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COLLATERALIZATION OF DESCENDING SPINAL PATHWAYS FROM RED NUCLEUS AND OTHER BRAINSTEM CELL GROUPS IN RAT, CAT AND MONKEY

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CONTENTS

CHAPTER I: GENERAL INTRODUCTION

I.1. Red Nucleus: anatomical aspects

I.1.a. Structure of the nucleus

The red nucleus is located centrally in the mesencephalic tegmentum. In the human brain the most conspicious gross feature of the red nucleus is its pinkish-red color in fresh sections. This is due to the high vascularity of the nucleus (Hough and Wolff, 1939) which distinguishes it from the surrounding structures. Its „capsule" is formed by fibers of the superior cerebellar peduncle, which surround and traverse the nucleus and by the oculomotor nerve which runs along the medial aspect of the nucleus (Carpenter, 1956). Using the Golgi and Nissl techniques, Cajal (1952) described three types of neurons in the red nucleus based on their size: large neurons, medium sized neurons and small neurons. Hatschek was the first who recognized that the red nucleus was divided in a magno- and parvicellular part (Hatschek, 1907). In humans the red nucleus is mainly composed of small neurons with only relatively few magnocellular neurons, the latter of which are located at the caudal pole of the nucleus (Ariens-Kappers et al., 1960; de Lange, 1912; Ten Donkelaar, 1976a; Masion, 1967). In monkey the red nucleus can readily be subdivided into a caudal magnocellular portion and a rostral parvicellular portion. At the meso-diencephalic junction the latter is located lateral to the fasciculus retroflexus. However, in lower mammals no such a strict distinction between a magnocellular and a parvicellular portion can be made, although also in these species large neurons are more numerous in the caudal parts of the nucleus and small neurons predominate in its rostral parts. In birds and reptiles the red nucleus consists of large cells only. Rats, cats, monkeys, apes and humans have a magno- and parvicellular part. From rat to cat, from cat to monkey and from monkey to human the relative size of the magnocellular part decreases, and the relative size of the parvicellular part increases.

These two principal subdivisions of the red nucleus have their own efferent pathways. Thus the caudal magnocellular part gives rise to projections to the cerebellum, the lower brainstem and the spinal cord, while the rostral parvicellular part projects to the inferior olive and possibly the thalamus. However, the segregation in a caudal red nucleus projecting to the cerebellum, the lower

brainstem and the spinal cord and a rostral red nucleus projecting to the inferior olive is a relative one, such that for the rubrospinal and rubrocerebellar neurons only a rostrocaudal gradient exists. This was shown by the fact that in cat rubrospinal and rubrocerebellar neurons are located throughout the rostrocaudal extent of the nucleus but that they are concentrated in the caudal half (Brodal and Gogstad, 1954; Pompeiano and Brodal, 1957), and that both sets of neurons comprise large as well as medium and small sized cells.

I.1.b. cytoarchitecture in rat

In cross-sections the caudal half of the nucleus is egg shaped and consists of large (soma diameter 40 μm, giant neurons) and medium sized (soma diameter 25-40 μm) neurons. The medium sized neurons are most numerous, but a considerable number of large neurons is also present, particularly in the caudal pole of the nucleus, where they are more numerous than in any other part (Reid et al., 1975). The rostral half of the nucleus is composed of predominantly small neurons (soma diameter 25 μm) with a scattering of medium sized ones and caudally in this rostral part also some large ones. In the most caudal part of this rostral half a population of neurons, designated the lateral horn (Reid et al., 1975) extends from the ventral surface of the nucleus dorsally along its lateral aspect. This subgroup contains predominantly small and medium sized neurons, but also some large ones. This lateral horn in rat is not analogous to the nucleus minimus of Von Monakow (1910) in cat, which subnucleus is located dorsolateral to the red nucleus and consists of very small neurons (soma diameter 6-8 μm) (Massion, 1967). The portion of the red nucleus which contains large neurons and therefore is designated the magnocellular portion, occupies the caudal three fourth of the nucleus. The rostral pole of the nucleus in rat blends imperceptively with the prerubral field and this part of the nucleus is cytoarchitectonically difficult to demarcate (Reid et al., 1975).

I.1.c. cytoarchitecture in cat and monkey

In cat the caudal magnocellular part of the red nucleus occupies the caudal two thirds of the nucleus but in monkey it seems relatively smaller and occupies only the caudal half. In both species the

caudal portion of the magnocellular part of the red nucleus is round in cross-section and contains large neurons which are dispersed between the tegmental fiber bundles. The rostral portion of the magnocellular red nucleus in monkey is also round in cross-section but contains mainly medium sized neurons. In cat this rostral portion of the magnocellular part as seen in cross-section has the shape of a flattened disc and contains both large and small neurons. According to earlier retrograde degeneration findings these various types of neurons in the magnocellular part of the red nucleus in cat and monkey all give rise to rubrospinal fibers (Kuypers and Lawrence, 1967; Poirier and Bouvier, 1966; Pompeiano and Brodal, 1957).

The rostral part of the red nucleus in monkey is better delineated against the surrounding reticular formation than in rat and cat. This part consists of relatively small neurons and therefore, as in other higher primates is called the parvicellular part. Rostrally this part of the nucleus is located lateral to the fasciculus retroflexus. In the ventral portion of the parvicellular part the cells are rather densely packed, while in its dorsal portion the cells form a loose network.[x] According to Fuse (1937) and Fukuyama (1940) the rostral red nucleus also comprises the subnucleus dorsomedialis (referred to as „Augenanteil des Roten Kerns") which extends dorsomedially from the red nucleus to the „Nebenokulomotorius Kerne" i.e. the medial accessory nucleus of Bechterew and nucleus of Darkschewitsch (Carpenter, 1956; Leichnetz, 1982). In monkey the parvicellular part extends rostrally close to the mesodiencephalic junction i.e. just beyond the level where the

[x] This dorsal portion of the parvicellular red nucleus in monkey is also called the rostral interstitial nucleus of Cajal or the rostral interstitial nucleus of the MLF (Büttner-Ennever and Büttner, 1978; Fukuyama, 1940; Leichnetz, 1982). However, it is of importance to emphasize, that these nuclei are distinct from the interstitial nucleus of Cajal, which projects to the spinal cord (Crutcher et al., 1978; Kuypers and Maisky, 1975, 1977; Peterson and Coulter, 1977; Vasilenko and Kostyukov, 1976).

fasciculus retroflexus (FR) traverses in full length the mesencephalic tegmentum. At these levels the parvicellular part is mainly located lateral to the FR. In monkey a small group of medium sized multipolar neurons extends from the caudal magnocellular part of the nucleus rostrally over a short distance along the lateral aspect of the parvicellular part, up to the level where the cross-section through the FR is located immediately ventral to the red nucleus.

In cat the most rostral part of the red nucleus is also situated lateral to the FR. However, in this animal this part of the nucleus is not truly parvicellular as in monkey, but consists of a loose network of small multipolar neurons which is difficult to delinate against the surrounding reticular formation. Yet, it seems reasonable to regard this portion of the red nucleus in cat as the counterpart of the parvicellular part in monkey since both structures give rise to the ipsilaterally descending rubro-olivary fibers (Condé and Condé, 1982; Courville and Otabe, 1974; Edwards, 1972; Strominger et al., 1979; Walberg, 1956).

I.1.d. efferents of red nucleus

Rubrobulbar and rubrospinal tract

This tract, is also called the bundle of Von Monakow after its discoverer Von Monakow (1883). It undergoes a complete decussation in the mesencephalic ventral tegmental decussation of Forel. After this decussation the bundle descends through the ventrolateral pontine tegmentum into the medulla oblongata, where it is located ventromedial to the spinal V complex and ventrolateral to the facial nucleus.

At the level of the pontine trigeminal nuclei, some fibers leave the rubro-bulbar and -spinal tracts and proceed dorsally to join the fibers of the superior cerebellar peduncle with which they ascend upstream into the cerebellum (Courville and Brodal, 1966; Hinman and Carpenter, 1959; Martin et al., 1974). These rubrocerebellar fibres terminate in the nucleus interpositus anterior (Courville and Brodal, 1966). In rat the cells of origin of this rubrocerebellar connection are located throughout the rostrocaudal extent of the red nucleus (Flumerfelt and Gwyn, 1974), whereas in cat they are located in its caudal two thirds (Brodal and Gogstad, 1954). In other words in both rat and cat rubrocerebellar fibers originate

from cells which are located in the same portion of the nucleus in which rubrospinal neurons are found (Condé and Condé, 1980; Murray and Gurule, 1979; Pompeiano and Brodal, 1957). In the lower pons and in the medulla oblongata fibers are distributed from the laterally descending rubral tract to the lateral part of the lateral tegmental field, to the lateral part of the cranial VII nucleus and to the supraspinal portion of the lateral reticular nucleus (i.e. to the part of the nucleus not in receipt of fibers from the spinal cord)(Bandler, 1978; Busch, 1961; Courville, 1966a; Edwards, 1972; Flumerfelt and Gwyn, 1974; Hinman and Carpenter, 1959; Kuypers, 1964, 1981; Martin, 1970b; Miller and Strominger, 1973; Waldron and Gwyn, 1971; Walberg, 1958). Further, the rubrospinal tract also distributes fibers to the principal nucleus V, some portions of the spinal V complex, the descending vestibular nucleus, the dorsal column nuclei cuneatus and gracilis and to cell groups x and z (Bandler, 1978; Edwards, 1972; Kuypers, 1964, 1981; Martin and Dom, 1970).

The rubrospinal tract in pigeon, opossum, rat, cat and monkey descends throughout the spinal cord in the dorsolateral funiculus and terminates in the dorsolateral part of the intermediate zone i.e. in the lateral part of Rexed's layers V and VI and in the dorsolateral part of layer VII (Brown, 1974a; Edwards, 1972; Kuypers, 1964, 1981; Martin and Dom, 1970a; Martin et al., 1974; Miller and Strominger, 1973; Nyberg-Hansen and Brodal, 1964; Petras, 1967; Rexed, 1952; Staal, 1961; Waldron and Gwyn, 1971; Wild, 1979). A crossed rubrospinal tract has also been demonstrated in the reptiles: turtle and lizard but not in the snake (ten Donkelaar, 1976a+b; 1978). In this respect it is of interest to note that the snake has no limbs. The frog in its tadpole stage also lacks extremities and in this stage (up to stage 57) likewise does not posses a rubrospinal tract. In this species rubrospinal neurons appear, simultaneously with the development of the limbs (ten Donkelaar, 1982).

In rat the cells of origin of the rubrospinal tract are located in the caudal three fourths of the nucleus (Murray and Gurule, 1979), while in cat and monkey they are located in the caudal two thirds of the nucleus (Condé and Condé, 1980; Kneisley, 1978; Pompeiano and Brodal, 1957). This interspecies difference may be a reflection of the difference in delineation of the rostral border of the red nucleus. Thus in rat, the population of neurons ventral and lateral to the fasciculus retroflexus (FR) at the meso-diencephalic junction, which neurons project to the inferior olive is normally not regarded as a part of the parvicellular red nucleus (Carlton et al., in press a+b; Cintas et al., 1980; Brown et al., 1977; Senba et al., 1981). On the other hand, in cat and monkey this population of neurons is regarded as part of the parvicellular red nucleus, and as in rat projects ipsilaterally to the inferior olive

(Condé and Condé, 1982; Courville and Otabe, 1974; Edwards, 1972; Strominger et al., 1979; Walberg, 1956).

The rubrospinal projection in rat, cat and monkey is somatotopically organized, such that the dorsomedial part of the red nucleus projects to the cervical enlargement, while its ventrolateral part projects to the lumbosacral enlargement (Flumerfelt and Gwyn, 1974; Gwyn, 1971; Hayes and Rustioni, 1981; Kneisley, 1978; Murray and Gurule, 1979; Pompeiano and Brodal, 1957b; Tsukahara, 1967). However, this somatotopic organization becomes less pronounced in lower animals. Thus, in opossum the rubrospinal tract displays a much less rigid somatotopic organization than in rat (Martin et al., 1974, 1981b) and in pigeon and reptiles no somatotopic organization is present at all (ten Donkelaar and de Boer-van Huizen, 1978; Wild, 1979), while in snakes the tract itself seems to be lacking (ten Donkelaar, 1976a+b).

Rubro-olivary tract

This uncrossed tract, which is well established in cat and monkey, descends along the lateral border of the trapezoid body in the central tegmental tract and terminates in the dorsal lamella of the principal inferior olive (Edwards, 1972; Miller and Strominger, 1973; Saint-Cyr and Courville, 1980, 1981; Walberg, 1956; Walberg and Nordby, 1981). Other meso-diencephalic nuclei, which also project to the inferior olive, are the interstitial nucleus of Cajal and the nucleus of Darkschewitsch, which nuclei project mainly by way of the medial tegmental tract (Busch, 1961; Ogawa, 1939; Walberg, 1974). In macaque including rhesus monkey a topographical arrangement between the rostral red nucleus and the inferior olive is described, such that the dorsal and medial parts of the rostral red nucleus project to dorsal regions of the principal olive, while the ventral and lateral parts project to ventral regions of principal olive (Courville and Otabe, 1974; Strominger et al., 1979). However, more recently, anterograde transport studies in cat (Edwards, 1972) indicate that rubro-olivary fibers terminate only in the dorsal lamella of the principal olive. In these studies the injection areas did not involve nuclei, which surround the parvicellular red nucleus, as the interstitial nucleus of Cajal and the nucleus of Darkschewitsch (c.f. Walberg and Nordby, 1981).

In cat retrograde HRP transport studies showed that the rubro-olivary neurons are located in the rostral one third of the red nucleus just lateral to the fasciculus retroflexus where they are continuous with the olivary projecting neurons located in the region of the interstitial nucleus of Cajal i.e. just dorsomedial to the red nucleus (Condé and Condé, 1982; Saint-Cyr and Courville, 1980, 1981; Walberg and Nordby, 1981). The neurons projecting to

the inferior olive, in the rostral red nucleus, the interstitial nucleus of Cajal, the nucleus of Darkschewitsch and in the ventral portion of the thalamic parafascicular nucleus form a continuous population (Saint-Cyr and Courville, 1980). Anatomical and physiological studies demonstrate only a very limited rostro-caudal overlap between the population of rubro-olivary neurons and that of rubrospinal neurons, the latter being located in the caudal two thirds of the red nucleus (Anderson, 1971; Condé and Condé, 1982).

The existence of a rubro-olivary tract in opossum and rat has been subject of discussion. Thus, retrograde transport studies in opossum and rat demonstrate only a limited population of rubro-olivary neurons in the dorsomedial rostral red nucleus (Brown et al., 1977; Carlton et al., in press [a]; Cintas et al., 1980; Henkel et al. 1975; Senba et al., 1981). On the other hand, in these animals many neurons were present in the area lateral to the fasciculus retroflexus, which area in cat and monkey is considered to be a part of the rostral red nucleus containing the cells of origin of the rubro-olivary tract (Condé and Condé, 1982; Kuypers and Lawrence, 1967). Based on these data one may suggest that the different delineation of the rostral part of the red nucleus in opossum, rat, cat and monkey underlies the difference in opinions about the existence of a rubro-olivary pathway as demonstrated by means of retrograde transport techniques. However, anterograde transport studies in opossum establish clearly the existence of a rubro-olivary tract to the principal olive (Martin et al., 1980).

Rubrothalamic pathway

Many conflicting data are available about the existence of an ascending rubrothalamic connection. Retrograde degeneration studies suggested the existence of ascending fibers originating from the red nucleus (Pompeiano and Brodal, 1957b). However, lesions placed in the red nucleus unavoidably interrupt cerebellothalamic fibers making it impossible to determine the precise origin of the resulting degeneration in the thalamus. One way of overcoming the problem is to interrupt these fibers and allow them to degenerate before making lesions or recordings in the red nucleus. From such an anatomical study it was concluded that in monkey no rubrothalamic projection exists (Hopkins and Lawrence, 1975). On the other hand, retrograde degeneration studies support the existence of an ascending rubral projection (Kuypers and Lawrence, 1967). In these studies it was found that many neurons in the rostral red nucleus ipsilaterally to the diencephalic lesion tended to be smaller than their contralateral counterparts and that some displayed acute retrograde changes. However, a direct effect of the lesion, which is very

close to the rostral red nucleus could be suspected (Kuypers and Lawrence, 1967). Electrophysiologically it was shown that stimulation of the thalamus produced excitation of red nucleus neurons. However, it has been shown that this excitation does not occur due to antidromic activity of red nucleus neurons, but that it occurs transsynaptically via an axon reflex in brachium conjunctivum fibers which send collaterals to red nucleus and also project to the thalamus (Anderson, 1971; Tsukahara, 1967). Anterograde autoradiographic transport studies also deny a rubrothalamic pathway (Edwards, 1972). However, more recent retrograde transport studies using HRP demonstrated the existence of a rubrothalamic projection in cat, originating from the rostral one third of the red nucleus (Condé and Condé, 1980). In this study it was shown that retrograde transport of HRP between the thalamic injection site and red nucleus was obtained by intra-axonal transport and not by extracellular diffusion or by the bloodstream, since no labeled neurons occurred between the injection site and red nucleus (i.e. the hypothalamic and subthalamic neurons). In addition neurons were only labeled in its rostral part. Further electrophysiological studies would be of interest to confirm whether a direct rubrothalamic pathway exists in addition to the cerebellothalamic pathway which gives off collaterals to the red nucleus (Tsukahara, 1967).

I.1.e. Afferents of red nucleus

Cortical afferents

The corticorubral connections are most extensively studied in monkey, in which corticorubral fibers to the parvi- and magnocellular red nucleus clearly originate from different cortical areas. Corticorubral fibers to the *parvicellular red nucleus* originate from Brodmann's area 4 in the precentral gyrus, which largely corresponds to the primary motor cortex, and from the premotor areas (area 6 and the frontal eye field area 8 in the prearcuate gyrus) (Brodmann, 1909; Kuypers and Lawrence, 1967). This projection is bilateral with an ipsilateral emphasis. The precentral projection to the parvicellular red nucleus is derived from the entire precentral gyrus including the „face area" (c.f. Woolsey, 1958) and also to a limited degree from postcentral and parietal areas (Kuypers and Lawrence, 1967). This projection is topically organized. Thus, the fibers from the upper one third of the precentral gyrus are distributed to the

ventrolateral portion of parvicellular red nucleus, those from the lower one third to the dorsomedial portion and those from the middle one third to the intermediate portion (Catsman-Berrevoets, 1979; Hartmann-von Monakow, 1979; Kuypers and Lawrence, 1967). The premotor area (area 6 and area 8) projects to the dorsomedial part of the parvicellular red nucleus. The supplementary motor cortex projects both to the dorsomedial and ventrolateral parts of the parvicellular red nucleus (Hartmann-von Monakow, 1979; Künzle, 1978; Kuypers and Lawrence, 1967). The terminal field of area 8 (prearcuate frontal cortex) in the subnucleus dorsomedialis of the parvicellular red nucleus (referred as „Augenanteil des Roten Kerns") continues into the „nebenokulomotorius Kerne" i.e. the medial accessory nucleus of Bechterew and the nucleus of Darkschewitsch (Carpenter, 1956; Fukuyama, 1940; Fuse, 1937; Kuypers and Lawrence, 1967; Leichnetz, 1982). Corticorubral fibers to the *magnocellular red nucleus* originate only from the upper two thirds of the caudal precentral gyrus. This projection is exclusively ipsilateral (Hartmann-von Monakow, 1979; Kuypers and Lawrence, 1967). Thus, in the cortico-rubro-spinal pathway the somatotopy is preserved, such that the cortical "upper limb area" (c.f. Woolsey, 1958) projects to the dorsomedial part of the red nucleus, which part in turn projects to the cervical cord. Moreover, the cortical "lower limb area" (c.f. Woolsey, 1958) projects to the ventrolateral part of the nucleus, which part projects to the lumbosacral cord (Catsman-Berrevoets, 1979; Hartmann-von Monakow, 1979; Kuypers and Lawrence,1967; Petras,1969). No "face area"(c.f. Woolsey, 1958) projection exists from area 4 (i.e. from the lower one third of the precentral gyrus) to the magnocellular red nucleus in contrast to the "face area" 4 projection to the parvicellular red nucleus (Catsman-Berrevoets, 1979; Hartmann-von Monakow, 1979; Kuypers and Lawrence, 1967).

