuypers 1976, Jones and Wise 1977, Toyoshima and Sakai 1982) contains branching corticospinal neurons the collaterals of which in all likelihood are distributed to the bulbar medial reticular formation (Fig. 9A), (Chapter IV, Kuypers 1958, 1960, Kuypers and Lawrence 1967). The corticospinal branch of these neurons terminate preferentially in the ventromedial part of the spinal intermediate zone (Kuypers and Brinkman 1970). The precentral motor areas which contain these branching neurons carry the representations of movements of the head and neck, shoulder and back (Woolsey et al. 1952, Kwan et al.1978, Wong et al. 1978; cf. Freund and Hummelstein 1984, 1985). Thus, there is a striking similarity in the organization of the projections of the branching corticospinal neurons from these areas in cat and monkey. The assumed similarity in cat and monkey may be carried one step further, since neurons projecting to the superior colliculus and the mesencephalic medial reticular formation in the monkey are also situated in the area containing branching corticospinal neurons (Catsman-Berrevoets 1979, Leichnetz et al. 1981, Fries 1984). Therefore it may be assumed that the rostral parts of the precentral area in the macaque contain highly branching neurons as in cat. In contrast, the caudal part of the motor cortex in monkey and the lateral and medial parts of the motor cortex in cat (Armands specific zones) do not contain such branching corticospinal neurons but give rise to much more focussed projections to the dorsolateral part of the contra-lateral spinal intermediate zone and in monkey also to the motoneuronal cell groups (Figs. 8D, 9B). These regions are involved in the steering of independent hand and finger movements.

It is of interest to note that corticospinal fibers in monkey distribute also collaterals to the dorsal column nuclei. These branching corticospinal neurons in the monkey are mainly situated in the somatosensory areas 1 and 2, but in addition are present in the caudal part of the precentral gyrus (area 4), especially in the anterior bank of the central sulcus. Some such branching neurons are also present in smaller numbers in areas 3a, 3b, the anterior part of area 5 and in SII (Fig. 9C, Bentivoglio and Rustioni 1986). In this respect the findings obtained in monkey differ from those in the cat.

The findings in monkey were also in other respects different to those obtained in the cat. For example, additional branching corticospinal neurons in the macaque were found also in the lower bank of the cingulate sulcus, the supplementary motor area, the precentral face representation area, the posterior bank

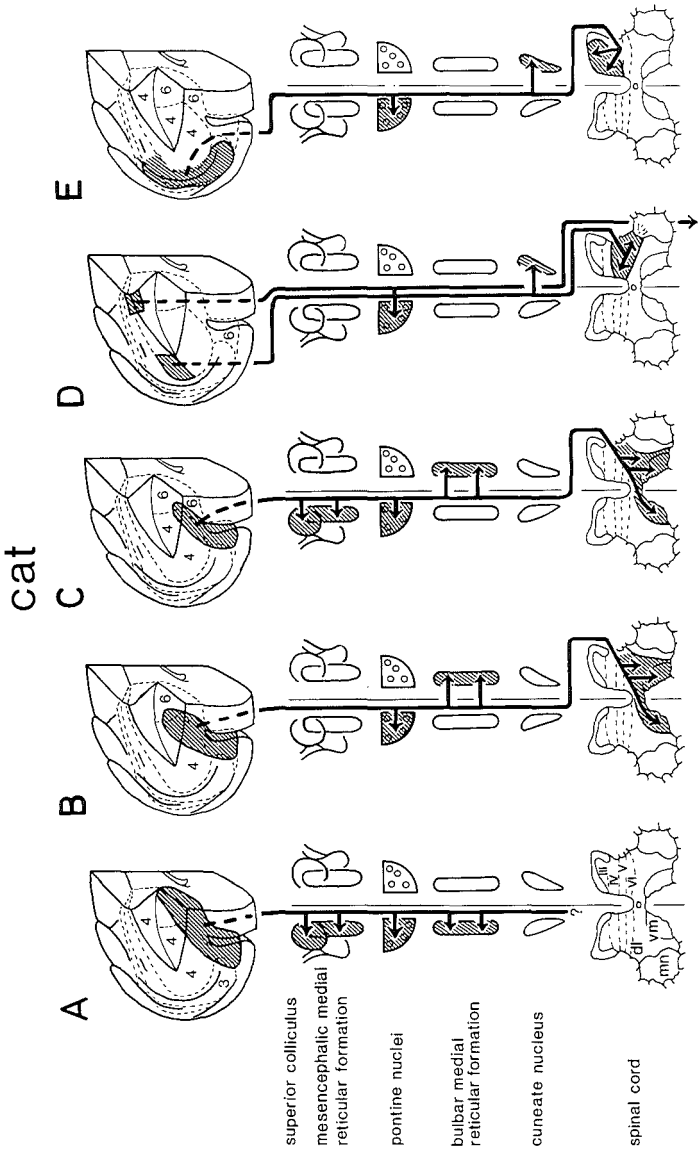
cat
A
B
C
D
E
superior colliculus
mesencephalic medial reticular formation
pontine nuclei
bulbar medial reticular formation
cuneate nucleus
spinal cord
dl
vm
mn