The magno- and parvi-cellular parts of the red nucleus receive their inputs not only from different cortical areas, but the cortical cells of origin of these rubral afferents are also situated in different cortical layers. Thus, the cells projecting to the parvicellular red nucleus, which are located in areas 4 and 6 are situated in the upper part of layer V (Brodmann) i.e. in area 4 above the Betz cells (Betz, 1948), while those projecting to the magnocellular red nucleus, which are located posteriorly in area 4, are situated deep in layer V i.e. at the level of the Betz cells (Catsman-Berrevoets, 1979).

In cat the corticorubral fibers to the caudal two thirds of the red nucleus, the part in which the rubrospinal neurons are located, originate from the primary sensori-motor cortex. As in monkey, the somatotopy in the cortico-rubro-spinal pathway is preserved, such that the fibers of the cortical "motor forelimb area" (c.f. Woolsey, 1958) terminate in the dorsal parts, and those

from the "motor hindlimb area" (c.f. Woolsey, 1958) in the ventral parts of the red nucleus (Mabuchi, 1966; Padel, 1973; Rinvik and Walberg, 1963; Pompeiano and Brodal, 1957b). The premotor area 6 in cat (see Hassler and Muhs-Clement, 1964) is located partly in the gyrus proreus. Only anterograde degeneration studies have revealed some data concerning corticorubral fibers from the gyrus proreus (Rinvik and Walberg, 1963). After lesion in this gyrus degenerating terminal fibers were seen bilaterally in the red nucleus. This was heaviest ipsilaterally. They did not state in which rostrocaudal part of the red nucleus these corticorubral fibers terminate. It would, of course, be of interest to know whether these fibers terminate in the rostral one third of the cat red nucleus (located lateral to the fasciculus retroflexus), which may be regarded to be analogous to the parvicellular red nucleus in monkey on the basis of the projections from this portion of the red nucleus to the inferior olive (Condé and Condé, 1982; Saint-Cyr and Courville, 1980; Walberg, 1956).

Originally the corticorubral fibers in rat were described to be distributed only to the ipsilateral parvicellular part of the red nucleus. They apparently do not distribute to the magnocellular part, which was considered to give rise to almost all rubrospinal fibers (Brown, 1974a; Flumerfelt and Gwyn, 1974; Gwyn and Flumerfelt, 1974). However, since rubrospinal neurons in rat also arise from more rostral parts of the red nucleus (Flumerfelt and Gwyn, 1974) the corticorubral fibers from the sensorimotor cortex in this animal are probably also distributed to rubrospinal neurons. As pointed out already, much of the controversy related to the efferent connectivity of the red nucleus may result from differences concerning the cytoarchitecture of the parvicellular part. If one accepts the cytoarchitectonic borders of Reid (1975) no rubro-olivary projection exists, since according to this author the parvicellular red nucleus in rat does not extend as far rostrally as the fasciculus retroflexus (Cintas et al., 1980). Yet, in this animal many fibers projecting to the inferior olive do arise from this specific area lateral to the fasciculus retroflexus, which in fact in cat and monkey is considered as part of the parvicellular red nucleus, projecting to the inferior olive (Condé and Condé, 1982; Courville and Otabe, 1974; Miller and Strominger, 1973; Saint-Cyr and Courville, 1980; Walberg, 1956). Therefore, if one considers the connectivity rather than the cytoarchitecture, the "parvicellular" red nucleus in rat extends further rostrally than indicated by Reid (1975) and as a consequence receives many fibers bilaterally from the sensorimotor cortex (Brown, 1974b) in the same way as in cat and monkey.

Cortical fibers in rat are distributed not only to the "parvicellular part" of the red nucleus, which projects to the inferior olive, but are distributed

also to the more caudal parts of the red nucleus, which project to the spinal cord. The same is true in opossum where the sensorimotor cortex projects to the area lateral to the fasciculus retroflexus, which area projects to the inferior olive (Henkel et al., 1975; Martin et al., 1980) and to the more caudal parts of the nucleus which project to the spinal cord (King et al., 1972; Martin, 1968).

As pointed out above in cat and monkey corticorubral projections to both the parvicellular and the magnocellular parts show a topic organization (Kuypers and Lawrence, 1967; Mabuchi, 1966; Rinvik and Walberg, 1963). However, no such a topic arrangement could be demonstrated in opossum (King et al., 1972; Martin, 1968). However, in opossum the somatosensori cortex projects only to the cervical and rostral thoracic spinal cord segments (Martin and Fisher, 1968), while the red nucleus projects throughout the spinal cord. Therefore, a pronounced somatotopic organization in the cortico-rubrospinal connections might not be expected (King et al., 1972; Martin, 1968). Moreover, in opossum the somatotopic organization in the rubrospinal part of the red nucleus is not so distinct as in cat and monkey (Martin et al., 1981b).

The corticorubral axon terminals in opossum, rat, cat and monkey contact mainly distal dendrites as was shown in anatomical electron microscopic (EM) studies (Brown, 1974; King et al., 1974b) and in electrophysiological studies (Humphrey, 1976; Tsukahara, 1967, 1968a). Cerebellorubral fibers on the other hand, terminate on or near the cell body as was shown in anatomical EM studies in opossum, rat and cat (Dekker, 1981; King, 1973; Nakamura, 1971) and in electrophysiological studies in cat (Tsukahara, 1967 and 1968a).

Cerebellar afferents

interposito-rubral connections

In monkey and cat, fibers from the nucleus interpositus anterior (NIA), which represent the main source of afferents to the caudal red nucleus, are distributed to that part of the red nucleus which projects to the spinal cord (Courville, 1966b; Flumerfelt and Otabe, 1973; Kievit, 1979; Voogd, 1964). This cerebello-rubral connection is somatotopically organized, such that the more rostral parts of the NIA project to the ventrolateral parts of the red nucleus, while the more caudal parts of the NIA project to its dorsomedial parts. Thus, according to electrophysiological findings of Pompeiano (1959)

and Massion (1961, 1963) and anatomical findings of Pompeiano and Brodal (1957) the "hindlimb" area localized in the rostral NIA is connected with the "hindlimb" area ventrolaterally in the red nucleus and the "forelimb" area localized in the caudal NIA is connected with the "forelimb" area located dorsomedially in the red nucleus. Electrophysiologically, it was also shown that in cat these interposito-rubral fibers are in part collaterals from axons which continue to the thalamus (Tsukahara et al., 1967). In addition to the fibers distributed from NIA to the red nucleus, a limited number of fibers is distributed from the nucleus interpositus posterior (NIP). These latter fibers terminate mainly in the medial edge of the caudal red nucleus (Voogd, 1964).

In rat and opossum the interposito-rubral fibers, which are distributed to the contralateral red nucleus are also distributed to the caudal red nucleus, which projects to the spinal cord (Gwyn and Flumerfelt, 1974; King et al., 1973). Electron microscopic studies in rat combining the anterograde intra-axonal transport of radioactive aminoacids and the retrograde intra-axonal transport of HRP showed that many rubrospinal neurons receive afferents from the cerebellar interpositus nucleus (Dekker, 1981).

dendato-rubral connections

In monkey the NIA and the dentate nucleus clearly distribute fibers to different parts of the red nucleus. The former nucleus projects to the magnocellular subdivision, while the latter sends its fibers to the parvicellular subdivision (Flumerfelt et al., 1973). In cat the dendato-rubral fibers are less numerous than in monkey and are distributed to the anterior one third of the red nucleus, which part projects to the inferior olive (Angaut and Bowsher, 1965; Condé, 1966; Courville, 1966b). In opossum approximately the same differential distribution of interposito-rubral and dendato-rubral fibers exists as in monkey, such that the former fibers distribute throughout the red nucleus but with an emphasis on the caudal one third and the latter distribute exclusively to a small dorsolateral portion, close to the meso-diencephalic junction (King et al., 1973). The fact that the interposito-rubral fibers are distributed to caudal magnocellular parts of the red nucleus and dendato-rubral fibers to rostral parvicellular parts seems to be in keeping with findings in humans. In this species the magnocellular part contains only a limited number of neurons, which occupy only the most caudal portion of the nucleus. Correspondingly the rubrospinal tract comprised only very few fibers (Massion, 1967; Nathan and Smith, 1982; Sie, 1956; Verhaart, 1938). This is in keeping with the findings of Hassler (1950) who stated that the vast majority of the cerebello-rubral fibers in the human brain are derived from the dentate nucleus, which from animal experiments (see above) is known to project preferentially to the pars parvicellularis.

I.2. Red Nucleus: physiological aspects

I.2.a. Function of rubrospinal pathway as one of the descending brainstem pathways

The descending pathways are derived from the cortex and the brainstem, the latter including the red nucleus. The corticospinal pathway terminates in the dorsolateral and ventromedial parts of the intermediate zone (Brown, 1971; Chambers and Liu, 1957; Kuypers, 1964; Martin and Fisher, 1968; Nyberg-Hansen and Brodal, 1963). In addition, the corticospinal fibers terminate (especially in the chimpanzee and human being) directly on contralateral motoneurons of distal extremity muscles and of girdle and proximal extremity muscles (Kuypers, 1964; Petras, 1968; Schoen, 1969). In lower species such as cat, no such direct corticospinal projection to motoneurons exists.

The descending brainstem pathways to the spinal cord may be divided in two groups (Kuypers, 1964, 1981): *the medially descending pathways* which terminate characteristically in the area of long propriospinal neurons located ventromedially in the ventral horn (Molenaar and Kuypers, 1978) and the *laterally descending pathways*, which terminate characteristically in the area of short propriospinal neurons located dorsolaterally in the intermediate zone (Molenaar and Kuypers, 1978). The medially descending pathways include the interstitiospinal pathway (Crutcher et al., 1978; Kuypers and Maisky, 1975, 1977; Nyberg-Hansen, 1966a; Staal, 1961), the tectospinal pathway (Castiglioni et al., 1978; Harting, 1977; Kuypers and Maisky, 1975; Martin, 1969; Nyberg-Hansen, 1964; Petras, 1967; Staal, 1961; Waldron and Gwyn, 1971), the vestibulospinal pathways (Crutcher et al., 1978; Kuypers and Maisky, 1975, 1977; Peterson and Coulter, 1977; Vasilenko and Kostyuk, 1976) and the reticulospinal pathways from the mesencephalic and pontine reticular formation and from the gigantocellular part of the medullary reticular formation at the level of the facial nucleus (Crutcher et al., 1978; Edwards, 1972; Kuypers and Maisky, 1975, 1977; Martin et al. 1979, 1982; Peterson et al., 1975; Petras, 1967; Tohyama et al., 1979a+b; Waldron and Gwyn, 1971; Zemlan and Pfaff, 1979). These pathways descend in the ventral and (or) ventrolateral funiculi of the spinal cord and most of them terminate preferentially in the ventromedial part of the intermediate zone bilaterally. The laterally descending pathways

on the other hand, include the pathways derived from the red nucleus (Edwards, 1972; Kuypers and Maisky, 1975, 1977; Nyberg-Hansen and Brodal, 1964; Petras, 1967; Staal, 1961; Tohyama et al., 1979a; Waldron and Gwyn, 1971), the Edinger-Westphal nucleus (Basbaum and Fields, 1979; Loewy and Saper, 1978a+b), the ventrolateral pontine tegmentum (Basbaum and Fields, 1979; Busch, 1961; Holstege et al., 1979; Kuypers and Maisky, 1975; Martin et al., 1979; Tohyama et al., 1979a+b) and raphe magnus, including the adjoining ventral reticular formation (Basbaum and Fields, 1978, 1979; Kuypers and Maisky, 1977; G.F. Martin et al., 1979, 1981a; R.F. Martin et al., 1978; Tohyama et al., 1979a+b). These pathways descend in the dorsolateral funiculus. The rubrospinal tract, which is derived from the contralateral red nucleus, terminates in the dorsolateral part of the spinal intermediate zone, while the others distribute their fibers to the dorsal horn.

In addition to the medially and laterally descending brainstem pathways described above, recent anterograde transport studies have revealed the existence of several other descending brainstem pathways, such as the (sub) coeruleospinal (Holstege et al., 1979, 1982; Martin et al., 1979; Nygren and Olson, 1977; Tohyama, 1979b) and the raphe spinal pathways (from raphe pallidus and adjoining reticular formation) (Basbaum and Fields, 1979; Holstege et al., 1982; Martin et al., 1978; Tohyama, 1979). These pathways descend superficially in the lateral and ventral funiculi of the spinal cord and terminate both in the intermediate zone as well as in the somatic and autonomic motoneuronal cell groups of the ventral and lateral horn, respectively. The exact functional role of the connection of these descending fibers to the somatic motoneuronal cell groups in the spinal ventral horn, is not yet established.

The findings after transsection of the laterally descending pathways at various brainstem levels of the medially descending pathways at the level of the abducens nucleus indicate that these pathways subserve different functions in motricity. Thus, these brainstem motor pathways in the rhesus monkey subserve control of total body-limb activity and independent limb movements (Lawrence and Kuypers, 1968 I). The medially descending pathways typically subserve steering of axial and proximal limb movements, maintenance of erect posture, steering of integrated movements of body and limbs and directing the course of progression (Lawrence and Kuypers, 1968 II). The laterally descending brainstem pathways on the other hand, are involved in independent use of the extremity particularly of the distal parts, which function is primarily subserved by the rubrospinal tract (Lawrence and Kuypers, 1968 II). This function of the laterally descending pathways is confirmed by a lesion study in monkey

in which the precentral corticospinal connections and both rubrospinal pathways were destroyed, while in contrast to the experiments of Lawrence and Kuypers (1968, II) the crossed pontospinal and uncrossed (sub)coeruleospinal pathways remained intact (Haaxma and Kuypers, 1975). The limb contralateral to the precentral lesion showed a defect similar to that observed by Lawrence and Kuypers (1968, II).

The corticospinal pathway, because of the way in which it terminates in the spinal cord, further amplifies the brainstem motor control and in addition provides the capacity of individual finger movements by way of direct corticomotoneuronal connections (Kuypers and Lawrence, 1968 II).

The findings in split-brain monkey confirm the above concenpt of the functional contributions of the various descending pathways to motor control, such that in these animals the seeing half of the brain exerts a visuo-motor control over individual arm, hand and finger movements contralaterally, but mainly over arm movements ipsilaterally (Brinkman and Kuypers, 1973). This may be explained by the distribution of the corticospinal fibers and by the fact that the cortical projections to the rubrospinal neurons are distributed only ipsilaterally, while those to the cells of origin of the medial brainstem system are distributed bilaterally (Hartmann-von Monakow et al., 1979; Künzle, 1978; Kuypers and Lawrence, 1967). The same visuo-motor disturbances were observed ipsilaterally in human patients, in whom surgical section of the commissures was carried out to treat severe convulsive disorders not controlled by medication (Sperry et al., 1969).

Anatomically, no direct connections exist from the medially and laterally descending brainstem pathways to motoneurons, in contrast to the corticospinal fibers as mentioned above. Electrophysiologically, however, it was shown that the reticulo- and vestibulospinal pathways in cat and monkey establish also some monosynaptic connections with motoneurons, preferentially those innervating axial and proximal hindlimbs. The rubrospinal pathway in monkey establishes also some monosynaptic connections with motoneurons preferentially those innervating distal muscles (Grillner et al., 1968, 1970; Hongo et al., 1969a; Peterson, 1979; Shapovalov et al., 1971; Shapovalov, 1972).

I.2.b. Physiology of the rubrospinal tract as the primary component of the laterally descending brainstem pathways

The facilitatory action of the red nucleus on contralateral flexor muscles was shown by Pompeiano (1957) (Massion, 1967) who found that stimulating the red nucleus induced a flexion of either the anterior or the posterior contralateral limb, depending on which part of the red nucleus was stimulated. However, microstimulation of the red nucleus in cat and monkey elicites contraction of both flexor and extensor muscles of the contralateral limbs (Ghez, 1975; Larsen and Yumiya, 1980). These studies also suggested that just as the motor cortex, the red nucleus contains colonies of neurons, which ultimately activate specifically the motoneuron pools of individual muscles (Ghez, 1975; Larsen and Yumiya, 1980). Intracellular recording studies demonstrated that after stimulating the red nucleus a predominance of EPSPs occurs in the flexor α motoneurons and IPSPs in the extensor α motoneurons (Hongo et al., 1965, 1969a; Sasaki et al., 1960). The pattern of the rubrospinal influence on static fusimotor neurons (γ motoneurons) to different muscles closely followed that on α motoneurons such that predominantly an excitation of flexor neurons and an inhibition of extensor neurons occurs (Appelberg et al., 1975). These studies further showed that stimulating the red nucleus excites interneurons of the contralateral side in Rexed layers VI and VII, which is in keeping with anatomical findings (Nyberg-Hansen and Brodal, 1964). These interneurons may be divided in two groups: i.e. interneurons excited by red nucleus stimulation alone and interneurons which are excited by the red nucleus but are also facilitated by primary afferents and thus are involved in spinal reflexes (Hongo et al., 1969b, 1972; Kostyuk and Pilyavsky, 1969). Flexion movements, which require the use of distal muscles, are more affected after red nucleus lesion than movements primarily involving more proximal muscles (Sybirska and Gorska, 1980). This is confirmed by unit recording in monkey red nucleus during skilled movement in which most neurons modulate their activity maximally preceding finger or wrist movements (Kohlerman et al., 1980). Red nucleus neurons thus modulate their activity prior to and during voluntary movement of the contralateral limb by which activity they contribute to the initiation of movement and development of force in particular muscles (Ghez and Kubota, 1977). In addition to involvement in steering of muscle activities

in the limbs as pointed out above, red nucleus neurons are also modulated in phase with locomotion as was shown by electrophysiological recording in red nucleus in awake cats. This modulation disappears after removing the cerebellum, which may indicate that this activity reflects peripheral or spinal locomotor rhythms (Orlovsky, 1972; Padel and Steinberg, 1978). The red nucleus receives its principal excitatory input from the interpositus nucleus (Angaut and Bowsher, 1965; Tsukahara, 1967). Sensory input data generated by movements represent the major determinant of the output of the interpositus nucleus (Burton and Onada, 1978), and the discharge of both interpositus and red nucleus neurons is modulated during voluntary and locomotor movements. In addition, red nucleus neurons have wide cutaneous receptive fields by way of the cerebellum and respond to deep pressure as well as joint rotation. These fields are present in those limbs, in which the corresponding neurons elicit muscle contraction as demonstrated by microstimulation (Ghez, 1975; Padel, 1981).

Interposito-rubral EPSPs compared with cerebral evoked EPSPs show much faster rise times (Tsukahara, 1967). This strongly suggests that corticorubral fibers make synaptic contact with the remote dendrites of red nucleus neurons, while at least some of the cerebellorubral fibers terminate closer to the soma or on it (Tsukahara, 1968a). This is based on a cable-like property of dendrites (Rall, 1977).