Fig. 8. Distribution of corticospinal neurons which project to different parts of the spinal cord and in addition distribute collaterals to different brain stem nuclei. A. Pyramidal tract fibers from area 6 and the rostral part of area 4 distribute collaterals to the superior colliculus, mesencephalic medial reticular formation as well as to the bulbar medial reticular formation. B. Corticospinal fibers from the lateral part of area 6 and the adjoining medial part of area 4 which project bilaterally to the ventromedial part (vm) of the spinal intermediate zone distribute collaterals to the bulbar medial reticular formation. C. Diagram showing that the rostromedial part of the pericruciate cortex in cat contains highly branching neurons which project bilaterally to the spinal cord and in addition distribute collaterals to the superior colliculus and the mesencephalic and bulbar medial reticular formation. Note that the branching neurons are concentrated in those regions from where axial and proximal limb movements can be elicited (see Introduction Chapter, Fig. 5). D. Corticospinal fibers from the fore- and hindlimb regions project preferential to the dorso-lateral (dl) parts of the intermediate zone. Note absence of collaterals to the brain stem nuclei which give rise to the medially descending brain stem pathways. Corticospinal fibers from the forelimb region distribute collaterals to the cuneate nucleus. E. Corticospinal fibers from somatosensory area 3 project to the dorsal part of the intermediate zone and the dorsal horn and in addition distribute collaterals to the cuneate nucleus. Note that the various cortical regions also give rise to corticospinal collaterals to the pontine nuclei.

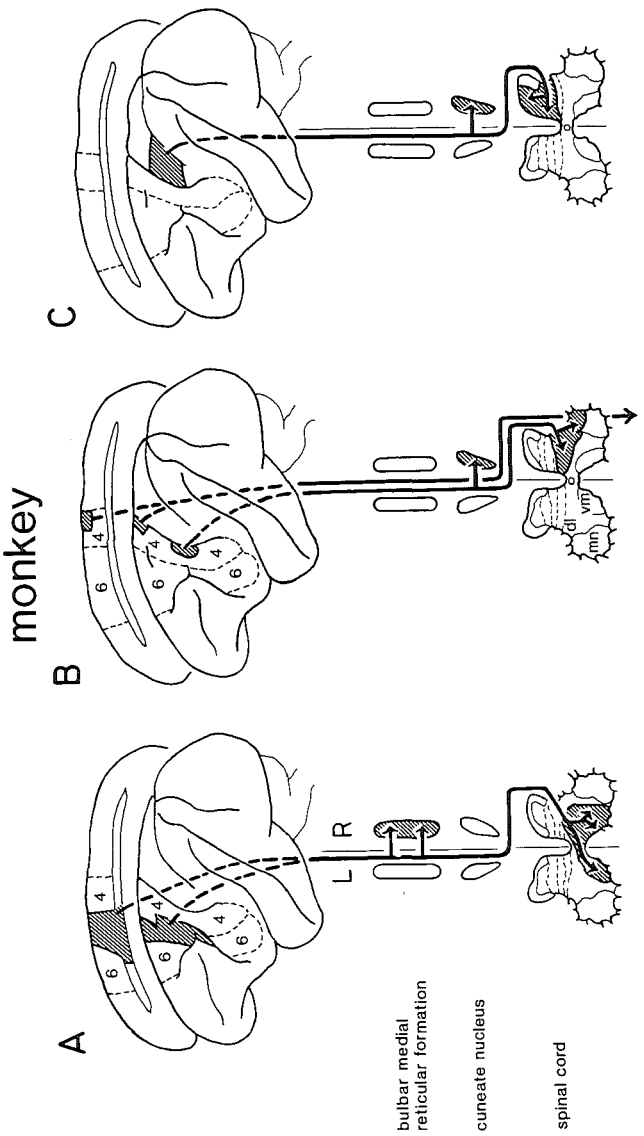
monkey
A
B
C
L
R
4
6
dl
vm
mn
bulbar medial reticular formation
cuneate nucleus
spinal cord

Fig. 9. Diagram showing the differences in spinal projections from the anterior and posterior parts of the precentral corticospinal area and the postcentral somatosensory area. A. Corticospinal fibers from the rostral part of area 4 and the caudal part of area 6 project bilaterally to the ventromedial (vm) parts of the intermediate zone. In addition several of the corticospinal neurons from the anterior part of the motor cortex and from the supplementary motor cortex give off collaterals to the bulbar medial reticular formation. B. Corticospinal fibers from the posterior parts of the precentral region project contralaterally to the dorsolateral (dl) parts of the intermediate zone and to the motoneuronal (mn) pool. Some of the corticospinal fibers from the hand region distribute collaterals to the cuneate nucleus. C. Corticospinal fibers from the postcentral somatosensory regions project typically to the dorsal part of the intermediate zone and to the dorsal horn. Many fibers from the hand representation area give off collaterals to the cuneate nucleus.

of the inferior limb of the arcuate sulcus and the insula (see Chapter IV, Fig. 1, and this Chapter, Fig. 9A).

The population of labeled corticospinal neurons in the lower bank of the cingulate sulcus was distinct from that in the SMA and extended more anteriorly (chapter IV). A relative large proportion of the corticospinal neurons in this region appeared to be branching neurons. This cingulate corticospinal region receives afferents from the postarcuate cortex (Barbas and Pandya 1987). This region projects to the precentral motor cortex as do the SMA, the postarcuate area and the cortex surrounding the precentral dimple (Muakassa and Strick 1979, Godschalk et al. 1984). The cingulate corticospinal region, roughly corresponding to area 24, is a subregion of the cingulate gyrus and as such regarded as part of the limbic system, which has been implicated in such behavioural functions as emotions, motivation, attention and memory (Papez 1937, Whitty 1966, Watson et al. 1973, Pandya et al. 1981). These findings suggest that the limbic system has a potential influence on the primary motor cortex and has direct corticospinal and cortico-bulbo-spinal projections, which for a considerable part are derived from the same cortical neuron.

The majority of the branching corticospinal neurons on the medial aspect of the hemisphere were present in the SMA (Woolsey et al. 1958, Mitz and Wise 1987). Thus, the connections of the SMA appear to be essentially different to those of the hand and foot representations of the precentral motor cortex. Several other observations suggest that the efferent connections of the SMA may be different from those of the precentral cortex. However, the present findings demonstrate clearly that a large number of the corticospinal neurons in the SMA distribute collaterals to the bulbar reticular formation, which does not apply to the hand and foot representations of the primary motor cortex. The presence of a mixture of cortical neurons projecting to the bulbar reticular formation and the spinal cord throughout the SMA, combined with branching corticospinal-bulbar neurons might explain that electrical stimulation of the SMA (Woolsey et al. 1958, Mitz and Wise) in particular after ablation of the motor cortex (Wiesendanger et al. 1973) elicits mainly proximal limb movements. The diffuse distribution of branching corticospinal neurons throughout the SMA might be related to the findings that in the SMA the representations of proximal and distal limb movements are intermingled (Mitz and Wise 1987). The diffuse organization of the SMA clearly differs from the organization in the precentral motor cortex with its proximal to distal representation of the limbs. Single cell recording from behaving monkeys strongly suggest that the SMA plays a more important role than the precentral motor cortex in the preparatory proces leading to correct initiation of trained movements (Tanji 1985). Thus, the SMA seems to be more remote from the spinal motor apparatus than the precentral motor cortex, the activity of which is tightly coupled to the spinal moto-neuronal activity (Lemon 1981, 1988). In keeping with the findings obtained in monkey, cerebral blood flow studies in humans showed that the SMA is activated during the preparation and the execution of a voluntary motor task that consists of complicated movement sequences (Ingvar and Philipson 1977, Roland et al 1980a).

Two views may be held regarding the role played by the premotor cortex in the control of movements. One regards the premotor cortex as mainly involved in the steering of axial and proximal movements, which is in keeping with the projections from the premotor cortex to the superior colliculus and the medial reticular formation of the brain stem (Catsman-Berrevoets et al. 1979, Kuypers 1981). In addition, lesions of premotor cortex in man are associated with "weakness of the contralateral hip and shoulder muscles and disturbance in the coordination of movements requiring the interaction of both arms or both legs" (Freund and Hummelstein 1984, 1985). According to the other view the premotor cortex represents an area of higher order which guides the activity of other executive areas, such as the primary motor cortex. This view is in keeping with two sets of data. Anatomical studies show that the premotor cortex projects to the precentral hand and foot representation areas (Muakassa and Strick 1979, Godschalk et al. 1984). In addition functional findings showed that unilateral destruction of these premotor areas interferes with visually guided movements (Fulton 1935, Woolsey et al. 1955, Moll and Kuypers 1975, 1977, cf. Matsumara and Kubota 1979, Muakassa and Strick 1979, Passingham 1985, Mitz and Wise 1987), and single cell recording from behaving monkeys show that the activity of many neurons in these non-primary motor areas is especially related to motor preparation (Tanji et al. 1980, Rizzolatti et al. 1981a, b, Weinrich and Wise 1982, Weinrich et al. 1984, Godschalk et al. 1981, Godschalk et al. 1985, Wise 1985, Tanji 1985). The observations of cerebral blood flow in humans are in keeping with this view, since the premotor cortex showed