The cortico-rubro-spinal pathway and the corticospinal pathways have an overlapping termination in Rexed laminae V-VII (Kuypers, 1964, 1981; Nyberg-Hansen and Brodal, 1963, 1964). In this respect it is of interest to note that both the pyramidal and rubrospinal tract in the cats hindlimb facilitate flexor motoneurons and inhibit extensor motoneurons (Hongo et al., 1965, 1969a; Lundberg, 1962; Pompeiano, 1957; Sasaki et al., 1960). At the cortical level there exists a collateral interaction between corticospinal neurons and nearby corticorubral neurons (projecting to the caudal red nucleus). This interaction is effected through recurrent axon collaterals in sensorimotor cortex. At the subcortical level pyramidal tract neurons inhibit or excite rubrospinal neurons through axon collaterals in the midbrain (Tsukahara et al., 1968b). The collateral interaction at both the cortical and subcortical levels influences the balance between cerebral and cerebellar control of the rubrospinal pathway (Tsukahara et al., 1968b).

In summary the cortico- and rubrospinal pathways have many features in common. In cat they both descend in the dorsolateral funiculus of the spinal cord (Nyberg-Hansen, 1966b; Nyberg-Hansen and Brodal, 1963, 1964) and terminate in largely overlapping areas of the spinal intermediate zone (Flindt-Egebak,

1977; Kuypers, 1964, 1981; Nyberg-Hansen and Brodal, 1963, 1964). Lesion experiments indicate that transsection of either of these two tracts produce similar deficits in motor function especially pertaining the distal limb movements (Kuypers, 1964; Sybirska and Gorska, 1980). However, in monkey, the movement capacity provided by the corticospinal tract shows a higher degree of motor resolution than the rubrospinal tract, since only the former tract provides the capacity to execute individual finger movements (Lawrence and Kuypers, 1968 I and II).

I.3. Aims and scope of the present investigations

The somatotopically organized rubrospinal pathway is the major component of the laterally descending brainstem pathways, and is especially involved in steering of fractionated movements of the distal parts of the limbs. Electrophysiological studies in cat showed that this fiber system, in contrast to the medially descending pathways, has a limited degree of collateralization in the spinal cord (Abzug et al., 1973 and 1974; Shinoda et al., 1977). The red nucleus projects also to the contralateral cerebellum (Brodal and Gogstad, 1954; Courville and Brodal, 1966). The collateralization of the rubrospinal neurons to the contralateral cerebellar interpositus nucleus is relatively high as indicated by anatomical and electrophysiological findings (Anderson, 1971; Brodal and Gogstad, 1954). Thus, the findings in these studies suggested that almost all rubrocerebellar fibers are collaterals from rubrospinal neurons.

In view of the above data it appears that the rubrospinal pathway represents a focussed system, which distributes its fibers to specific groups of spinal segments. On the other hand, the rubrocerebellar pathway probably represents a direct, tightly coupled return projection to the interpositus nucleus, which is the main source of afferents to the rubrospinal neurons (Courville, 1966b; Dekker, 1981; Flumerfelt et al., 1973; King et al., 1973; Tsukahara et al., 1967).

In the present anatomical study an attempt has been made to demonstrate anatomically the existence of collaterals in the rubrospinal pathway and to compare quantitatively the degree of this collateralization in rat, cat and monkey. This was done with the aid of

the multiple retrograde fluorescent tracer technique. This technique will be described in chapter II. In this anatomical study one fluorescent tracer was injected in the cervical grey and another in more caudal segments of the cord. In these experiments the distribution of single and double labeled neurons in red nucleus was studied. The descending pathways from the ventrolateral pontine tegmentum and from the raphe magnus, including the adjoining ventral reticular formation, also descend in the dorsolateral funiculus in rat, cat and monkey and also terminate in the dorsal grey (Basbaum and Fields, 1978, 1979; Holstege et al., 1979; Kuypers and Maisky, 1977; R.F. Martin et al., 1978; G.F. Martin et al., 1979 and 1981a; Tohyama et al., 1979a+b). Therefore, in these retrograde fluorescent double labeling studies the degree of the collateralization in these descending tracts was compared to that of the rubrospinal pathway in each of three mammalian species (i.e. rat, cat and monkey). In addition, in cat an attempt was made to determine the location of the rubro-olivary neurons in relation to the rubrospinal ones and to establish whether the rubro-olivary neurons give collaterals to the spinal cord. These studies will be presented in Chapter III and IV. In these studies attention was also paid to the rubro-cerebellar connections. Specifically an attempt has been made to determine whether these connections are established by collaterals of rubrospinal neurons. These findings will be presented in Chapter V. Electron microscopic studies in rat, combining the anterograde and retrograde intra-axonal transport techniques, showed that many rubrospinal neurons receive afferents from the cerebellar interpositus nucleus (Dekker, 1981). Some of the fluorescent tracers used in the present study can be employed both retrogradely to demonstrate parent cell bodies as well as anterogradely to demonstrate the fiber terminals. Using the fluorescent tracers in this fashion it could be demonstrated in light microscopy that the cerebellar interposito-rubral fibers establish contact with rubrospinal neurons. These data are also presented in Chapter V.

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CHAPTER II: FLUORESCENT NEURONAL TRACERS

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I. Introduction

In the past the neuroanatomical techniques for tracing fiber connections in the brain were primarily based on retrograde and anterograde degenerative changes which occur in neuronal cell bodies and axons (nerve fibers) after injury to the neuron (Brodal, 1940, 1957; Glees, 1946; Nauta and Gygax, 1954; Fink and Heimer, 1967). Recently several new tracing techniques have been developed, which are based on intraneuronal transport of substances. Such techniques are more effective in tracing fiber connections in the brain than the earlier degeneration techniques (Holstege et al., 1979, 1982; Martin et al., 1979, 1982). The present chapter will deal with the intraneuronal transport of some fluorescent substances. Originally, such fluorescent tracers were developed in order to demonstrate the existence of divergent axon collaterals. However, later it was found that they could also be used for other purposes and in combination with other techniques.

During the fourties Weiss and Hiscoe (1948) showed that there exists in the neuron a proximo-distal 'axonal flow'. Later the characteristics of this anterograde axonal flow were studied in detail (Grafstein and Forman, 1980). In the late sixties it became widely recognized that the anterograde axonal flow of radioactively labeled substances could be used to trace fiber connections in the brain. Thus tritiated aminoacids when injected in the brain are taken up by nerve cell bodies and incorporated into proteins. Subsequently the radioactive label is transported down the axons to their terminals. The distribution of the radioactive label can be demonstrated by means of the autoradiographic technique (Lasek et al., 1968; Cowan et al., 1972). Hendrickson (1972) was the first to show that in this way the labeled terminals of a fiber system can also be demonstrated in EM using EM autoradiography. Kristensson and Olsson (Kristensson, 1970; Kristensson and Olsson, 1971; Kristensson et al., 1971) and the LaVails (1972) showed that there also exists a retrograde axonal flow by means of which certain substances, i.e. bovine albumen and the enzyme horseradish peroxidase (HRP) can be transported retrogradely through axons to their parent cell bodies. The presence of HRP in the cell body can be demonstrated histochemically by means of several methods (Graham and Karnovsky, 1966; Mesulam, 1978; Mesulam and Rosene, 1979; Mesulam et al., 1982).

Classic Golgi studies (Cajal, 1952) showed that many axons in the brain give off collaterals. However, with the degeneration or intra-axonal transport techniques, it is very difficult to distinguish axon collaterals from unbranched fibers. Therefore in the late seventies several attempts were made to demonstrate the existence of axon collaterals by developing different retro-

grade tracers, which after retrograde transport through the stem axon and through a collateral respectively, can be demonstrated independently in the parent cell body. The following three methods for such retrograde double labeling of neurons are available.

A) The HRP-tritiated apo-HRP technique (Hayes and Rustioni, 1979, 1981). This technique is based on the fact that both HRP and tritiated apo-HRP are transported retrogradely to the cell body (Kristensson and Olsson, 1971; LaVail and LaVail, 1972; Geisert, 1976). The presence of HRP in the cell body is then demonstrated histochemically, while the presence of the tritiated-apo-HRP is demonstrated autoradiographically. This technique therefore requires a combined histochemical-autoradiographic procedure which can be applied to frozen sections. However, because the autoradiographic technique can only demonstrate the presence of a tritiated label in the upper 3 μm of the section (Sidman, 1970), double labeled neurons can only be demonstrated in these upper 3 μm.

B) The HRP-Iron dextran technique (Olsson and Kristensson, 1978; Cesaro et al., 1979). These two tracers are demonstrated histochemically in the parent cell body by first processing the sections for HRP and then demonstrating the presence of ferric ions by means of the Perl's reaction. However, double labeling may be underestimated when only a few granules of iron dextran or HRP are present in the cell body, because they tend to be masked by a massive accumulation of the other reaction product. This can be prevented by adapting the survival times to the fiber systems under study (Cesaro et al., 1979).

C) The fluorescent double labeling technique. This technique makes use of the fact that several fluorescent substances are transported retrogradely through divergent axon collaterals to the same parent cell body where they can be visualized independently by means of fluorescence microscopy. The present chapter will deal with this fluorescent double labeling technique (Kuypers et al., 1977). A general survey of the various fluorescent retrograde tracers, as they were tested in our laboratory, will be provided and a guideline will be given for the use of these tracers in animal experiments.

II. Development of the multiple retrograde fluorescent tracer technique for demonstrating axon collaterals.

The first steps towards the development of a multiple fluorescent tracer technique were made by Kristensson (1970) and by Stewart and Scoville (1976) who demonstrated that *Evans Blue (EB)* combined with bovine albumen (BA), is

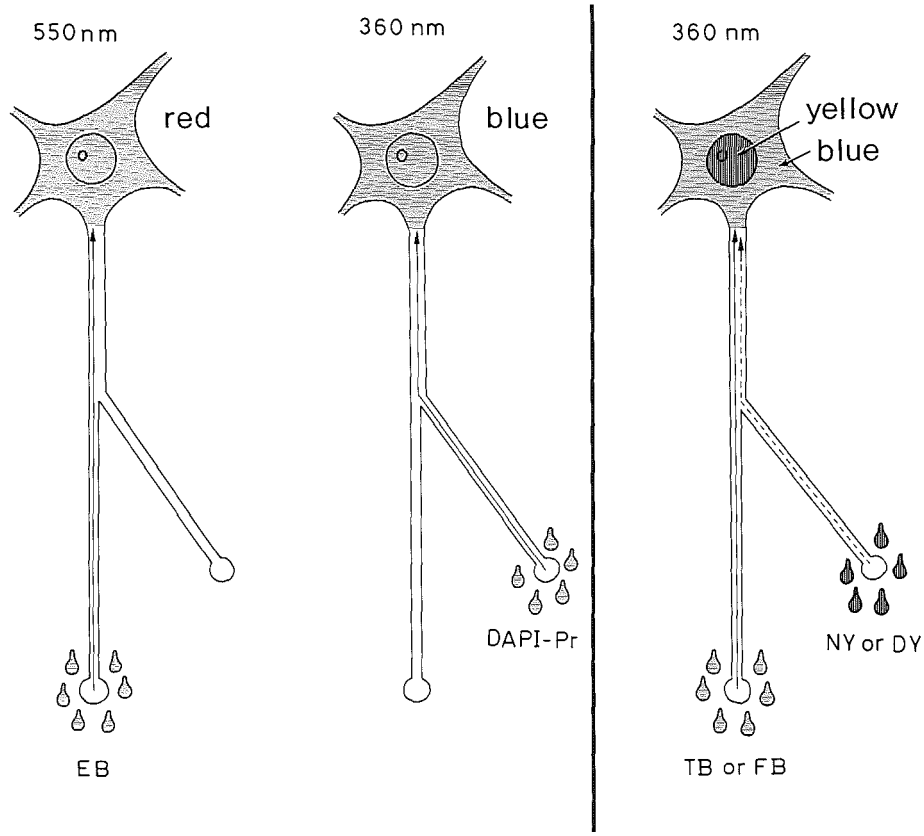

Fig. 1. This diagram shows two different fluorescent tracer combinations, which are suitable for use in double labeling experiments aimed at demonstrating the existence of divergent axon collaterals. On the left the combination „Evans Blue" (EB) and „DAPI/Primuline" (DAPI/Pr) is shown. EB after retrograde transport labels the parent cell body red at 550 nm (EB) and DAPI/Pr labels the parent cell body blue with golden fluorescent granules in the cytoplasm at 360 nm. On the right the combination „True Blue" (TB) or „Fast Blue" (FB) and „Nuclear Yellow" (NY) or „Diamidino Yellow" (DY) is shown. TB and FB after retrograde transport, label the cytoplasm of the parent cell body blue at 360 nm, and NY and DY label the nucleus of the parent cell body yellow at the same 360 nm excitation wavelength. DY refers both to Diamidino Yellow dihydrochloride (DY.2HCl) as well as to Diamidino Yellow diaceturic acid (DY.2aa).

transported retrogradely through axons to their parent cell bodies in the same way as HRP. EB can be demonstrated in these cell bodies by means of fluorescence microscopy because in formaline fixed material the EB labeled cell bodies show a flaming red fluorescence when illuminated with light of 550 nm wavelength (Kristensson, 1970; Kristensson et al., 1971; Steward and Scoville, 1976). Therefore the retrograde neuronal labeling by means of Evans Blue (EB) was studied in more detail (Kuypers et al., 1977). The findings showed that EB without bovine albumen (BA) may give as good or even better retrograde labeling of neuronal cell bodies than EB combined with BA. Thus when injections of EB (10% in water) in the tongue or in the caudate putamen in rat was followed by one or more days survival the hypoglossal neurons in the one case and the neurons in the centre median and the substantia nigra pars compacta (SNC) in the other were flaming red fluorescent. This red fluorescence was studied in frozen sections cut from formalin fixed material, which sections were mounted on slides and air dried, but were not coverslipped. They were studied with the aid of a Leitz Ploemopack fluorescence microscope, which was equipped with a high pressure lamp of 100 Watt and with filter mirror systems: A, D, and N2, which provide excitation light of approximately 360 nm, 390 nm and 550 nm wavelengths. The flaming red fluorescence of the EB labeled neurons could be clearly observed with filter mirror system N2 (550 nm wavelength) but only at higher magnifications (i.e. objectives of 25x or higher). However, lightly EB labeled neurons were difficult to differentiate from not labeled neurons, which at 550 nm wavelength show a red granular autofluorescence.

The efficacy of the combination of EB with HRP for double retrograde labeling of neurons was tested by injecting these substances together in caudate-putamen of rats followed by one day survival. In these cases many double labeled neurons were present in the substantia nigra, which neurons must have been double labeled through single axons. When viewed with filter mirror system A (360 nm excitation wavelength) the neurons displayed HRP granules in the cytoplasm, but when viewed with filter mirror system N2 (550 nm excitation wavelength) they showed only red fluorescence of the nucleus. The absence of the red fluorescence from the cytoplasm was probably due to the oxydation of EB by H_2O_2 in the presence of HRP, which accumulates in the cytoplasm, but does not enter the nucleus. However, since the red fluorescence of the nucleus in the EB-HRP double labeled neurons was sometimes difficult to detect, the combination of EB and HRP was regarded as less than satisfactory for retrograde double labeling.

In a new series of experiments an attempt was made to find another tracer which could be combined with EB in double labeling experiments. For this purpose the retrograde transport of a large series of substances was tested (Kuypers

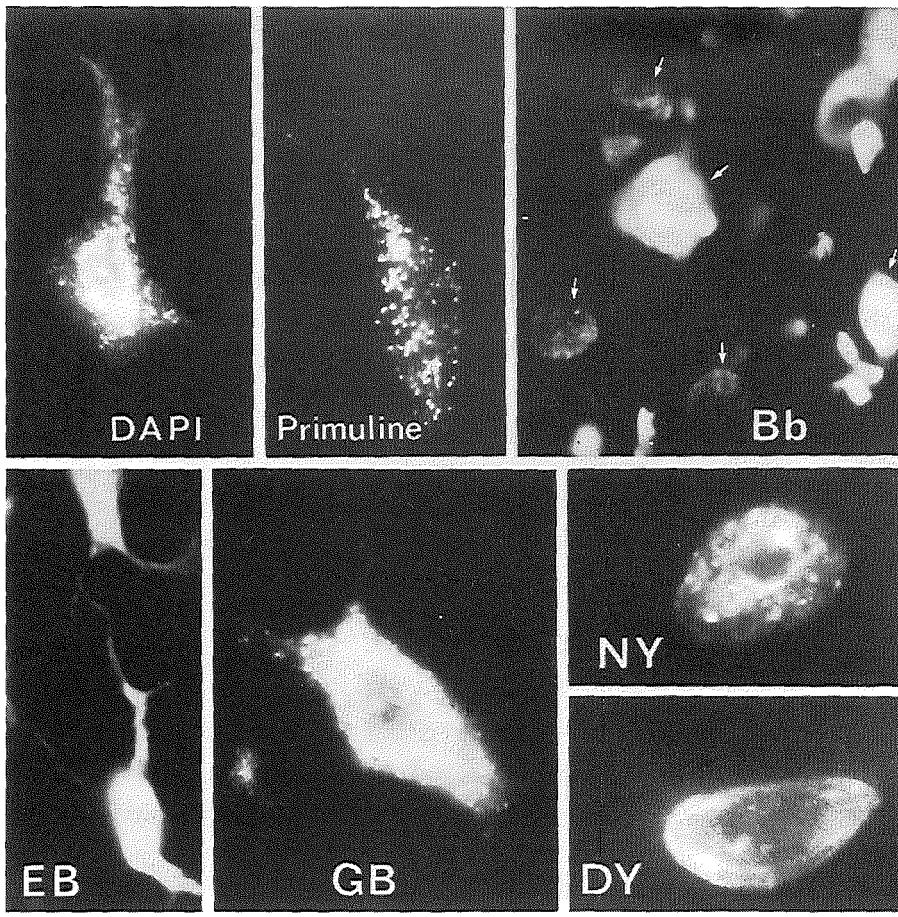

Fig. 2. Photomicrographs in *upper row* show neurons in the central nervous system single labeled with DAPI, Primuline and Bisbenzimide (Bb). Photomicrographs in *bottom row* show neurons single labeled with Evans Blue (EB), Granular Blue (GB), Nuclear Yellow (NY) and Diamidino Yellow (DY).
Note that around the retrogradely Bb-labeled neurons Bb-labeled glial nuclei are present, which are absent when using NY with restricted survival times or when using DY. Arrows indicate Bb-labeled neuronal nuclei.

et al., 1977) using the rat nigro-striatal system as a model. All these tracers are transported retrogradely both after injections in termination areas of fiber systems as well as after injections in the fiber bundles themselves. In the fluorescence microscope these tracers can be observed only at higher magnifications (i.e. objectives of 25x or higher). Thus, the retrograde transport of *DAPI* (2.5% in water) and *Primuline* (10% in water) was discovered.

Neurons, retrogradely labeled with DAPI display a bright blue fluorescence of the nucleus including the nucleolus and a duller blue fluorescence of the cytoplasm when viewed with filter mirror system A (360 nm excitation wavelength). Moreover, the labeled neurons are frequently surrounded by blue fluorescent glial nuclei. When viewed with filter mirror system D (390 nm excitation wavelength) DAPI labeled neurons are green fluorescent.

Primuline labeled neurons when viewed with filter mirror systems A and D display golden fluorescent granules in the cytoplasm but no labeling of the nucleus. The Primuline fluorescent granules can be differentiated easily from the autofluorescent clumps, which are also located in the cytoplasm, but which fluoresce brown-yellow at 360 nm. However, in neurons in which the cytoplasm is filled with these autofluorescent clumps, the Primuline granules may be difficult to detect. A similar labeling is obtained with the retrograde fluorescent tracer SITS, which in contract to the vast majority of the other tracers, seems to be taken up by terminals only and not by broken axons (Schmued and Swanson, 1982). The combination Primuline and DAPI (DAPI/Pr) has the advantage that both the nucleus (with DAPI) and the cytoplasm (with DAPI and Pr) are labeled. When EB and DAPI/Pr were injected together in rat caudate-putamen many double labeled neurons were present in centremedian-parafascicular complex and SNC. These neurons, which must have been double labeled through single axons, displayed a flaming red EB fluorescence when viewed with filter mirror system N2 (550 nm excitation wavelength) and a blue and golden granular fluorescence when viewed with filter mirror system A (360 nm excitation wavelength) (Figs. 1 and 2).