and increase in activity when the subject performs non-routine voluntary movements, executed under visual, somatosensory and auditory guidance (Roland and Larsen 1976, Ingvar and Philipson 1977, Roland et al. 1980b).

However, the premotor cortex as defined by Fulton (1935) overlaps with the precentral motor area (MI) of Woolsey (1952) in particular with those regions carrying the representations of axial and proximal movements (Woolsey et al. 1952, cf. Freund and Hummelstein 1985). This region contains the "premotor" neurons related to movements of the hand and especially of the foot described by Kurata and his collaborators (1985). It coincides also with the location of branching cortico-spinal neurons in the anterior part of the precentral corticospinal area. However, this region may subserve a dual function: a. a particular role in the execution of axial and proximal movements, by way of its descending connections with the brain stem reticular formation and the spinal cord, and b. visual guidance of hand and finger movements, by way of the premotor connections to the hand area of the precentral motor cortex. These two functions may actually be tightly coupled since the execution of visually guided independent hand and finger movements, in general takes place against a background of postural movements in the stabilization of the reaching limb. The entire sequence of this complex movement presumably takes place under visual guidance.

Considering these various findings and concepts, it is striking to note that many of the questions raised by todays psychological experiments were initially derived from observations of the human patient in clinical neurology. This is especially exemplified by Liepmann's seminal descriptions of the apraxia in the beginning of this century (Liepmann 1900, 1906).

SUMMARY

The present study deals with the collateralization of the descending pathways from the cerebral cortex to the brain stem and the spinal cord in cat and monkey. The distributions of the branching cortical neurons were studied using retrograde fluorescent tracers. In addition, a new retrograde fluorescent tracer is described.

The existence of axon collaterals was demonstrated by Ramon Y Cajal (Cajal 1911). He showed by means of the Golgi silver impregnation technique, that pyramidal tract fibers emit axon collaterals projecting to the tegmentum of the brain stem. However, the cortical origin and the target areas of these collaterals in the brain stem could in many instances not be determined. In the seventies three anatomical techniques became available for demonstrating divergent axon collaterals (c.f. chapter I.2.). In the present study the retrograde fluorescent double-labeling technique has been used to study the collateralization of the cortical descending pathways. This technique makes use of several fluorescent substances, which after being retrogradely transported through both collaterals can be demonstrated independently in the parent neuronal cell body. The retrogradely labeled neurons can be visualized by fluorescence microscopy. An historical overview and a description of the retrograde fluorescent double-labeling technique is given in chapter I.3.

In 1981 when the present study was initiated, the retrograde fluorescent double-labeling technique made use mainly of the combination of Nuclear Yellow (NY) and True Blue (TB) in rat, and of NY in combination with Fast Blue (FB) in cat and monkey. TB and FB produce a blue fluorescent labeling of the cytoplasm at 360 nm excitation wavelength, while NY produces a yellow fluorescence of the nucleus at this same wavelength. After long survival times NY migrates out of the retrogradely labeled neurons as exemplified by labeling of neighbouring glial nuclei, producing false neuronal labeling. This can be prevented by restricting the NY survival time. However, TB and FB have much longer survival times than NY. Thus, TB and FB must be injected first and NY later, i.e. a short time before the animal is sacrificed. This problem can be circumvent when NY is replaced by Diamidino Yellow dihydrochloride (DY.2HCl, i.e. DY), the characteristics of which are described in chapter II. In conclusion, DY is transported over long distances in rat, cat and monkey (chapter IV), and produces a yellow fluorescence of the neuronal nucleus at 360 nm excitation wavelength, resembling that obtained with NY. It can be used succesfully in combination with TB and FB in double labeling experiments, demonstrating divergent axon collaterals. It migrates much more slowly out of the retrogradely labeled neurons than NY and its survival time is comparable to those of TB and FB.

Earlier anatomical studies demonstrated by means of the retrograde transport of horseradish peroxidase (HRP), that the cells of origin of the cortical fibers

descending to the brain stem and the spinal cord are situated exclusively in deep pyramidal layer V of the cerebral cortex. Moreover, the distributions of the different populations of neurons showed considerable overlap in the cerebral cortex (c.f. chapter I). Therefore, the question arose whether part of the descending fibers are derived from branching cortical neurons which project by means of axon collaterals to more than one brain region. For a better understanding of the anatomical and functional implications of the double-labeling findings presented in chapters III-VI, the anatomical, physiological and functional aspects of the descending brain stem pathways, the corticospinal pathway and the motor areas of the cerebral cortex are described in chapters I.4. and I.5.

The projections from the cerebral cortex to the pontine nuclei and the superior collicus are dealt with in chapter I.6.

Some remarks on the cortical and subcortical projections of Meynert cells are given in chapter I.7.

Chapters III and IV deal with the cortical distributions of corticospinal neurons with collaterals to the bulbar reticular formation in cat and monkey respectively. In these studies the retrograde fluorescent tracers NY, DY and FB were used. After unilateral injections of one tracer in the spinal cord at C2 and injections of the other tracer in the ipsilateral medial tegmentum of the lower brain stem, in the contralateral hemisphere large numbers of single-labeled corticospinal and single-labeled corticobulbar neurons were present. In addition, a substantial number of double-labeled, branching neurons were found. In cat these branching corticospinal neurons are present in the rostral part of area 4 and the adjoining part of area 6, contralateral to the spinal and bulbar injections. In these regions the percentages of branching corticospinal neurons with a collateral to the lower brain stem ranged from 5% laterally to 30% medially. These regions carry the representations of movements of the back, neck and shoulder. Virtually no branching corticospinal neurons were present in the areas, which carry the representations of movements of the wrist, forepaw and hindlimb. In monkey, a similar distribution of branching corticospinal neurons with collaterals to the bulbar reticular formation was obtained. Thus, branching corticospinal neurons were present in the rostral part of the precentral corticospinal area, corresponding to the rostral part of area 4 and the caudal part of area 6. This region carries the representations of movements of the head, neck, shoulder and back. In the caudal part of the precentral corticospinal area, which carries the representations of movements of the hand and fingers, the fore- and hindlimb, virtually none of these branching neurons were present. Thus, there is a striking similarity in the organization of the projections of the branching corticospinal neurons from these areas in cat and monkey. However, in monkey branching corticospinal neurons are also present in the rostral portion of the "cingulate cortico-spinal area" in the lower bank of the cingulate sulcus, the supplementary motor area (SMA), the upper portion of the precentral face representation, the caudal bank of the inferior limb of the arcuate sulcus and the insula. In these areas 10% to 30% of the corticospinal neurons distribute collaterals to the lower brain stem reticular formation.

The rostral part of the motor cortex in cat contains also branching corticospinal neurons which distribute collaterals to the mesencephalon. The findings suggest that the rostral part of the motor cortex in both cat and monkey contains highly branching corticospinal neurons, which project to the ventromedial part of the spinal intermediate zone and distribute collaterals to the colliculi, and the mesencephalic and bulbar reticular formation. The functional implications of the presence of branching corticospinal neurons in these areas are discussed in chapter VII.

The central issue of chapter V is the double-labeling of corticopontine neurons after injections of DY in the pontine grey and of FB in the colliculi. The distributions of the double-labeled corticopontine neurons could only be studied outside the cortical pyramidal tract area, because the pyramidal tract fibers are involved in the pontine injection area (see chapter V).

The parietal and the cingulate areas together contained three quarters of all labeled corticopontine neurons outside the pyramidal tract area. In the parietal areas roughly 25% of them were double-labeled and in the cingulate area 14%. In the visual areas 18 and 19 a much larger percentage (30%-60%) was double-labeled. In the remaining areas, i.e. the frontal granular cortex, the Clare-Bisshop area, SII, the lower bank of the anterior ectosylvian sulcus, the insular cortex and in the periphery of the auditory regions the percentages of double-labeled corticopontine neurons ranged from 20% to 29%. In a related study in the cat, it was found that the pyramidal tract fibers originating from the

sensorimotor cortex distribute an abundance of collaterals to the pontine grey. Thus, a considerable proportion of all corticopontine connections in the cat appear to be established by branching neurons which also distribute fibers to other brain stem regions and the spinal cord. The cerebellum is regarded to be involved in the coordination of movements. For this purpose the cerebellum serves as a listening post which is continuously informed about the peripheral inputs and the central instructions, from the cerebral cortex. The present findings, that part of the corticopontine connections are established by collaterals of the cortical pathways descending to the brain stem and the spinal cord are in keeping with this view. It might be assumed that these branching neurons when transmitting information to subcortical cell groups or the spinal cord, transmit simultaneously a copy of these instructions via the pons to the cerebellum.