In view of the above findings the combination of EB and DAPI/Pr seemed suitable for use in double labeling experiments aimed at demonstrating the existence of divergent axon collaterals. In order to test this, the fiber projections from the mammillary bodies to the thalamus and mesencephalon were used as a model (Van der Kooy et al., 1978) because according to Cajal they are at least in part established by divergent axon collaterals. In rats EB was injected in the anterior thalamus and DAPI/Pr in the mesencephalic midline. After a survival time of 4 days many EB-DAPI/Pr double labeled neurons were

present in the lateral mammillary nucleus and in the medial portion of the medial nucleus. Also when EB and DAPI/Pr were injected in the left and the right anterior thalamus, respectively, many double labeled neurons were present in the mammillary nucleus on both sides.

The above findings clearly showed that EB and DAPI/Pr could be used as retrograde tracers in double labeling experiments. However, in double labeled neurons the EB fluorescence and the DAPI/Pr fluorescence is less pronounced than in single labeled ones. EB and DAPI/Pr were also used in experiments aimed at studying the existence of axonal branching in the ascending raphe and nigral projections (Van der Kooy and Kuypers, 1979) and in experiments aimed at clarifying the existence of axonal branching in the nigra-fugal connections (Bentivoglio et al., 1979a). In this latter study, it was demonstrated that the projections from the pars reticulata of the substantia nigra to the tectum and the thalamus are at least in part established by divergent axon collaterals of the same neurons as has been confirmed in electrophysiological experiments (Niijma and Yoshida, 1982). Further, in this study (Bentivoglio et al., 1979a) the efficacy of EB and DAPI/Pr in retrograde labeling of pars reticulata neurons from thalamus and from superior colliculus was found to be comparable to that obtained with HRP as demonstrated by the DAB technique (Graham and Karnovsky, 1966; Mesulam et al., 1982).

In several, largely unpublished, experiments long distance transport of DAPI/Pr was found to be somewhat inconsistent especially in cat, because in some cases beatiful labeling of cortical and brain stem neurons was obtained while in others no such labeling occurred. Therefore the search for other fluorescent retrograde tracers was continued. Thus it was found that *Bisbenzimide (Bb)* and *Propidium Iodide (PI)* are transported retrogradely over long distances, e.g. from the thoracic spinal cord to the sensorimotor cortex in rat and cat (Kuypers et al., 1979). These two substances, however, produce an entirely different type of retrograde neuronal labeling.

Nigral neurons, which are retrogradely labeled after injections of Bisbenzimide (Bb, 10% in water) in caudate-putamen display a yellow-green granular fluorescence of the neuronal nucleus which also shows a pronounced yellow-green fluorescence of its membrane and a pronounced yellow-green fluorescent ring around its nucleolus. In heavily labeled neurons, in addition, bright yellow fluorescent granules were present in the cytoplasm. This type of fluorescence is obtained when viewing the neurons with filter mirror system D (390 nm excitation wavelength), but, when using filter mirror system A (360 nm excitation wavelength) the nucleus showed a blueish green instead of yellow-green fluorescence (Fig. 2). In these experiments with long survival

times relative to the transport distance (e.g. several days for transport from striatum to substantia nigra in rats), also many fluorescent glial nuclei were present around the retrogradely labeled neurons (Fig. 2). These glial nuclei can be easily distinguished from the neuronal nuclei because the latter characteristically show a fluorescent ring around the nucleolus. The labeling of the glial nuclei was thought to be due to Bb migration from the neuronal cell body into the glial cells, associated with these neurons, in which glial cells Bb, because of its affinity to nucleotides, labeled primarily the nuclei. At that time it was not realized however, that this migration of Bb may produce false labeling of neurons (see later). Bb fluorescent glial nuclei also occur along axons which proceed from the area of the retrogradely labeled neurons to the injection area. These also occur along axons which proceed from the injection area to their terminations in other cell groups. It was therefore concluded that Bb proceeds both retrogradely and anterogradely through axons. However, after anterograde Bb transport through axons no transsynaptic labeling of recipient neurons was observed. Yet, later it was found that Bb (and Nuclear Yellow (NY) which is related to Bb) after very long survival times may produce transsynaptic neuronal labeling (Bentivoglio et al., 1980b; Aschoff and Holländer, 1982).

Propidium Iodide (PI) produces an entirely different retrograde labeling than Bb. Strongly PI labeled neurons in SNC after injection of PI (10% in water) in caudate-putamen display a brilliant orange-red fluorescence of cell body and proximal dendrites when viewed with filter mirror system N2 (550 nm excitation wavelength), but show little fluorescence when viewed with filter mirror systems A or D. Retrograde PI labeled neurons show very little nuclear labeling except for an orange-red fluorescence of the nucleolus. After long survival times relative to the transport distance (i.e. longer than the survival times in table I) PI fluorescent glial nuclei appear around the retrogradely labeled neurons.

From the characteristics of the retrograde labeling produced by Bb and PI it was inferred that they could be used in double labeling experiments demonstrating the existence of divergent axon collaterals. This was tested in the mammillary bodies of rats (Kuypers et al. 1979) by injecting Bb in one thalamus and PI in the other. Many PI-Bb double labeled neurons were present in the lateral mammillary nucleus on both sides in the same way as observed with EB and DAPI/Pr (see above). These double labeled neurons displayed an orange-red PI fluorecent cytoplasm when viewed with filter mirror system N2 (550 nm excitation wavelength) and a yellow-green (Bb) fluorescent nucleus when viewed with filter mirror systems A and D. Since then several investi-

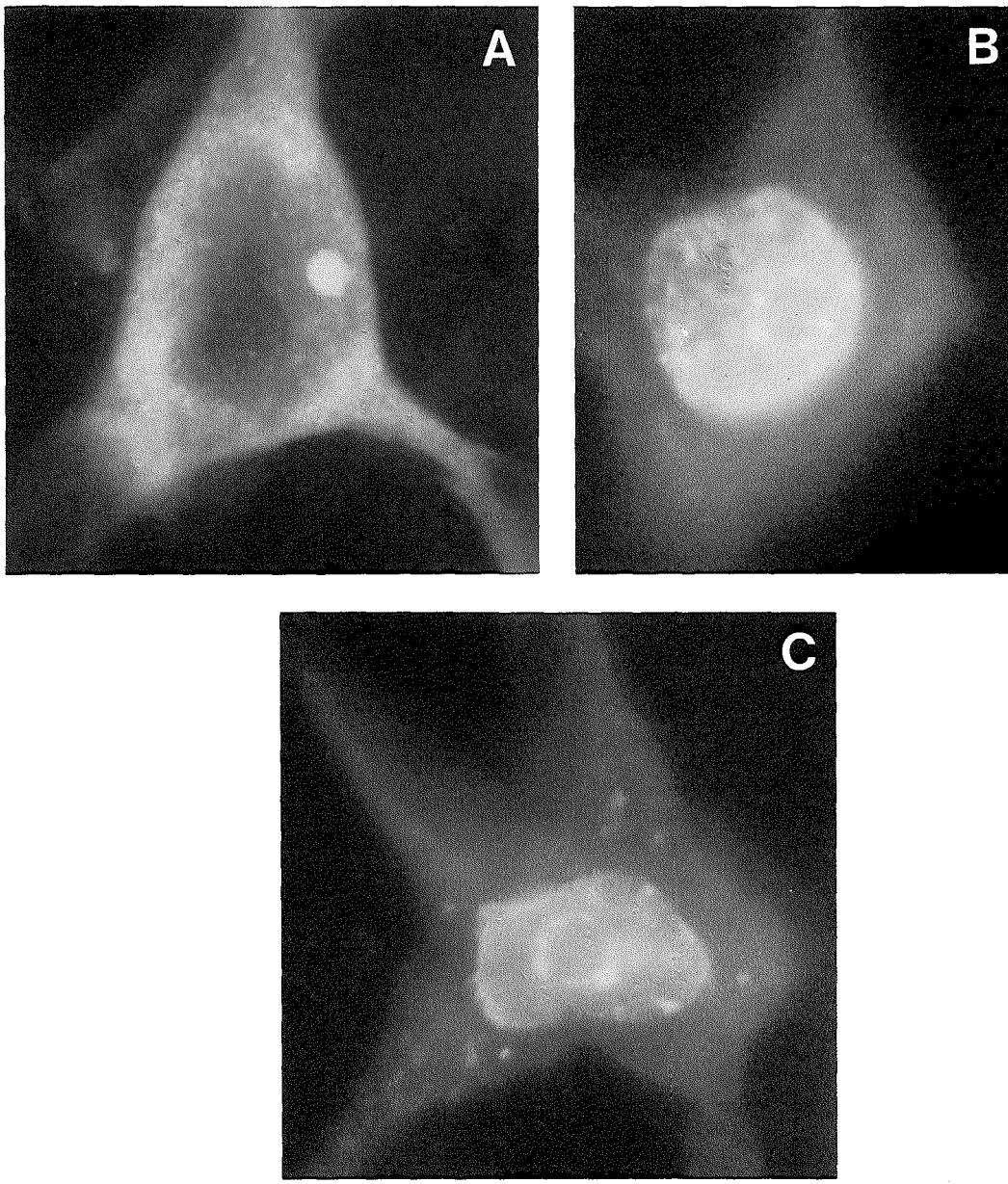

Fig. 3 Colorphotomicrographs of red nucleus neurons, retrogradely labeled from the spinal cord.
A: single TB-labeled neuron
B: single NY-labeled neuron
C: TB-NY double labeled neuron

gators have used PI for double retrograde labeling and even for triple retrograde labeling of neurons. In addition, PI has been used in combination with histochemical and immunohistochemical techniques (Olmos and Heimer, 1980; Van der Kooy and Wise, 1980; Björklund and Skagerberg, 1979a+b; Steinbusch et al., 1981; Brann and Emson, 1980; Hökfelt et al., 1979a+b, 1980). However, in our laboratory PI has seldom been used because it seems rather toxic and lightly PI labeled neurons are very difficult to distinguish from unlabeled ones. This latter difficulty, which is also encountered with EB, is due to the fact that even in a normal brain many neurons, when viewed with filter mirror system N2, display some orange-red autofluorescent granules in the cytoplasm. This makes lightly PI labeled neurons difficult to distinguish from unlabeled ones although PI labeled neurons show an orange-red fluorescent nucleolus.

In our experiments PI was not transported effectively over long distances, especially not in cat (Kuypers et al., 1979). Therefore the search for other retrograde tracers was continued. In view of the findings obtained with DAPI, special attention was paid to other diamidino compounds, all of which bind with RNA and DNA. A large series of diamidino compounds, synthesized in Dr. Dann's laboratory, was tested. Many of them give retrograde fluorescent labeling of neurons. The following two compounds seemed useful as retrograde tracers, i.e. *True Blue (TB)*[x] and *Granular Blue (GB)* (Bentivoglio et al., 1979; Rosina et al., 1980). In rats they are transported effectively over long distances (from the spinal cord to the cerebral cortex) and produce a blue fluorescent labeling of the neuronal cytoplasm in cell body and proximal dendrites when viewed with filter mirror system A (360 nm excitation wavelength). True Blue also gives a pronounced blue labeling of the nucleolus and Granular Blue produces an accumulation of blue silver granules in the cytoplasm (Figs. 2 and 3 and Chapter III, Fig. 3). Moreover, after long survival times relative to the transport distance

[x] In addition to TB mentioned in this chapter which is a chloride, there is also another TB compound available (i.e. TB-aceturate), which is more water soluble. However, this soluble-TB gives a smaller number of retrogradely labeled neurons as compared to the original TB.

(8 days, c.f. Table I) in the nigro-striatal system, some blue fluorescent glial nuclei are present around the retrogradely labeled neurons. These and further findings have been shown that TB is a very effective retrograde tracer in double and triple labeling experiments in rat (Olmos and Heimer, 1980; Swanson et al., 1980; Swanson and Kuypers, 1980a+b).

TB, despite its very favorable characteristics as a retrograde tracer, has the disadvantage that it is not transported effectively over long distances in cat. However, an other 'blue' diamidino compound was found, i.e. *'Fast Blue' (FB)* (Bentivoglio et al., 1980a) which is more soluble in water than is TB and is rather effectively transported retrogradely over long distances in rat, cat and monkey (Kuypers et al., 1980; Huisman et al., 1982). Retrogradely FB labeled neurons display a blue fluorescent cytoplasm when viewed with filter mirror system A, but the FB fluorescence is a little duller and slightly more greyish than the TB fluorescence (Chapter IV, Figs. 7 and 15). After long survival times (much longer than cited in table I) the blue cytoplasm contains some orange fluorescent granules and glial nuclei surrounding the retrogradely FB labeled neurons, become fluorescent. Moreover, contrary to the findings with TB, some very heavily FB labeled neurons with a brightly blue fluorescent cytoplasm occasionally display a white to blueish fluorescent nucleus (Bharos et al., 1981; Huisman et al., unpublished observations). In the central nervous system this phenomenon was rarely observed and only when a relatively long survival time was combined with a short transport distance. However, in the peripheral nervous system apparently this phenomenon is more frequently observed (Illert et al., in press)(see later).

The labeling characteristics of the 'blue' tracers suggested that Bb could be combined with TB or FB in double labeling experiments, because the double labeled neurons when viewed in the filter mirror system A (360 nm) would show a blue fluorescent cytoplasm and a yellow fluorescent nucleus. In addition in these cases the neuronal autofluorescence would not interfere with the detection of the tracer fluorescence since with filter mirror system A (360 nm excitation wavelength) the autofluorescence appears as brown-yellow granules in the blue labeled cytoplasm. The effectiveness of such double labeling was demonstrated in experiments using the efferent connections of the mammillary bodies in rat and cat as a model (Kuypers et al., 1980). However, Bb also provides a yellow-green cytoplasmic labeling which might obscure the blue TB or FB labeling. Therefore, in such double labeling experiments another benzimidazole, which was provided by Dr. H. Loewe of the Hoechst Company, i.e. *Nuclear Yellow (NY)* is regularly used (Bentivoglio 1980a), which gives primarily a nuclear labeling similar to that obtained with Bb

(Figs. 2 and 3). In respect to this type of double labeling it may be argued that the combination of FB and NY carries the disadvantage that in some cases single FB labeled neurons show some blue to white FB labeling of the nucleus which could make such neurons difficult to distinguish from FB-NY double labeled ones. However, in our experiments in the brain only very seldom a strong FB labeling of the neuronal nucleus was obtained (Bharos et al., 1981; Huisman et al., unpublished observations) and this blue to white FB labeling could always be clearly distinguished from the yellow to green NY (or DY, see below) labeling. Therefore the combination of FB and NY has been consistently used in double labeling experiments in cat and monkey (Fig. 1 and chapter IV, Figs. 7 and 15) and the combination FB, NY and EB has been used in triple labeling experiments in rat (Bentivoglio and Molinari, 1982).

In one of the first studies in which the TB-NY combination was used it became obvious however, that the NY and Bb migration out of the retrogradely labeled neurons might give rise to false labeling of neurons. The aim of this first study was to determine whether corticospinal neurons in rat possess callosal collaterals (Catsman et al., 1980). For this purpose NY (10% in water) was injected in one hemisphere and TB (2% in water) was injected ipsilaterally in the spinal cord followed by approximately 6 days survival time. In these experiments many of the TB labeled corticospinal neurons in the non-injected hemisphere were TB-NY double labeled. However, in electrophysiological experiments (Catsman-Berrevoets et al., 1980) no indication of the existence of corticospinal callosal collaterals could be obtained. Moreover, with other tracer combinations: e.g. EB and GB, no unequivocal double labeling of corticospinal neurons was found. It was therefore concluded that in the TB-NY experiments some false NY labeling of corticospinal neurons had occurred, probably due to migration of NY out of the retrogradely labeled callosal neurons as indicated by the presence of NY labeled fluorescent glial nuclei around these neurons (c.f. Kuzuhara et al., 1980). In a new set of experiments (Bentivoglio et al., 1980b) it was found that the NY and Bb migration out of retrogradely labeled neurons occurs gradually during the survival period, such that first the nucleus is labeled and then the cytoplasm plus the surrounding glial nuclei which after longer survival times become progressively more brilliantly fluorescent. These experiments further showed that the migration of Bb and NY from the retrogradely labeled neurons may be prevented by using 1% Bb in water, or 1% NY in water (instead of 10%) and by restricting the survival times, such that the glial nuclei around the retrogradely labeled neurons either are non-fluorescent or are only dull fluorescent i.e. much duller than the retrogradely labeled neurons. When in rat the injections in the spinal cord and the hemis-

phere were repeated in this manner such that first TB was injected in the spinal cord followed by a 6-day survival time, and NY (1% in water) was injected later in the hemisphere: 28 hours before the animal was sacrificed (28 hours NY survival time), none of the corticospinal neurons were double labeled. However, this was not due to a failure of the TB-NY combination to double label neurons because, when this same procedure was applied to the mammillothalamic connections, many double labeled neurons were present in the lateral mammillary nucleus even after very short NY survival times. In restricting the survival times NY was preferred above Bb, because NY requires a slightly longer transport time than Bb and migrates more slowly out of the retrogradely labeled neurons than Bb, which allow for a somewhat longer survival time.

The necessity to inject the two tracers at different times during the survival period, however, makes the double labeling with TB or FB in combination with NY a somewhat cumbersome procedure. Nonetheless, the combination of NY with TB or FB (Figs. 1 and 3) is still preferred above the combination of a red and a blue tracer because, as pointed out already, when using the red tracers, lightly labeled neurons are difficult to distinguish from non-labeled ones.

The complication that TB or FB and NY have to be injected at different times during the survival period would be avoided if NY could be replaced by another tracer, which also labels mainly the nucleus but does not migrate out of the retrogradely labeled neurons. Such a tracer could be injected together with TB or FB in the same session. In order to find such a tracer several diamidino compounds synthesized in Dr. Dann's laboratory were tested. The diamidino compound No 28826 was found which will be called *Diamidino Yellow dihydrochlorid (DY.2HCl)*[x]. This compound, which is related to GB, TB and FB,

x
Two DY compounds are available from Dr. Dann's laboratory and are manufacturized by Dr. Illing: DY dihydrochlorid (DY.2HCl) and DY diaceturic acid (DY.2aa). The latter compound, which produces neuronal labeling similar to that obtained with DY.2HCl is easier to purify. 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 less suitable for injections in the brain, because a) it often cloggs the injection needle or the glasspipette, 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 phenomenon may be avoided by using a 2% DY.2aa suspension in 0.2 M phosphate buffer (pH 7,2) under which circumstances the DY precipitates. Secondly, after short distance transport DY.2aa migrates more rapidly out of the retrogradely labeled neurons than DY.2HCl. Therefore it has been concluded that DY.2HCl should be preferred above DY.2aa but that in case of long distance retrograde transport a 2% DY.2aa suspension in 0.2 M phosphate buffer (pH 7,2) might be used (Keizer et al., in press).

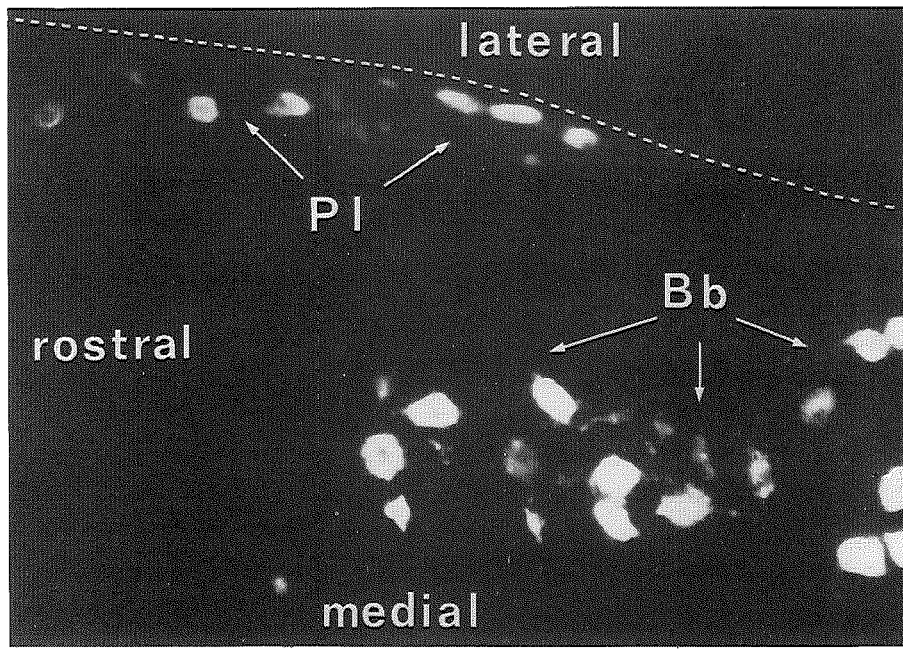

Fig. 4. Photomicrograph of two motoneuronal populations retrogradely labeled with Bisbenzimide (Bb) and Propidium Iodide (PI). Used with permission from Illert.

appears to meet the above requirements and soon will be commercially available (Keizer et al., in preparation). At 360 nm excitation wavelength DY.2HCl gives a diffuse golden yellow labeling of the neuronal nucleus with a lightly fluorescent ring around the nucleolus and a diffuse yellow labeling of the cytoplasm which sometimes also contains golden yellow fluorescent granules (Fig. 2 and chapter V, Figs. 4 and 5). DY.2HCl is effectively transported over long distances in rat and cat (Fig. 1) and can be successfully combined with TB and FB in double labeling experiments aimed at demonstrating the existence of divergent axon collaterals (Keizer et al. in preparation).

When trying to obtain double labeling of neurons by way of divergent axon collaterals it should be realized that the number of double labeled neurons is determined by the sensitivity of the weakest tracer. Therefore a combination of the most effective tracers should be used, because if one tracer is much less

sensitive than the other, i.e. labels much fewer neurons, only a small percentage of the neurons which give rise to divergent axon collaterals will be double labeled. In light of this the combination of TB or FB and NY or DY is preferred.

The fluorescent and the histological features of the injection areas produced by the various tracers reported in this chapter (EB, DAPI/Pr, PI, GB, TB, FB, Bb, NY, DY) are described in the corresponding publications (Kuypers et al., 1977, 1979, 1980; Bentivoglio et al., 1979, 1980a+b; Catsman-Berrevoets and Kuypers, 1981; Keizer et al., in preparation). These injection areas in general consist of several concentric fluorescent zones which surround the end of the needle track where the tracer has been deposited in the brain tissue. The findings in the various experiments indicate that the uptake and the retrograde axonal transport of the different tracers mainly occurs from the central zones of the injection areas both from fiber termination areas as well as from broken axons. However, as already mentioned SITS seems to be taken up by terminals only and not by broken axons (Schmued and Swanson, 1982).

The injection of the tracers produces tissue necrosis in the centre of the injection area. This is most pronounced with DAPI, GB, TB, FB and DY.2HCl. However, the occurrence of such necrosis does not interfere with the retrograde axonal transport of the tracers as indicated by the fact that DY.2HCl, which produces a relatively considerable necrosis in the centre of the injection area, gives frequently a retrograde labeling of a larger number of neurons than NY which produces relatively little necrosis.

The preceding description of the retrograde neuronal labeling obtained with the different tracers is based on observations in frozen section material of the brain. In this respect, it is of importance to emphasize that in different parts of the central nervous system and after different histological procedures a different type of labeling may be obtained. For example, as described by Björklund and Skagerberg (1979a+b) in freeze dried material TB and PI are present in the neuronal cytoplasm as iceblue and red fluorescent granules instead of being distributed diffusely as observed in frozen section material (Fig. 5). Further, under certain conditions the type of retrograde labeling of motoneurons observed in frozen section material after transport of the tracers through peripheral nerves (Illert et al., in press) differs from that obtained in neuronal systems in the brain. For example after dipping branches of the cats radial nerve in 10% FB dissolved in ethylenglycol (Illert et al., in press) a bright blue fluorescent labeling of both the motoneuronal cytoplasm and the motoneuronal nucleus was obtained which was so bright that it could be observed under low magnification (objective 10x).

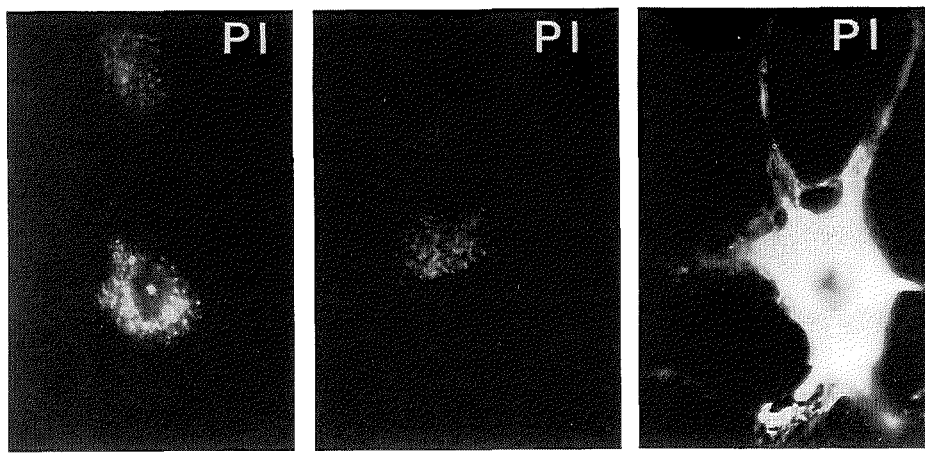

Fig. 5. Photomicrographs of retrogradely labeled neurons with Propidium Iodide (PI) in freeze dried material (left and middle photomicrograph). *Note* the granular distribution of PI in freeze dried material (used with permission from Björklund and Skagerberg) and the diffuse distribution of PI in frozen section material. Note also the labeled nucleolus in the left and the right photomicrographs.

Moreover, after intramuscular injections of 2% NY dissolved in ethylenglycol a strong predominantly white-blue cytoplasmic NY labeling of motoneurons occurred which also could be observed under low magnification, while only a soft yellow NY labeling of the nucleus was present (Illert et al. in press). Thus, under those specific circumstances NY labeled motoneurons resemble FB labeled ones. This has led Illert and his collaborators to the conclusion that when trying to differentially label motoneurons from different peripheral nerves according to their procedure, tracers with different emission spectra should be used (Fig. 4). The unusual FB, NY and Bb retrograde labeling obtained in motoneurons by Illert and his collaborators is probably due to the fact that by using their procedure these neurons are almost entirely saturated by the tracers. This probably results from the fact that large quantitities of the tracers are injected in the muscles and that the peripheral nerves are dipped in high concentrations of the tracers dissolved in ethylenglycol. This is indicated by the observations of Lemon and Muir (personal communications) who obtained a FB motoneuronal labeling similar to that observed in neuronal systems in the brain, after dipping the deep branch of the monkeys ulnar nerve in 5% FB (instead of 10%) which was dissolved in water with 2% DMSO (instead of in

ethylenglycol).

The large intramuscular injections as used by Illert and his collaborators produced not only a very heavily labeling of the motoneurons but produced also labeling of the endothelial cells lining the bloodvessels in the spinal cord. This endothelian labeling is only very rarely observed after small injections in the central nervous system and in all likelihood is due to the fact that the dye had entered the bloodstream.

III. Differential retrograde labeling of different members of a neuronal population by means of fluorescent tracers

Many cell groups in the brain distribute fibers to several target areas. Such projections are either established by divergent axon collaterals of individual neurons or by axons which are derived from different subsets of neurons. In the latter case injecting fluorescent retrograde tracers in the various target areas, as done when studying double retrograde labeling, will also reveal which subsets of neurons project to which target area. For this purpose the same combinations of fluorescent tracers can be used as in double labeling studies. Thus EB may be combined with DAPI/Pr, GB, TB, FB, Bb, NY or DY.2HCl and GB, TB and FB may be combined with Bb, NY and DY.2HCl. In this way it was demonstrated that the neurons in the pars reticulata of the substantia nigra project mainly to the thalamus and those in the pars compacta to the caudate-putamen (Bentivoglio et al., 1979a) and that the projection from the medial mammillary nucleus to the thalamus is exclusively ipsilateral (Van der Kooy et al., 1978). Further, it was shown in rat that different subsets of neurons in the paraventricular nucleus of the hypothalamus give rise to the projections to the pituitary gland on the one hand and to the brain stem-spinal cord on the other (Swanson and Kuypers, 1980), that the callosal and the spinal projections from the sensorimotor cortex are derived from different neurons which are frequently intermingled and that both in rat and cat corticospinal fibers and cortical fibers to the lateral thalamic nuclei are derived from different subsets of cortical neurons (Catsman et al., 1980, 1981). In this way it has also been demonstrated that the neurons in the intralaminar thalamic nuclei distribute their fibers to specific cortical areas (Bentivoglio et al., 1981) and that different cell populations in the red nucleus project to the cervical and the lumbosacral cord, respectively (Huisman et al., 1981, 1982) (Chapter III, Fig. 5), in agreement with earlier retrograde degeneration findings (Flumerfelt and Gwyn, 1974; Pompeiano and Brodal, 1957).

Similar findings are obtained in other cell groups as in the rat dorsal raphe and parabrachial nucleus, the cat Edinger-Westphal nucleus, the rat and primate globus pallidus and the pigeon thalamus (Burde et al., 1982; Van der Kooy and Hattori, 1980; Van der Kooy and Carter, 1981; Miceli and Repérant, 1982; Parent and de Bellefeuille, 1982; Voshart and Van der Kooy, 1981). Using this same technique it was also possible to clarify the precise topographical inter-relationship between two neighbouring populations of spinal motoneurons which distribute their fibers through different peripheral nerves to different muscles (Illert et al., in press). (Fig. 4).

IV. The use of retrograde fluorescent tracers in studying developmental changes in fiber connections in the brain.

The blue retrograde fluorescent tracers i.e. GB, TB and FB remain visible in the neuronal cell body for a rather long time and can still be demonstrated e.g. after a survival time of two months (Innocenti, 1981). This makes it possible to determine whether certain fiber connections in the brain which are present early in development are maintained at later stages. For this purpose two different tracers are injected in the same structure at different ages. Double retrograde labeling of neurons would indicate that the fibers which are distributed from these neurons to or through the injection sites at the time of the first injection are still present at the time of the second injection.

In this way developmental changes in the cortical callosal connections have been demonstrated in cat and rat. Thus, in cat FB injections were made at postnatal day 3 in the visual cortical areas 17 and 18 and NY injections were made in these same areas at day 27. In these animals in the non-injected hemisphere single FB labeled callosal neurons labeled only by the early injection were present throughout areas 17 and 18, while FB-NY double labeled neurons which were labeled by both injections were present only at the area 17 - area 18 border (Innocenti, 1981). O'Leary et al., (1981) showed in this same way that after TB injections in the rat parietal cortex on postnatal day 3 and NY injections in the same area on postnatal day 17, the non-injected hemisphere contained many single TB labeled callosal neurons throughout the parietal cortex but contained only columnar patches of TB-NY double labeled neurons. These findings have been interpreted to indicate that in rat and cat cortex at the early stages of development callosal connections of the parietal and visual areas are derived from neurons throughout these areas but that during

further development only the callosal fibers from restricted sets of neurons are maintained. However, in regard to such experiments it has to be kept in mind that the diamidino compounds (GB, TB, FB and DY.2HCl) because they become attached to DNA and RNA (Bentivoglio et al., 1979b; 1980a; Keizer et al., in preparation) may interfere with cell processes in which DNA and RNA are involved. As a consequence, in such double labeling experiments 'in time' it may be necessary to demonstrate that a failure to obtain double labeling does not result from a functional impairment of the neuron due to the presence of the first tracer (TB or FB). However, in the above experiments this probably does not apply since in HRP experiments in adult animals the retrograde HRP labeled neurons in areas 17 and 18 and in the parietal cortex are distributed in virtually the same way as the double labeled neurons in the retrograde fluorescent tracer study (Innocenti, 1981; Ivy et al., 1979, 1981; Wise and Jones, 1976).

V. Anterograde axonal transport of fluorescent tracers

As pointed out already, Bb and NY are transported also anterogradely through axons as indicated by the presence of fluorescent glial nuclei along their trajectory and in their termination area. However, this anterograde transport of Bb and NY does not produce a green or yellow fluorescent labeling of the axons. Yet, the retrograde and anterograde axonal transport of TB and FB does produce a blue fluorescent staining of the axons through which these tracers are transported including their terminal arborizations especially when using FB (Kuypers and Huisman, 1982; Huisman et al., in press; Rosina in press). Such anterogradely FB labeled axons and their terminal arborizations frequently show fluorescent varicosities reminiscent of those observed in histofluorescent monoamine containing axons (Björklund and Skagerberg, 1979a+b). The anterograde FB labeling of axons has been obtained both by injection of FB in the cell population which give rise to the fibers in question as well as by injecting FB in the fiber bundles. In both cases the neurons in question are probably damaged. Therefore some of the varicosities observed along the blue fluorescent axons may represent degenerative changes.

The anterograde fluorescent FB labeling of nerve fibers can be visualized at 360 nm excitation wavelength and the intensity of their fluorescence can be increased by perfusing the animal with 30% formaline instead of with 10% formaline (c.f. section VII).

Anterograde FB labeling of axons has been observed in several fiber systems in the brain. After FB injections in the caudal medulla oblongata in rat FB labeled axons were seen to proceed into the spinal funiculi and could be traced from the funiculi to the motoneuronal cell groups of the ventral horn (C5-C8)(Kuypers and Huisman, 1982). After FB injections in the inferior olive in cat FB labeled mossy fibers and their rosettes were seen in the cerebellar cortex (Kuypers and Huisman, 1982). After FB injections in the pericruciate cortex in cat FB labeled fibers could be traced through the cerebral peduncle and the pyramidal tract to their termination areas in the brain stem where their blue fluorescent terminal arborizations could be observed (Rosina, in press). After FB injections in the interpositus nucleus in cat FB labeled fibers could be traced from the cerebellum to the contralateral red nucleus where their terminal arborizations were blue fluorescent (Huisman et al., in press).

The anterograde fluorescent FB labeling of axons and their terminal arborizations can be combined with retrograde NY or DY.2HCl labeling of the recepient neurons. For example after FB injections in the cerebellar interpositus nucleus and NY injections in the contralateral dorsolateral funiculus of the spinal cord in cat, blue FB fluorescent fibers could be traced from the cerebellum to the contralateral red nucleus. The blue FB fluorescent terminal arborizations of these fibers could be observed to make contact with the cell body and the dendrites of the retrogradely NY labeled rubrospinal neurons (Huisman et al., Brain Research, in press) (Fig. 6).

VI. Combination of the retrograde tracers with other techniques

A. Formaldehyde induced histofluorescence technique for monoamines

The formaldehyde induced histofluorescence technique and its glyoxilic acid modification can be used to demonstrate the presence of different monoamines in nerve cell bodies and their axons (Axelsson et al., 1973; Björklund et al., 1972; Dahlstrom and Fuxe, 1964, 1965; Falck et al., 1962). In such material the catecholamines fluoresce yellow-green and the indolamines fluoresce brown-yellow (Björklund et al., 1968; Falck et al., 1962). This histofluorescence technique can be combined with retrograde fluorescent labeling, which makes it possible to determine the fiber connections of the monoamine containing neurons. This combined technique in general has been applied to freeze-dried material (Björklund and Skagerberg, 1979a+b; Albanese and Bentivoglio, 1982b) but can also be applied to chilled vibrotome sections

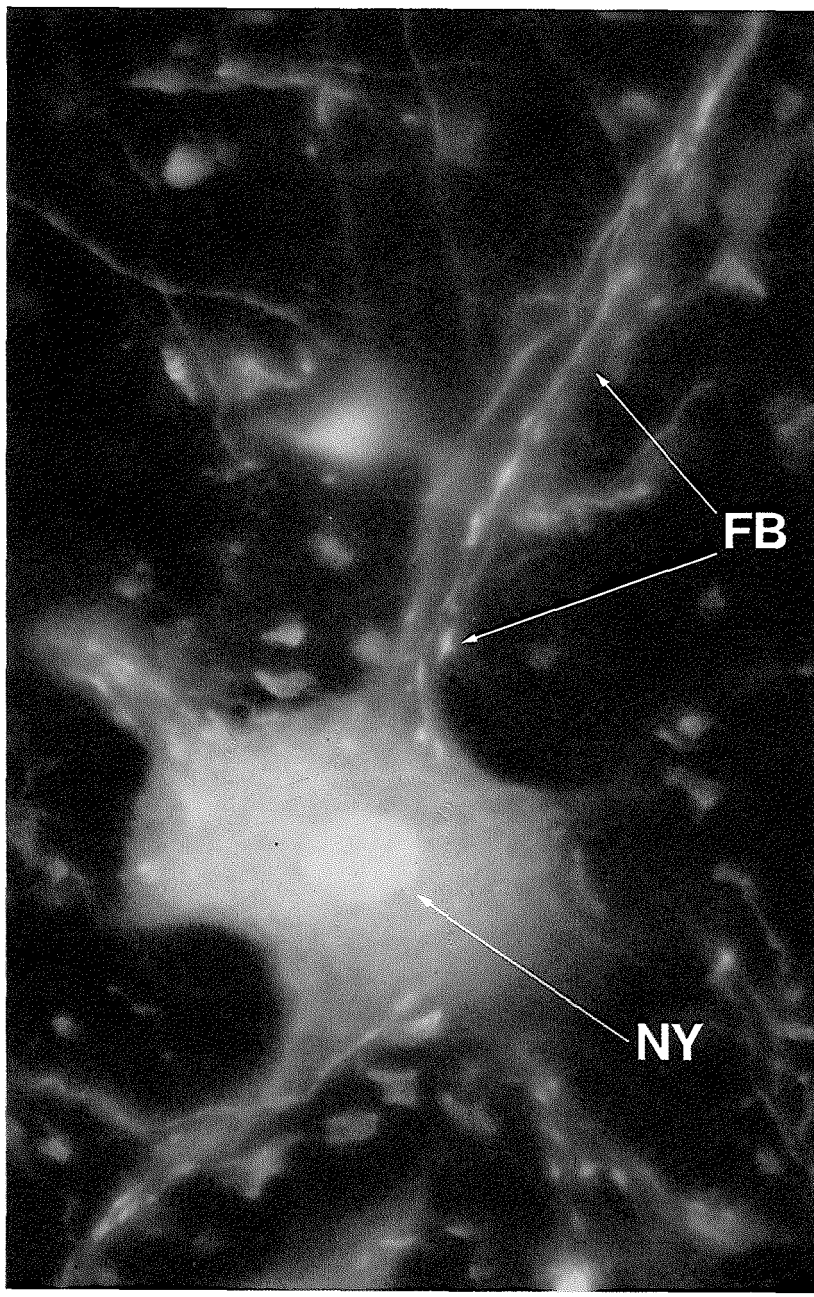

Fig. 6. Colorphotomicrograph of FB fluorescent fibers in cat red nucleus, which are anterogradely labeled from the contralateral cerebellar interpositus nucleus. *Note:* the fiber varicosities, which seem to be in contact with the surface of cell body and dendrites of the single NY labeled rubrospinal neuron.