Chapter VI deals with a curiosity. The large solitary cells of Meynert are situated in layer VI of the macaque striate cortex. These cells project to the posterior bank of the superior temporal sulcus (visual area V5) and in other studies it appeared that they also project to the superior colliculus. After FB injections in the superior colliculus and DY injections in the posterior bank of the superior temporal sulcus it was found that 40% to 50% of the Meynert cells were double-labeled with FB and DY. Thus Meynert cells distribute collaterals to a distant cortical and a subcortical structure.

SAMENVATTING

Dit proefschrift beschrijft de collateralisatie van de afdalende banen van de hersenschors naar de hersenstam en het ruggemerg van de kat en de aap. De distributie van neuronen in de hersenschors die axoncollateralen distribueren naar de hersentam en het ruggemerg, werd bestudeerd met behulp van retrograde fluorescerende tracers. Tevens wordt in het proefschrift een nieuwe retrograde fluorescerende tracer beschreven.

Axoncollateralen werden voor het eerst beschreven aan het begin van deze eeuw door Ramon Y Cajal (Cajal 1911). Hij toonde aan, gebruik makend van de Golgi zilver impregnatie techniek, dat vezels in de piramidebaan axoncollateralen afgeven die naar het tegmentale veld van de hersenstam projecteren. Echter, de cellen van oorsprong in de hersenschors en de eindigingsgebieden van deze axoncollateralen in de hersenstam konden niet met zekerheid worden vastgesteld. In de zeventiger jaren kwamen drie anatomische technieken beschikbaar waarmee de cellen van oorsprong van axoncollateralen konden worden aangetoond (zie hoofdstuk I.2.). In deze studie is gebruik gemaakt van de retrograde fluorescerende dubbel-labeling techniek. Deze techniek maakt gebruik van verschillende fluorescerende verbindingen, die nadat ze retrograad door beide axoncollateralen zijn getransporteerd, onafhankelijk van elkaar in het cellichaam kunnen worden aangetoond. De retrograad gelabelde neuronen kunnen zichtbaar worden gemaakt door middel van fluorescentie microscopie. Een beschrijving van deze techniek wordt gegeven in hoofdstuk I.3.

Bij de aanvang van deze studie in 1981, maakte de retrograde fluorescentie techniek hoofdzakelijk gebruik van de combinatie Nuclear Yellow (NY) en True Blue (TB) in de rat en van NY in combinatie met Fast Blue (FB) in de kat en de aap. TB en FB geven een blauwe fluorescentie van het cytoplasma bij excitatielicht met een golflengte van 360 nm, terwijl NY een gele fluorescentie geeft van de celkern bij dezelfde golflengte. Na lange overlevingstijden migreert NY uit de retrograad gelabelde neuronen. Dit is zichtbaar, doordat de celkernen van naburige gliacellen ook worden gelabeld, waardoor vals positieve labeling van neuronen kan optreden. Dit kan worden voorkomen door de overlevingstijd van NY te beperken. Echter, de overlevingstijden van TB en FB zijn veel langer dan die van NY. Dientengevolge moeten eerst TB en FB worden geïnjiceerd en NY op een later tijdstip, vlak voordat het dier wordt geofferd. Dit probleem kan worden omzeild indien NY wordt vervangen door Diamidino Yellow dihydrochloride (DY.2HCl, kortweg DY), waarvan de eigenschappen worden beschreven in hoofdstuk II. Samenvattend wordt DY getransporteerd over lange afstanden in de rat, de kat en de aap (hoofdstuk IV) en geeft het een gele fluorescentie van de celkern bij excitatielicht van 360 nm golflengte, gelijkend op dat van NY. Het kan succesvol worden gebruikt in combinatie met TB en FB in dubbel labeling experimenten voor het

aantonen van axon-collateralen. Het migreert veel langzamer uit de retrograad gelabelde cellen dan NY en de overlevingstijd van DY is vergelijkbaar met die van TB en FB.

Eerdere anatomische studies hebben aangetoond door middel van het retrograde transport van mierikswortel peroxidase (horseradish peroxidase, HRP), dat de cellen van oorsprong van de afdalende banen van de hersenschors naar de hersenstam en het ruggemerg uitsluitend gelegen zijn in de diepe piramidale laag V van de hersenschors. Bovendien bleek, dat de distributie van de verschillende populaties van neuronen elkaar aanzienlijk overlappen in de hersenschors (zie hoofdstuk I). De intrigerende vraag kwam naar voren of een deel van de afdalende vezels van de hersenschors wellicht afkomstig zou zijn van corticale neuronen, die door middel van axoncollateralen naar verschillende delen van het centraal zenuwstelsel projecteren. Voor een beter begrip van de anatomische en functionele implicaties van de "dubbel labeling" bevindingen beschreven in de hoofdstukken III-VI, worden de anatomische, fysiologische en functionele aspecten van de afdalende hersenstam banen, de corticospinale baan en de motorische velden van de hersenschors beschreven in de paragrafen I.4 en I.5.
De projecties van de hersenschors naar de ponskernen en de colliculus superior worden behandeld in paragraaf I.6.
Enkele opmerkingen over de corticale en subcorticale projecties van Meynert cellen staan in paragraaf I.7.

De hoofdstukken III en IV behandelen de corticale distributie van corticospinale neuronen die axoncollateralen distribueren naar de bulbaire reticulaire formatie in respectievelijk de kat en de aap. In deze studies werd gebruik gemaakt van de fluorescerende tracers NY, DY en FB. Na unilaterale injecties van de ene tracer in het ruggemerg op niveau C2 en injecties van de andere tracer in het ipsilaterale, mediale tegmentum van de lagere hersenstam, waren grote hoeveelheden enkelvoudig gelabelde corticospinale en enkelvoudig gelabelde corticobulbaire aanwezig in de contralaterale hemisfeer. Bovendien werd een aanzienlijk aantal dubbel gelabelde neuronen gevonden. In de kat waren corticospinale neuronen die axoncollateralen distribueren naar de lagere hersenstam aanwezig in het voorste deel van area 4 en het aangrenzende deel van area 6, contralateraal aan de spinale en bulbaire injecties. In deze gebieden varieerde het percentage dubbel gelabelde neuronen van 5% lateraal in area 4 en 30% mediaal in area 4 en in 6. Deze gebieden zijn betrokken bij bewegingen van nek, rug en schouders. Er waren vrijwel geen dubbel gelabelde neuronen aanwezig in de motorische gebieden die betrokken zijn bij bewegingen van de pols, klauwen en achterpoot. In de aap werd een overeenkomstige distributie gevonden van corticospinale neuronen met collateralen naar de bulbaire reticulaire formatie. Dus, dubbel gelabelde corticospinale neuronen waren aanwezig in het voorste deel van het precentrale corticospinale gebied, overeenkomend met het voorste deel van area 4 en het achterste deel van area 6. Dit gebied is betrokken bij bewegingen van hoofd, nek, schouders en rug. In het achterste deel van het precentrale motorisch gebied, betrokken bij bewegingen van de hand en vingers en het distale deel van voor- en achterpoot, werden vrijwel geen dubbel gelabelde neuronen gevonden. Er is sprake van een opvallende overeenkomst in de organisatie van corticospinale neuronen met axoncollateralen naar de lagere hersenstam tussen de kat en de aap. Echter in de aap zijn deze corticospinale neuronen ook aanwezig in het voorste deel van het "cingulate corticospinale gebied" in de onderste wand van de gyrus cinguli, het supplementaire motor gebied, het bovenste deel van de precentrale gelaatsrepresentatie, de achterste wand van het onderste been van de sulcus arcuatus en de insula. In deze gebieden distribueert 10% tot 30% van de corticospinale neuronen een axoncollateraal naar de reticulaire formatie van de lagere hersenstam.
Het voorste deel van de motorcortex in de kat bevat tevens corticospinale neuronen die collateralen distribueren naar het mesencephalon. De huidige gegevens suggereren dat het voorste deel van de motor cortex, zowel in de kat als in de aap, corticospinale neuronen bevat die zowel projecteren naar het ventromediale deel van de intermediaire zone van het ruggemerg en tevens collateralen afgeven naar de colliculus superior, en naar de reticulaire formatie van het mesencephalon en de lagere hersenstam. De functionele betekenis van deze neuronen in het voorste deel van de motorische schors wordt behandeld in hoofdstuk VII.