(Van der Kooy and Wise, 1980). The retrograde fluorescent tracers TB, PI and EB are most suited for retrograde labeling of neurons in combination with monoamine fluorescence histochemistry, because these tracers and the monoamine fluorophores can be visualized independently in the same cell. This is facilitated by the fact that, as has been pointed out earlier, in freeze dried material TB and PI show a granular distribution throughout the cytoplasm (Fig. 4) while the monoamine fluorophores, show a more diffuse distribution. Retrograde double labeling through divergent axon collaterals by means of PI or EB in combination with TB can be combined with monoamine histofluorescence in which case three fluorescent markers (i.e. two retrograde tracers and one transmitter derived fluorophore) are visualized in one and the same cell (Björklund and Skagerberg, 1979a+b).

B). Acetylcholinesterase histochemistry

The enzyme acetylcholinesterase (AChE) when present in neurons can be demonstrated histochemically (Koelle, 1954; Butcher et al., 1975). This method can be combined with retrograde HRP labeling, which makes it possible to study the efferent connections of AChE neurons (Hardy et al., 1976). Recently it was shown that the procedure may be simplified by using retrograde fluorescent tracers instead of HRP, because under such circumstances only one histochemical procedure is required. Both the AChE brown reaction products and the fluorescent tracer can be observed in one and the same cell body by using bright field illumination and fluorescence microscopy, respectively (Albanese and Bentivoglio, 1982a). Applying this combined method in the rat substantia nigra it was shown that after injecting TB or FB (360 nm excitation wavelength) or EB (550 nm excitation wavelength) in the striatum some of the retrogradely labeled nigrostriatal neurons also contain AChE reaction products (Albanese and Bentivoglio, 1982a).

C. Immunohistochemical technique

Hökfelt and collaborators (Hökfelt et al., 1979a+b, 1980) were the first to combine the immunohistochemical technique with fluorescent retrograde tracers. Employing this technique Sawchenko and Swanson favor in particular TB out of the group of fluorescent tracers (Sawchenko and Swanson, 1981, 1982). However, some pitfalls are present, because during the immunohistochemical procedure several of the fluorescent tracers diffuse out of the retrogradely labeled neuron. This is probably due to the fact that the light fixation as used for the immunohistochemical procedure does not adequately anchor the tracer in the retrogradely labeled neurons (Sawchenko and Swanson, 1981). Under these circum-

stances it is therefore necessary to photograph the retrogradely labeled neurons before the immunohistochemistry is applied. By means of this combined technique the transmitter histochemistry of several pathways has now been demonstrated (Hökfelt et al., 1979a+b, 1980; Dalsgaard et al., 1982; Sawchenko and Swanson, 1982; Chesselet et al., 1982). When PI, Primuline or DY. 2HCl are used as retrograde tracers it is not necessary to photograph the retrogradely labeled neurons before the immunohistochemical procedure, because these tracers withstand the immunohistochemistry (Steinbusch et al., 1981; Brann and Emson, 1980; Dalsgaard et al., 1982; Skirboll et al., in preparation; Steward, 1981). However, when using TB a longer fixation is necessary to prevent TB from leaking out of the retrogradely labeled cell body during the immunohistochemical procedure (Sawchenko and Swanson, 1981). Skirboll et al. (in preparation) prefer a short fixation, because of the immunohistochemistry, which implies that the TB retrogradely labeled neurons have to be photographed before this procedure. Moreover, according to these investigators (Skirboll et al., in preparation) all data about retrogradely labeled neurons are preserved by photographing and plotting of the material prior to the immunohistochemical procedure since during this procedure the tracer may disappear out of the labeled neurons, which may cause a loss of 5% - 15% of the labeled neurons (Sawchenko and Swanson, 1981; Van der Kooy and Sawchenko, 1982).

D). Simultaneous steroid autoradiography and retrograde labeling of neurons

The steroid autoradiography makes it possible to localize the steroid target cells in the brain (Kim et al., 1977; Morrell et al., 1975). Combining the steroid autoradiography with the retrograde tracer technique makes it possible to establish both the location of steroid target cells and their fiber connections. For this purpose the retrograde fluorescent tracers Primuline, True Blue and Granular Blue may be used, since these tracers survive the autoradiographic procedures (Arnold, 1980; Morrell and Pfaff, 1982).

VII. Methods for using the fluorescent tracers Evans Blue (EB), DAPI, Primuline (Pr), DAPI-Primuline mixture, Propidium Iodide (PI), Granular Blue (GB), True Blue (TB), Fast Blue (FB), Nuclear Yellow (NY), Diamidino Yellow (DY).

When one begins to use the fluorescent tracers it is of importance first to gain some experience with the type of retrograde labeling they produce. For this purpose the rat nigrostriatal system can be used as a reliable testing ground. Therefore when starting to use the fluorescent tracers one should first in a few rats inject 0,4 µl to 0,6 µl of the tracer in the head of the caudate followed by 3 days survival time. Under these circumstances the neurons of the substantia nigra pars compacta are consistently labeled.

The tracers are dissolved or suspended in distilled water with the aid of an ultrasonic waterbath (for % solution or suspension used for the different tracers, see table I). In general each time 0.1 ml of a solution or suspension is made up which may be used for 2 weeks, and which is stored in a small phial at 4^{o}C. However, the use of fresh solutions or suspensions is preferred.

The tracers are injected either by means of a glass micropipette (tip diameter 40-80 µm), which is connected to an oil filled pressure system, or by means of a Hamilton microsyringe equipped with a 22 Gauge needle. When suspensions are injected by means of a micropipette a pipette with a tip diameter of more than 60 µm should be used, because with smaller tip diameters the pipette tends to become clogged. The smallest injection area obtained after injecting 0,1 µl TB in rat spinal cord grey matter with a micropipette of a tip diameter of 60 µm measured in 1 mm in diameter (chapter III, Fig. 12). The same result has been obtained after injections of FB and DY in the cortex (Bullier, personal communications). However, smaller injection areas (diameter of 0.5 mm) can be obtained by means of iontophoresis or chronic implantations of micropipettes (Adelheid and Carlsen, 1982).

The micropipette or Hamilton microsyringe with which the tracers are injected is held a few minutes in situ after injection. This helps to minimize spreading of the tracer liquid up into the needle track. For each tracer a separate pipette or needle is used in order to avoid contamination which may produce false double labeling.

All tracers are transported retrogradely from terminal fields, but they are also transported retrogradely from damaged axons. Dissolving the tracers in 2% dimethylsulfoxide (DMSO) enhances their transport from damaged axons, passing through the injection area (Huisman et al., 1982). In order to detect retrograde neuronal labeling by means of the fluorescent tracers, the survival

	Code number of the tracers	Filter Mirror System A providing excitation light of 360 nm wavelength	Filter Mirror System N2 providing excitation light of 550 nm wavelength
Evans Blue (EB)			cytoplasm + nucleus: red
DAPI/ Primuline	Serva,Heidelberg 18860/Eastman, 1039	cytoplasm + nucleus: blue/ cytoplasm : light golden granules	
Propidium Iodide (PI)	Sigma; P-5264		cytoplasm + nucleolus: orange-red
Granular Blue (GB)	Diamidino [x] Compound 186/134	cytoplasm: blue, with silver golden granules	
True Blue (TB)	Diamidino [x] Compound 150/129	cytoplasm + nucleolus: Blue	
Fast Blue (FB)	Diamidino [x] Compound 253/50	cytoplasm + blue with fine silver granules	
Nuclear Yellow (NY)	Benzimidazole [xx] Compound Hoechst S769121	nucleus + nucleolar ring: golden yellow (granular)	
Diamidino Yellow (DY)	Diamidino [x] Compound 288/26	nucleus + nucleolar ring: golden yellow (diffuse)	

[x] The code numbers were given by the institute of Pharmacy and Food chemistry of the Friedrich-Alexander University in Erlangen (F.R.G.) where these substances have been synthetized. For research purposes samples can be obtained from Prof.Dr. Illing, Warthweg 14-18 Postfach 1150 D-6114 Gross Umstadt Germany

[xx] For research purposes small samples of NY can be obtained from Dr. H. Loewe, Hoechst Aktiengesellschaft, Postfach 800320, 6230 Frankfurt am Main 80, F.R.G.

Table I: shows for the tracers, which are still used, the code numbers, the label characteristics at 360 and 550 nm, the percentages of tracer solutions, and the survival times, necessary for proper labeling. *Note* the relatively short survival times, which are used for Nuclear Yellow (NY).

	SURVIVAL TIME, necessary for proper labeling				
% tracer solution (w/v)	RAT Caudate/Put to Nigra (SNC)	RAT spinal C5 to Red Nucleus	RAT spinal T1 to cortex	CAT spinal C5 to red Nucleus	CAT spinal T1 to cortex
10%	24-48h				
2,5%/10%	4d				
3%	2d	7d	(7d) light labeling		
5%	2-4d	5-7d	7-9d		
2%	2-4d	5-7d	7-9d		
3%	2d	4d	4d	3-4w	3-4w
1%	6h	24h	40h	±46h	±70h
2%	2-3d	7d	10d	3w	4w

time has to be chosen such that enough tracer has accumulated in the cell body. Therefore two factors determine the optimal survival time, i.e. the type of tracer and the transport distance. Moreover, proper retrograde labeling with the same tracer over the same distance may require different survival times in different fiber systems. Therefore when applying the tracers in a given fiber system, it is necessary to do a few preliminary experiments in order to determine the optimal survival time (see table I for survival times in striatal-nigral system and descending pathways in rat and cat). The survival times for NY must be relatively short, because at longer survival times NY migrates out of the retrogradely labeled neurons which produces bright fluorescence of the nuclei of adjoining glial cells and may result in false retrograde labeling of surrounding neurons (Bentivoglio et al., 1980b). Therefore the NY survival times are titrated such that at the most a dull glial staining occurs around the retrogradely labeled neurons. This implies that when NY is used in combination with tracers which require a longer survival time e.g. TB or FB, they are injected first and NY is injected later a short time before the animal is sacrificed. In this way a relatively long TB or FB survival time is combined with a relatively short NY survival time. This somewhat complicated procedure may be avoided by using DY instead of NY, since during the relatively long survival times required by TB or FB, DY does not leak out of retrogradely labeled neurons (Keizer et al., in preparation). In general TB is used in rat because it is more blue fluorescent than FB. On the other hand FB is preferred above TB in cat and monkey, because FB is more effectively transported over long distances as required in these animals (c.f. table I).

After the appropriate survival time the animals are sacrificed with an overdosis Nembutal (6%) and are transcardially perfused with saline followed by formalin. When the fluorescent tracers are combined with HRP, the perfusion with glutaraldehyde diminishes drastically the intensity of the fluorescence and changes its characteristics. Therefore, under these circumstances a formaline perfusion should be used as advocated by Kevetter and Willis (1982). In general the perfusion solutions are pumped through the circulation with a speed of 80 cc a minute. Rats are perfused with 0.5 liter hypertonic NaCl (1.5%) followed by 1 liter cacodylate or citrate buffered formalin (10%, pH 7,2) and the brain and spinal cord are then stored overnight in cacodylate or citrate buffered sucrose (30%, pH 7,2) at 4^{o}C. Cats and monkeys are transcardially perfused with 2 liter hypertonic NaCl (2.7%) followed by 3 liter cacodylate or citrate buffered formaline. In general a solution of 10% formaline (pH 7,2) is used. However, with FB 30% formaline (pH 7,2) is used since this stronger fixation enhances the FB fluorescence. Brain and spinal cord of

cats and monkeys are not stored overnight in sucrose. These animals, following the formaline perfusion, are perfused with cacodylate or citrate buffered sucrose (8%, pH 7,2) instead and the brain and spinal cord are dissected and immediately cut in frozen sections. In our studies the tissue is frozen to approximately -30°C on a freezing microtome and then cut in frozen sections of 30 µm thick. In order to prevent freeze artefacts the temperature is kept constant during cutting of the sections. The material does not have to be cut immediately but first can be stored at very low temperature (-80°C). Injected spinal segments of a rat spinal cord are coated with carboxymethylcellulose in order to preserve the sections containing the needle tracks. Especially in the case in which NY is injected a rapid processing of the material is important because when the sections are kept in water for some time NY may migrate out of retrogradely labeled neurons (Bentivoglio et al., 1980b). Therefore the sections, after being cut, are immediately mounted from distilled water. The mounted sections are air dried and not coverslipped. The material is studied with a Leitz Ploemopack fluorescence microscope with a high pressure lamp of 100 Watt. The microscope is equipped with a filter mirror system A, D and N2, providing excitation wavelength of 360 nm, 390 nm and 550 nm, respectively. The retrogradely labeled neurons are only clearly visible with objectives of 25x or higher. These objectives require the use of immersion oil, which is applied directly to the air dried sections. The distributions of the single and double labeled neurons in the sections are plotted with the aid of an X-Y plotter, which is connected with transducers attached to the microscope stage. In order to localize exactly the plotted neurons the sections after being plotted are counterstained with cresyl violet. Fluorescent counterstains may also be used (Schmued et al., 1982). Pictures (black and white or color) are taken with the aid of a Leitz-vario-orthomat which regulates the exposure time automatically. For black and white pictures an Agfapan 100 film is used and for color pictures a Kodak Ektachrome 200 film is used. Also a Kodak Ektachrome 400 or 800 film can be used. When illuminating the sections the fluorescence in the retrogradely labeled neurons gradually fades. This is especially obvious in lightly TB or FB labeled neurons. The fluorescence also diminishes with time, especially in slides covered with oil. In order to slow down this fading of the fluorescence the sections are stored in the dark and at 4°C. In this way sections without oil may be kept for several weeks. After long storage the tissue starts to display a diffuse glistering fluorescence, which tends to obscure the fluorescence of the retrogradely labeled neurons.

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CHAPTER III: QUANTITATIVE DIFFERENCES IN COLLATERALIZATION OF THE DESCENDING SPINAL PATHWAYS FROM RED NUCLEUS AND OTHER BRAINSTEM CELL GROUPS IN RAT AS DEMONSTRATED WITH THE MULTIPLE FLUORESCENT RETROGRADE TRACER TECHNIQUE

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QUANTITATIVE DIFFERENCES IN COLLATERALIZATION OF THE DESCENDING SPINAL PATHWAYS FROM RED NUCLEUS AND OTHER BRAIN STEM CELL GROUPS IN RAT AS DEMONSTRATED WITH THE MULTIPLE FLUORESCENT RETROGRADE TRACER TECHNIQUE

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Key words: red nucleus — nucleus raphe magnus — ventrolateral pontine tegmentum — multiple fluorescent tracers — collateralization

SUMMARY

In 8 rats 'True Blue' was injected into dorsal half of C5-C8 spinal grey, 5 days later 'Nuclear Yellow' was injected in midthoracic, upper lumbar, lumbosacral and sacral cord respectively. The animals were sacrificed about 43 hours after NY injections. The distribution of retrogradely labeled neurons was studied in Red Nucleus, in Ventrolateral Pontine Tegmentum and in Nucleus Raphe Magnus, all of which project to spinal dorsal grey.

In Red Nucleus large populations of single TB-labeled neurons and single NY-labeled ones occurred in the dorsomedial and ventrolateral part, respectively. In addition, about 8% of the neurons labeled with TB from C5-C8 were double labeled with NY from L5-S1, and 35% from T7-8, which percentages resemble those of electrophysiological studies[15]. However, in ipsilateral Nucleus Raphe Magnus about 40% of the TB-labeled neurons were double labeled from L5-S1. This percentage resembles the 66% obtained in electrophysiological studies of reticulospinal collaterals[10]. These findings in rat support electrophysiological findings in cat and show, that rubrospinal neurons distribute their fibers primarily to the grey matter of specific groups of spinal segments, while many of the raphe spinal neurons distribute fibers throughout the spinal cord.

INTRODUCTION

Classic anatomical studies using the Golgi technique[1-4] demonstrated that many axons in the central nervous system give off axon collaterals along their trajectory

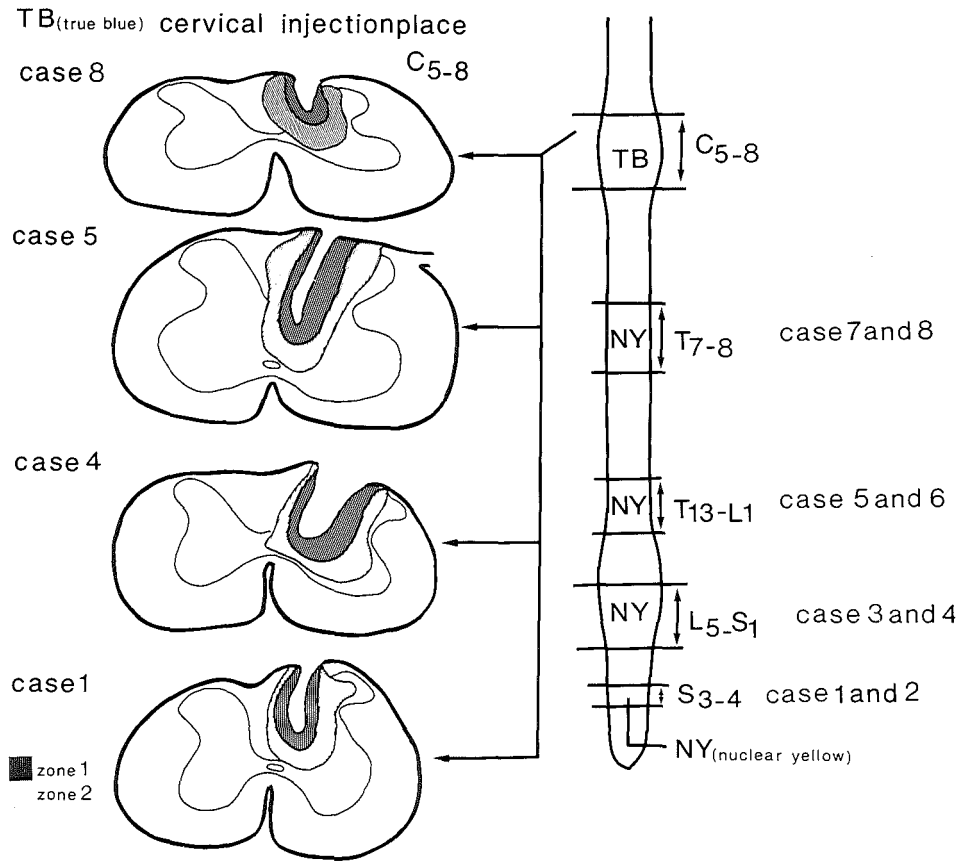


Fig. 1. Diagram of the injection sites in the 8 rats reported in this paper. True Blue (TB) was injected in all 8 cases in the dorsal part of C5–C8 spinal gray matter. For characteristics of zones 1 and 2 see text. The TB injection was combined with a Nuclear Yellow (NY) injection in cases 1 and 2 at S3–4, in cases 3 and 4 at L5–S1, in cases 5 and 6 at T13–L1 and in cases 7 and 8 at T7–T8.

through the brain. For a long time little attention has been paid anatomically to these collaterals, mainly because modern tracing techniques[15,20,36] did not make it possible to distinguish divergent axon collaterals from unbranched fibers. However, during recent years electrophysiological studies have reopened the issue of the existence of axon collaterals especially in respect to the descending pathways[1,2,27,40,45]. Recently the existence of divergent axon collaterals has also been studied anatomically by means of the retrograde neuronal double labeling techniques using for example two fluorescent retrograde tracers which, after being transported retrogradely through an axon and its collateral, label in different colors different features of the parent cell[31]. Similar results can be obtained by means of HRP and [^{3}H]apo-HRP[24]. In the present study the former technique has been used to study the collaterals of the brainstem pathways in rat, which descend from red nucleus (RN), ventrolateral pontine tegmentum (VLPT) and nucleus raphe magnus (NRM) to spinal cord where they all

terminate in the dorsal half of the spinal gray matter[7,12,18,26,34,37,41,50]. The retrograde fluorescent tracers True Blue (TB)[9] and Nuclear Yellow (NY)[10,11] were used. TB produces a blue fluorescent labeling of the cytoplasm and the nucleolus while NY, after short survival times relative to the transport distance[11], produces only a golden yellow fluorescence of the nucleus both tracers at 360 nm excitation wavelength. In all animals first TB was injected in the dorsal half of the C5–8 spinal gray. Subsequently in the various animals NY was injected at more caudal levels in the cord (Fig. 1). In all cases in which the TB deposit was restricted to the gray matter, the number of TB-labeled neurons and the number of TB–NY double-labeled ones in the above brain stem cell groups was counted. These data made it possible to approximate both the number of neurons in the three different brain stem cell groups in rat which distribute fibers to the dorsal half of C5–8 spinal gray and the percentages of these cells, which distribute fibers to the various, progressively more caudal portions of the neuraxis.