Het centrale thema van hoofdstuk V is het aantonen van dubbel gelabelde corticopontiene neuronen na injecties van DY in de basale pons en van FB in de colliculus superior en inferior. De distributie van dubbel gelabelde corticopontiene neuronen kon alleen worden bestu-

deerd buiten het oorsprongsgebied van de piramidebaan, omdat de vezels van de piramidebaan door het injectiegebied van de pons lopen (zie hoofdstuk V).
De parietale gebieden en de gyrus cinguli samen bevatten driekwart van alle gelabelde corticopontiene neuronen buiten het oorsprongsgebied van de piramidebaan. In de parietale schorsgebieden waren ongeveer 25% van deze neuronen dubbel gelabeld en in de gyrus cinguli 14%. In de visuele gebieden 18 en 19 bleek een veel groter percentage, 30%-60%, dubbel gelabeld. In de overige gebieden; de frontale granulaire schors, het Clare-Bisshop gebied, SII, the onderste wand van de voorste sulcus ectosylvius, de insula en in de periferie van de auditieve gebieden varieerde het percentage dubbel gelabelde neuronen van 20% tot 29%. In een gerelateerde studie in de kat werd gevonden dat ook piramidebaan vezels afkomstig van de motorische en somatosensibele delen van de hersenschors een grote hoeveelheid axoncollateralen distribueren naar de basis pontis. De gegevens van deze twee studies tonen aan dat een aanzienlijk deel van de verbindingen van de hersenschors, via de pons, met het cerebellum afkomstig zijn van corticale neuronen, die via collateralen tevens projecteren naar het ruggemerg en celgroepen in de hersenstam die oorsprong zijn van de afdalende hersenstam banen. Het cerebellum wordt beschouwd betrokken te zijn bij de coordinatie van bewegingen. Voor dit doel dient het cerebellum als luisterpost die voortdurend wordt geinformeerd over de perifere toestand in spieren, gewrichten en huid en de centrale instructies, afkomstig van de motor cortex. De bevinding dat een deel van de corticopontiene verbindingen bestaan uit collateralen van de afdalende vezels van de hersenschors naar de hersenstam en het ruggemerg is geheel in overeenstemming met de veronderstelde functie van het cerebellum. Men mag aannemen dat als informatie van de hersenschors via de afdalende banen naar subcorticale structuren of het ruggemerg wordt gestuurd, er tevens een copie van deze informatie via de pons het cerebellum bereikt.

In hoofdstuk VII wordt een curiositeit beschreven. De grote solitaire cellen van Meynert zijn gelegen in laag VI van de visuele hersenschors in de aap. Deze cellen projecteren naar de achterste wand van de sulcus temporalis superior (visueel gebied V5). Volgens andere studies blijkt dat deze cellen ook naar de colliculus superior projecteren. Na injecties van FB in de colliculus superior en van DY in de achterste wand van de sulcus temporalis superior bleek dat 40% tot 50% van de Meynert cellen dubbel gelabeld waren met FB en DY. Dit betekent dat Meynert cellen axoncollateralen distribueren naar een afgelegen corticaal gebied en naar een subcorticale structuur.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd op 11 januari 1957 te Winschoten geboren. Hij bezocht de Winschoter Scholengemeenschap en hij legde in 1975 het eindexamen Atheneum B met goed gevolg af. In datzelfde jaar begon hij de studie geneeskunde aan de Medische Faculteit te Groningen, waar hij in 1980 het doctoraal examen behaalde. Tijdens de medische studie vervulde hij student-assistentschappen op de afdeling Anatomie (Prof. Dr. A.G. de Wilde) en de Kliniek voor Obstetrie en Gynaecologie (DR. R. van Lunsen) in het kader van onderwijs aan junior-coassistenten. Door het werk op de afdeling anatomie kwam hij in contact met Dr. R. Haaxma (staflid afdeling Neurologie van het Academisch Ziekenhuis te Groningen), die met veel enthousiasme zijn belangstelling voor "het Brein" aanwakkerde.
Van februari 1981 tot augustus 1985 (tot februari 1984 aanstelling via ZWO/Fungo, resterende periode in dienst van de Erasmus Universiteit) was hij werkzaam als wetenschappelijk medewerker op de afdeling neuro-anatomie van de Erasmus Universiteit te Rotterdam, waar hij onder leiding van Prof. Dr. H.G.J.M. Kuypers experimenteel onderzoek verrichtte naar de collateralisatie van de corticale, afdalende banen naar de hersenstam en het ruggemerg. De resultaten hiervan zijn beschreven in dit proefschrift. In september 1985 vervolgde hij zijn medische studie aan de Medische Faculteit te Rotterdam en behaalde het artsexamen, cum laude, in december 1986. In januari 1987 volgde een aanstelling als arts-assistent op de afdeling Neurologie van het Academisch Ziekenhuis Dijkzigt te Rotterdam (hoofd Prof. Dr. A. Staal) en sinds januari 1988 is hij in opleiding in het specialisme neurologie.