MATERIALS AND METHODS

In 8 rats, anesthetized with Nembutal (6%), low cervical laminectomies were made and in each rat 5 glass micropipette injections of 0.1 μl 2% True Blue (TB) (=0.5 μl in total)[9] were made in a rostrocaudal row into the dorsal horn and the dorsal part of the intermediate zone of the C5, C6, C7 and C8 segments (Figs. 1 and 2). After 5 days the animals were operated for a second time. Under Nembutal, new laminectomies were made and 1 μl 1% Nuclear Yellow (NY)[10,11] was injected in the spinal white and gray matter at more caudal levels of the cord. These NY injections were made by means of multiple micropipette penetrations to damage a large number of fibers. This was in order to obtain NY transport by fibers passing through the injected segment and damaged by the penetrations, as well as by fibers terminating there. In two rats NY injections were made in the sacral cord (cases 1 and 2), in two rats in the lumbosacral cord (cases 3 and 4), in two rats in the upper lumbar cord (cases 5 and 6) and in two rats in the midthoracic cord (cases 7 and 8) (Fig. 1). NY injections at the different levels were made 45, 44, 43 and 40 h prior to the perfusion of the animals, respectively. These short survival times were used in order to avoid migration of NY from the retrogradely labeled neurons in the brain stem[11]. During the entire postoperative period the animals were given morphine 0.5 mg/day.

The rats were sacrificed with an overdose of Nembutal (6%). They were transcardially perfused with 1 liter NaCl (1.5%) followed by 1 liter cacodylate-buffered formaline (10%, pH 7.2). The brains and the injected segments were stored overnight in cacodylate-buffered sucrose (30%, pH 7.2) at 4 °C and were cut transversally in frozen sections 30 μm thick on a freezing microtome. The injected segments, before being cut, were coated with carboxymethylcellulose in order to keep the sections containing the needle tracks intact. The sections, after being cut, were immediately mounted from distilled water and air dried at room temperature. In general one out of every three sections was mounted.

The material was studied with a Leitz Ploemopack fluorescence microscope equipped with a filter mirror system A providing excitation wavelength of 360 nm wavelength. Subsequently some of the sections were counterstained with cresyl violet.

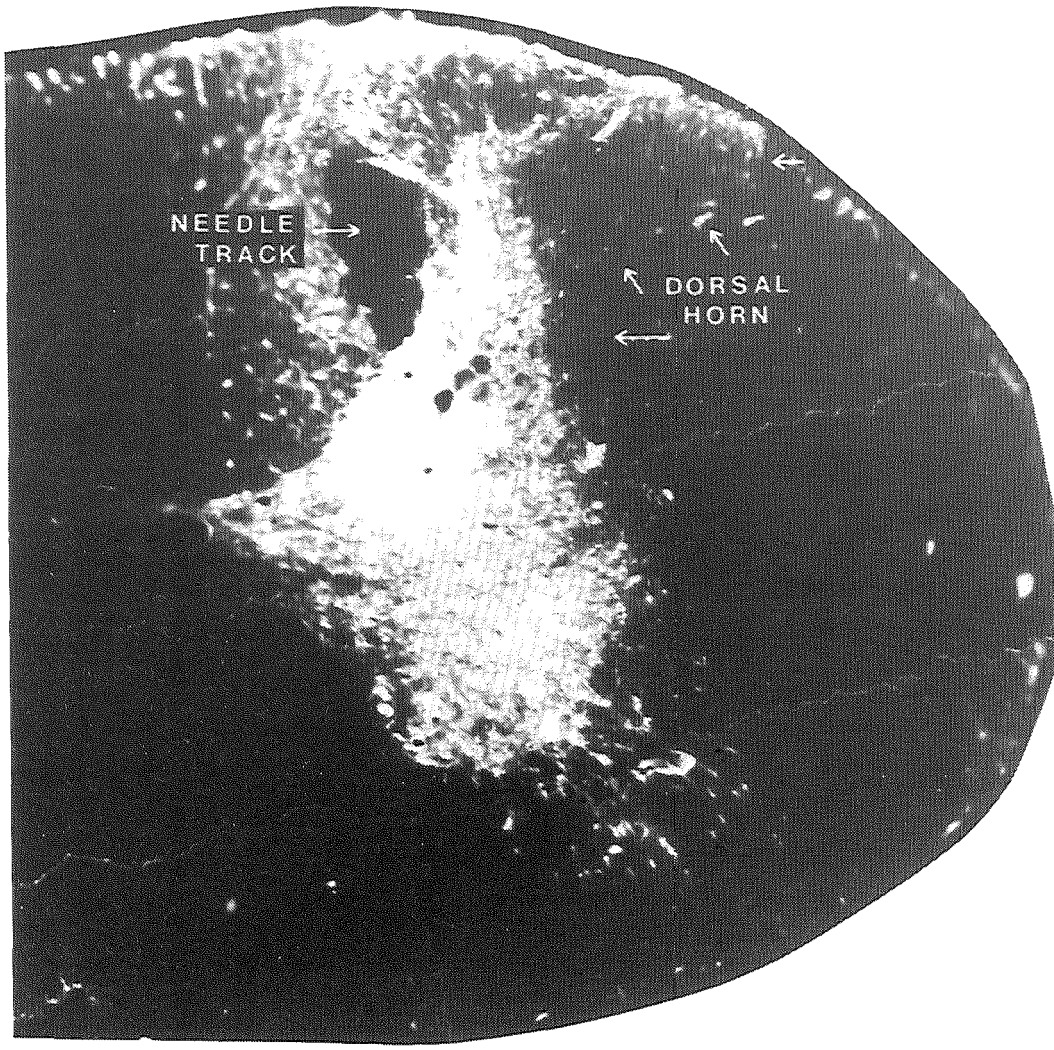


Fig. 2. Photomicrograph of one half of a cross-section between C5 and C8 with TB injection site in the spinal gray matter. Note that fluorescence extends through ipsilateral spinal gray matter but that no fluorescence is present in the dorsolateral funiculus.

The distribution of the single TB- and single NY-labeled neurons and of the TB–NY double-labeled one in the contralateral red nucleus (RN), the contralateral ventrolateral pontine tegmentum (VLPT) and the ipsilateral nucleus raphe magnus (NRM) was charted with the aid of an X–Y plotter connected with transducers attached to the microscope stage.

At 360 nm excitation wavelength, the retrograde single TB-labeled neurons (Fig. 3) showed a deep blue fluorescent cytoplasm and a blue fluorescent nucleolus, the retrograde single NY-labeled neurons after short survival time relative to the transport distance (Fig. 3) showed a golden yellow fluorescent nucleus with a clear fluorescent

ring around the nucleolus[10,11] and the NY–TB double-labeled neurons (Fig. 3) showed all mentioned features[31]. In the present experiments either no fluorescent glial nuclei or only a few dull fluorescent ones were present around the single NY-labeled neurons as well as around the NY–TB double-labeled ones. This indicates minimal migration of NY from the retrogradely labeled neurons[11].

RESULTS

Injection areas

In the 8 rats the TB fluorescent injection areas involved the dorsal horn with the intermediate zone and extended ventrally into the ventral horn (Figs. 1 and 2). In the animals reported here no fluorescence occurred in those parts of the dorsolateral funiculus in which the rubrospinal, raphe magnus spinal and VLPT-spinal fibers are located[6,30,34].

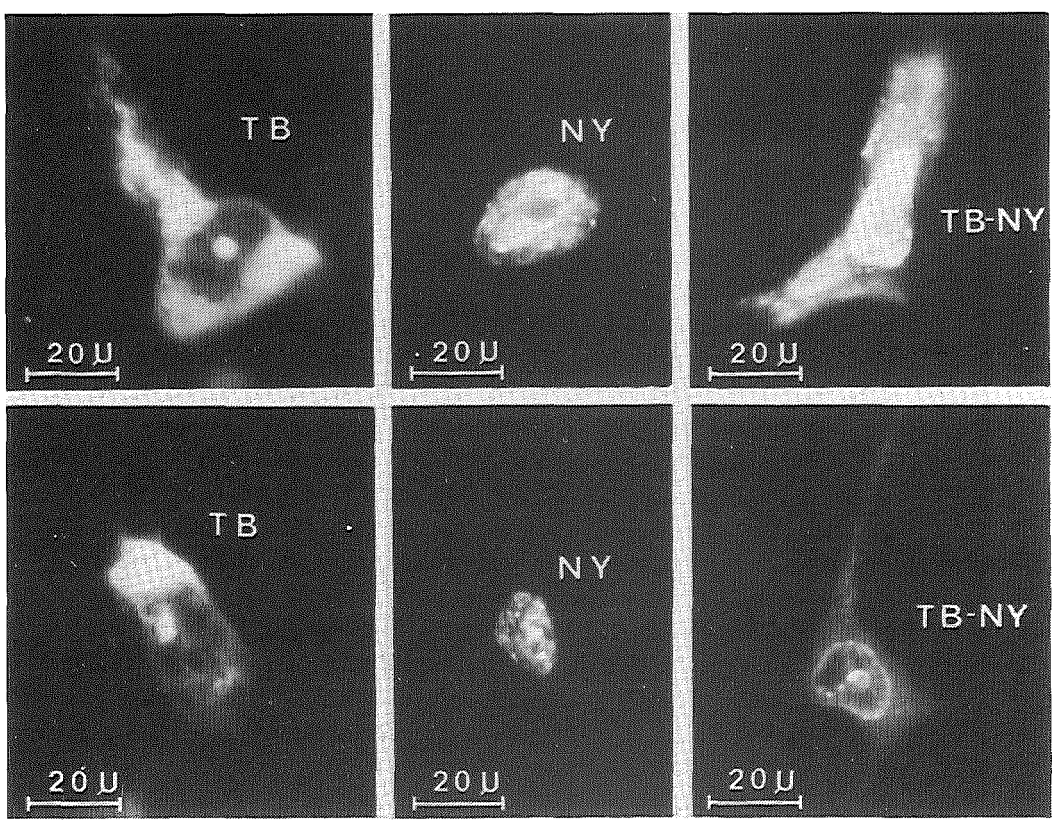


Fig. 3. Photomicrographs of TB single-, NY single- and TB–NY double-labeled neurons in red nucleus (upper row) and the nucleus raphe magnus (bottom row). The TB single-labeled neurons show a fluorescent labeling of cytoplasm and nucleolus and the NY single-labeled ones a fluorescent nucleus and a clear fluorescent ring around the nucleolus. The TB–NY double-labeled neurons display all features.

The *TB injection area* around the needle tracks showed two concentric fluorescent zones with different features. The first zone, immediately surrounding the needle tracks, contained a bright blue fluorescent cellular debris and a dense accumulation of fluorescent glial nuclei. In counterstained sections this area showed no further cytoarchitectural structure. The second zone contained fluorescent glial nuclei, which decreased in number and brightness towards the periphery of this zone. Peripherally in this zone also blue-labeled neurons and fibers occurred. The second zone faded into the area of the normal tissue with little or no fluorescence. In the present cases zone II extended laterally up to the border of the gray matter (Figs. 1 and 2), rostrally to the rostral border of C5 and caudally to the caudal border of C8.

The *NY injection area* displayed 3 concentric zones around the needle tracks. The first zone was very narrow and contained yellow tissue fluorescence and a dense accumulation of bright yellow-white glial nuclei. The second zone, which was much wider than the first, also displayed yellow tissue fluorescence but contained fewer fluorescent glial nuclei. The third zone showed no tissue fluorescence but did contain dull fluorescent glial nuclei. The first and second zones involved the injected half of the cord, while the third zone extended into the contralateral half of the spinal cord. Rostro-caudally the first and second zone involved the injected segments, while the third zone extended into the segments rostrally and caudally bordering the injected ones.

Red nucleus

In all rats a population of single TB-labeled rubrospinal neurons was present in the contralateral red nucleus and, in addition, a separate population of single NY-labeled ones occurred. In the caudal portion of the RN the single TB-labeled neurons were present mainly in the dorsomedial part, while the single NY-labeled ones were present in its ventrolateral part (Figs. 4 and 5). In the more rostral part of the nucleus the population of labeled rubrospinal neurons formed a shell in which the populations of single TB- and of single NY-labeled neurons to some degree became interdigitated (Fig. 4). Some TB–NY double-labeled neurons were present caudally in the red nucleus, between the dorsomedial TB-labeled part and the ventrolateral NY-labeled part. However, the bulk of the double-labeled neurons was observed rostrally in the nucleus intermixed with the single TB- and the single NY-labeled ones. In general, when the NY injections were made progressively more rostrally in the spinal cord the number of TB–NY double-labeled neurons in the RN increased in number (Table I, histogram 1). In order to compare the present findings with earlier electrophysiological observations[44] the number of TB-fluorescent neurons in the contralateral red nucleus, which were labeled from C5 to C8, were counted and the percentages of these neurons which were TB–NY double-labeled were computed. For this purpose the following procedure was followed. In each case the TB-labeled neurons as well as the TB–NY double-labeled ones were counted in every mounted section; these numbers were added and then treated according to the following formula:

$$\frac{\text{TB–NY double-labeled neurons}}{\text{single TB+TB–NY double-labeled neurons}} \times 100\%$$

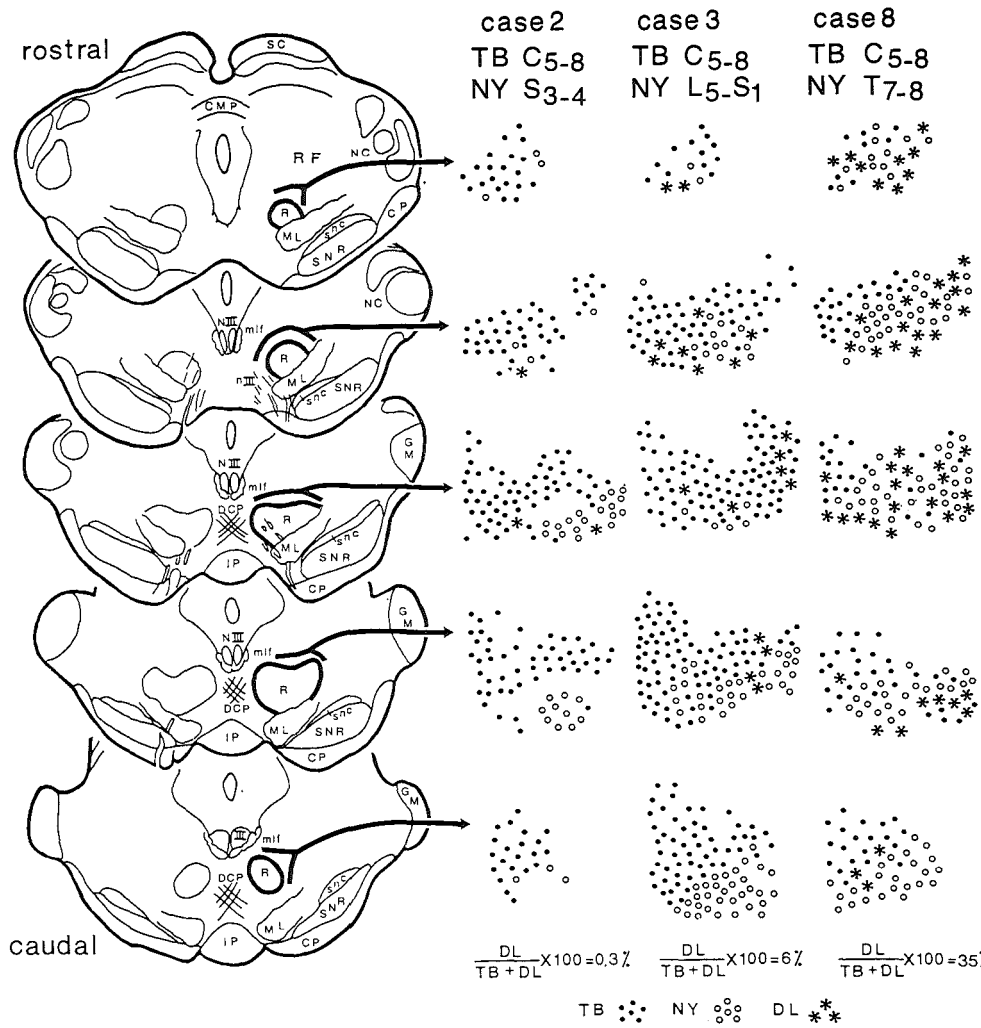


Fig. 4. Semidiagrammatic representation of the distribution of retrogradely labeled neurons in the red nucleus after spinal injections in case 2: True Blue (TB) in C5–8 and Nuclear Yellow (NY) in S3–4; in case 3: TB in C5–8 and NY in L5–S1; in case 8: TB in C5–8 and NY in T7–8. Abbreviations: CMP, posterior commissure; CP, cerebral peduncle; DCP, decussation of superior cerebellar peduncles; GM, medial geniculate body; IP, interpeduncular nucleus; ML, medial lemniscus; mlf, medial longitudinal fasciculus; NC, cochlear nuclei; NIII, oculomotor nucleus; nIII, oculomotor nervus; R, red nucleus; RF, reticular formation; SC, superior colliculus; SNR, substantia nigra, pars reticulata; snc, substantia nigra, pars compacta.

The total number of TB-labeled neurons, including the TB–NY double-labeled ones, in the different cases ranged from 300 to 683. Thus in the two rats with NY injections at S3–S4 the percentages were 0% and 0.3%, in the two rats with NY injections at L5–S1 they were 6% and 9.7%, in the two rats with NY injections at T13–L1 they were 15% and 25% and in the two cases with NY injections at T7–T8 the percentages were

35% and 35% (Table I, histogram 1). These findings imply that the red nucleus neurons, which project to the C5–C8 spinal gray matter, also distribute branches to more caudal parts of the spinal cord. They further indicate that a high proportion of these neurons gives rise to branches which pass through or terminate in midthoracic cord (35%), that a small proportion of neurons give branches which pass through or terminate in lumbosacral cord (6% and 9.7%), and that only one out of 300 neurons gives rise to branches which pass through or terminate in low sacral cord.

Nucleus raphe magnus (NRM) and ventrolateral pontine tegmentum (VLPT)

Nucleus raphe magnus. This nucleus, which contains many serotonergic neurons[16,17] also projects by way of the dorsolateral funiculus[30,33,49] to the dorsal horn and the dorsal part of the intermediate zone[6,34]. This nucleus comprises a population of neurons in the ventral part of the rostral medullary tegmentum immediately

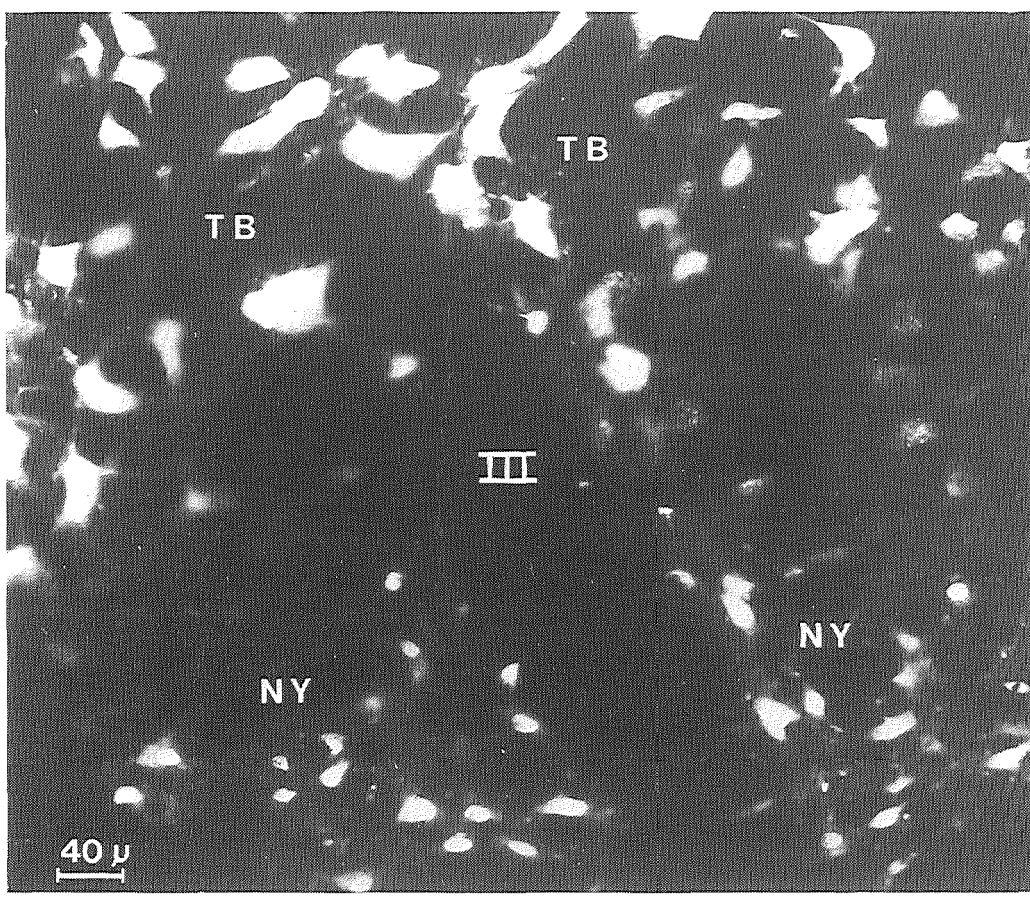
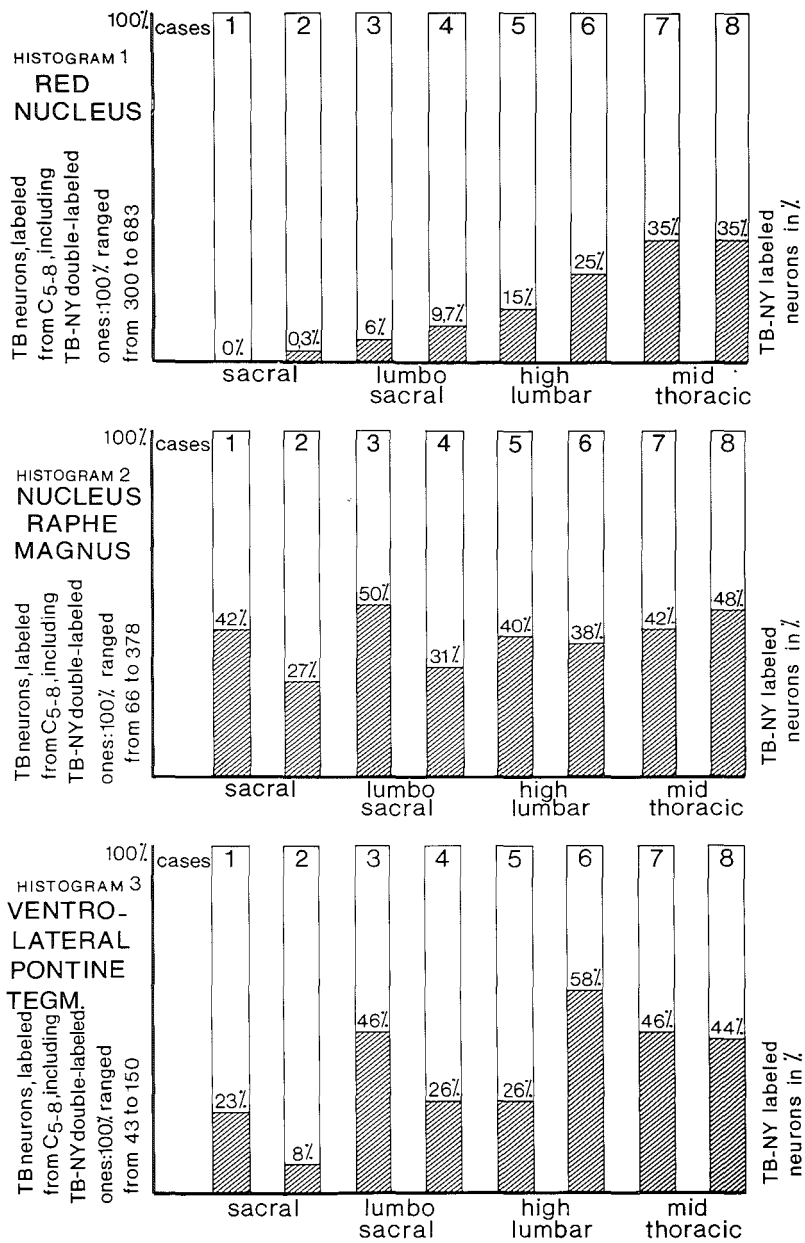


Fig. 5. Photomicrograph of caudal part of red nucleus. The True Blue (TB) single-labeled neurons are located in the dorsomedial part and the Nuclear Yellow (NY) single-labeled ones in the ventrolateral part. The oculomotor nerve (III) passes through red nucleus at this level.

TABLE I

$$\textit{The percentages}\left(\frac{\textit{TB-NY double-labeled neurons}}{\textit{TB single} + \textit{TB-NY double-labeled neurons}}\right) \times 100\%$$

Histogram 1: contralateral red nucleus; histogram 2: ipsilateral nucleus raphe magnus; histogram 3: contralateral ventrolateral pontine tegmentum.



adjoining the medial lemniscus and the pyramidal tract at the level of the facial nucleus. On the basis of the distribution of the serotonergic neurons[16,17], as well as on the basis of the retrograde HRP labeling after HRP injections in the dorsolateral funiculus[33,48,49], the nucleus appears to be less restricted to the raphe than suggested on the basis of the cytoarchitecture[47]. Thus, on the basis of these findings, the nucleus raphe magnus extends laterally along the pyramidal tract, where it includes the nucleus reticularis magnocellularis ventralis. In the present study therefore the nucleus raphe magnus was defined as that accumulation of neurons in the ventral part of the medullary tegmentum at the level of the facial nucleus, which is labeled retrogradely by HRP injections in the dorsolateral funiculus[7,33].

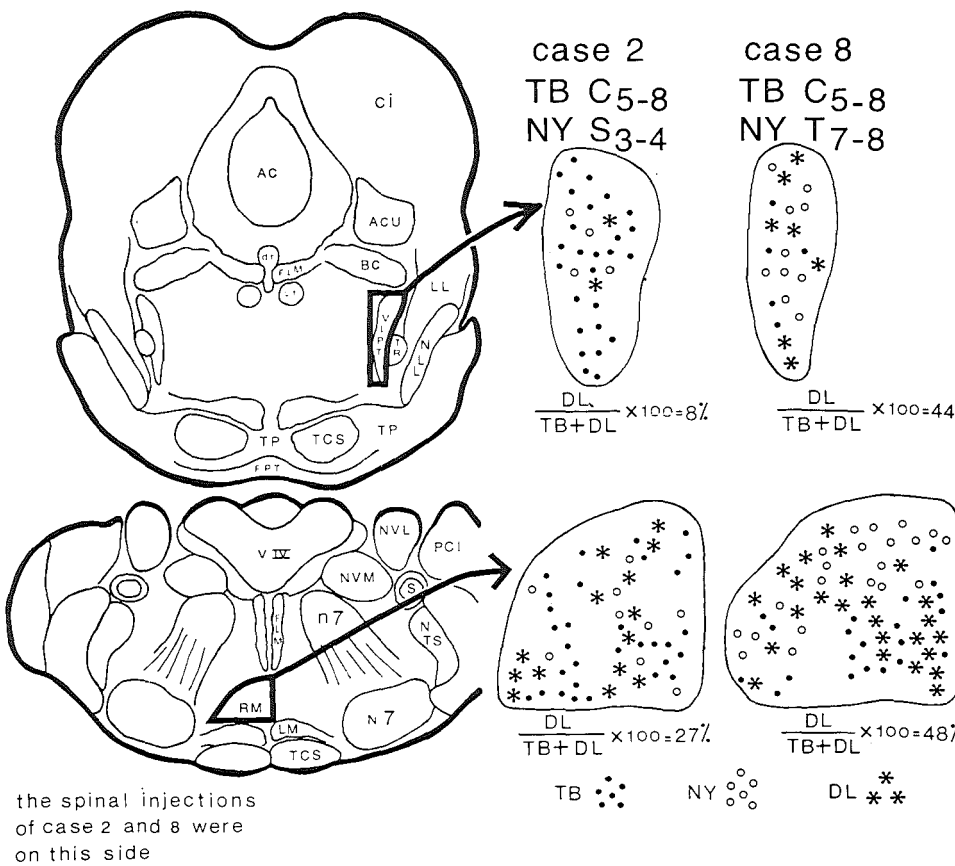


Fig. 6. Semidiagrammatic representation of the distributions of retrogradely labeled neurons in the ventrolateral pontine tegmentum and the nucleus raphe magnus after spinal injections. In case 2: True Blue (TB) in C5–C8 and Nuclear Yellow (NY) in S3–S4 and in case 8: TB in C5–C8 and NY in T7–T8. Note the relatively high number of double-labeled neurons especially in nucleus raphe magnus, even in case 2 with NY injection at S3–4. Abbreviations: AC, cerebral aquaduct; ACU, area cuneiformis; BC, brachium conjunctivum; CI, inferior colliculus; dr, nucleus dorsalis raphe; FLM, medial longitudinal fasciculus; FPT, transverse pontine fibers; LL, lateral lemniscus; LM, medial lemniscus; NLL, nucleus of lateral lemniscus; NTS, nucleus of spinal V tract; NVL, lateral vestibular nucleus; NVM, medial vestibular nucleus; N7, facial nucleus; n.7, facial nerve; PCI, inferior cerebellar peduncle; RM, nucleus raphe magnus; S, nucleus and tractus solitarius; TCS, corticospinal tract; TR, rubrospinal tract; VLPT, ventrolateral pontine tegmentum; vt, ventral tegmental nucleus; VIV, fourth ventricle.

Ventrolateral pontine tegmentum (VLPT). Retrograde HRP studies revealed a group of neurons adjoining the area of the rubrospinal tract (TRS)[7,29,33], which gives rise to mainly crossed spinal fibers which descend in the dorsolateral funiculus throughout the spinal cord[30,48]. These fibers seem to correspond to the crossed pontospinal tract of Busch[13] and the lateral reticulospinal tract of Papez[39]. Anterograde labeled amino acid transport studies[26,34] showed that this crossed descending pathway, which extends throughout the whole length of the spinal cord, terminates in the dorsal part of the spinal gray.

Retrograde labeling in nucleus raphe magnus and ventrolateral pontine tegmentum. The distribution of the single TB-labeled neurons and the TB–NY double-labeled ones in the NRM and the VLPT was also charted in the 8 rats reported here. The findings in these nuclei showed that, in contrast to the arrangement in the RN, the single TB-, single NY- and the TB–NY double-labeled neurons in the NRM and the VLPT did not display any topographical distribution but were randomly intermixed with one another (Fig. 6). In these nuclei the number of the TB-fluorescent neurons labeled from C5–C8, including the TB–NY double-labeled ones, were counted. In the NRM ipsilateral to the spinal injections they ranged from 66 to 378 and in the VLPT contralateral to the spinal injections they ranged from 43 to 150. Subsequently, the percentages of the neurons which were double-labeled with NY in these nuclei were calculated in the same way as in the red nucleus. Histograms 2 and 3 in Table I show that the percentages in these nuclei after NY injections at different spinal levels displayed an entirely different distribution than in the RN (histogram 1, Table I) such that in all cases a considerable percentage of the TB-labeled neurons in NRM and VLPT was double labeled. This was particularly striking in the NRM. Moreover, in this nucleus the percentages of the TB–NY double-labeled neurons were always roughly of the same magnitude, independent of the spinal level of the NY injections. Comparing histogram 1 with histograms 2 and 3 (Table I) clearly demonstrates that this arrangement is strikingly different from that in the RN, which is especially clear when comparing the percentages of the double-labeled neurons in the different nuclei after sacral and lumbosacral NY injections.

The present findings therefore suggest that different groups of RN neurons to a large extent project preferentially to certain levels of the spinal cord and distribute only few fibers to the other levels, while many of the NRM neurons distribute descending fibers throughout the length of the spinal cord, an arrangement which also seems to prevail in the VLPT.

DISCUSSION

The use of multiple retrograde fluorescent tracers seems to be one of the few anatomical techniques suitable to study the existence of collaterals in brain pathways. Therefore this technique has been used to try to clarify anatomically possible quantitative differences in collateralization in the rat rubrospinal tract, raphe spinal tract from the nucleus magnus and crossed pontospinal tract, all of which descend

from the brain stem through the dorsolateral funiculus and terminate in the dorsal half of the spinal gray[7,12,18,26,34,37,41,50]. However, applying this technique to the descending pathways in spinal cord is difficult, especially in rat, due to the close proximity between the area of termination of the collaterals in the gray matter and the fibers of the descending pathways in the adjoining funiculus. It is therefore imperative that the tracer injection is entirely restricted to the gray matter, because injection of some of the tracer in the lateral funiculus may result in its retrograde transport through damaged fibers. This in turn will lead to retrograde labeling of neurons in the red nucleus and the other brain stem cell groups which may not distribute collaterals to the injected part of the spinal gray. This difficulty only applies to the TB injections in C5–C8 segments. The NY injections at more caudal spinal levels were intentionally made to damage many fibers of the lateral funiculus in order to obtain NY retrograde transport through a maximum number of fibers descending in this funiculus at the NY injected levels.

In light of the above considerations, in all cases each of the 5 TB injection areas in C5–C8 gray were studied histologically in detail and only those cases were selected in which the TB fluorescent zones did not involve the lateral funiculus, while the remainder of the cases (about 20) were discarded. As a consequence the present study is based on the 8 selected cases in which no involvement of the lateral funiculus had occurred (Figs. 1 and 2). Sparing of the lateral funiculus in these 8 cases was confirmed by the fact that the population of TB-labeled neurons in the caudal part of the contralateral red nucleus was always restricted to its dorsomedial part, while the population of NY-labeled neurons was always located separately in the ventrolateral part (Figs. 4 and 5). This is in keeping with retrograde degeneration and retrograde HRP findings[21,35]. However, in the discarded cases with involvement of the lateral funiculus many either TB single- or TB–NY double-labeled neurons were also present in the ventrolateral part of the red nucleus.

Sparing of the rubrospinal tract also implies sparing of the two other descending pathways, since their fibers are grouped together with the rubrospinal tract in the dorsolateral funiculus, where the raphe-spinal tract is located in the most peripheral zone. Correspondingly, in the discarded cases with TB injections in the lateral funiculus not only the contralateral red nucleus, but also the ipsilateral nucleus raphe magnus and the contralateral ventrolateral pontine tegmentum contained an exceptionally large number of TB single- or TB–NY double-labeled neurons.

Earlier electrophysiological studies[45] of the collaterals of the descending pathways reported the number of red nucleus neurons which could be antidromically invaded from the C3–8 cervical gray matter, as well as their percentage that could also be antidromically invaded by stimulation at other spinal levels (i.e. T3 and L1). In order to facilitate a comparison with these earlier electrophysiological data, the present data were expressed in the similar manner, such that the number of TB-labeled neurons in the respective brain stem cell groups was counted and the percentage of these neurons which were double labeled was computed (Table I).

The retrograde labeling findings in the red nucleus (Figs. 4 and 5) confirm its somatotopic organization as demonstrated by means of the retrograde degeneration

technique in rat and cat[21,42] and the retrograde HRP transport technique in rat[35]. However, the present findings also demonstrate some degree of somatotopic organization in the rostral shell-shaped portion of the red nucleus. This rostral portion consistently contained the highest number of double-labeled neurons (Fig. 4), which indicates that it harbors the bulk of the neurons distributing collaterals to different levels throughout the spinal cord. The presence of this considerable number of branching neurons in the rostral part of the red nucleus probably explains the fact that its somatotopic organization has escaped detection by means of retrograde degeneration and HRP transport technique[21,35,42].

The electrophysiological findings[45] and the present anatomical findings appear quantitatively roughly of the same magnitude. In fact, the electrophysiological findings show that 5% of neurons projecting to C3–C8 distribute collaterals to L1 or beyond and 50% to T3 or beyond, while the present findings show that 15–25% of the neurons projecting to C5–C8 distribute collaterals to L1 or beyond and 35% to T7–8 or beyond. The slight differences between these physiological and anatomical findings may be due to the fact that different cervical and thoracic segments were studied, that possibly a different rostrocaudal extent of the red nucleus was explored in the two studies and that the two techniques are of a different nature. However, the differences in the two sets of findings may also reflect differences between cat and rat, as suggested by some of our preliminary findings in the former animal.

It may be assumed that the red nucleus neurons projecting to the lumbosacral enlargement behave in the same way as those projecting to the cervical enlargement, which were the main subject of the present study. If this is correct, the percentage distribution of the double-labeled neurons in the present cases (Table I, histogram 1) indicate that the population of red nucleus neurons which project to a certain group of segments distribute only relatively few collaterals to other groups of segments in the spinal cord.

The retrograde labeling findings in red nucleus thus suggest that the rubrospinal connections represent a highly focussed system. This would be in keeping with functional findings[22,28,32,46] which indicate that in cat and monkey this system is especially involved in controlling relatively fractionated extremity movements. The highly focussed nature of the rubrospinal system is further emphasized by a comparison of the findings in red nucleus with those in nucleus raphe magnus at the level of the facial nucleus. Thus, in the latter nucleus a relatively small population of single NY- and single TB-labeled neurons occurred which were largely intermingled and which also were intermixed with a large number of double-labeled neurons, such that 31–50% of the TB-fluorescent neurons labeled from C5–C8 were NY double-labeled from the lumbosacral enlargement or beyond. In this respect, the raphe spinal neurons in the nucleus raphe magnus resemble the reticulospinal ones because, according to electrophysiological findings[40], 66% of the reticulospinal neurons projecting to the cervical cord also distribute collaterals to the lumbar cord or below. In the present study the high percentage of double-labeled neurons in the NRM was found to be independent of the level of the NY injections. This indicates that a large percentage of the raphe spinal fibers projecting from nucleus raphe magnus to the cervical cord distributes

branches throughout the length of the spinal cord. This arrangement may explain the difficulties encountered in demonstrating a somatotopic organization in the nucleus raphe magnus[51]. It may also suggest that the raphe spinal system in question comprises a focussed component consisting of neurons projecting to the two enlargements, respectively, and a diffusely organized component which distributes throughout the length of the spinal cord and probably gives off collaterals at various spinal levels. This idea of a subdivision of the raphe spinal system would be strengthened if it could be shown that only the latter component mainly comprises serotonergic neurons. In that case this component may represent the anatomical substratum of the pain modulating function of the raphe spinal pathway[3–8,19,23,25,39], which function presumably is subserved by serotonergic neurons[16,17,43,44]. However, the diffuse component may also represent the raphe spinal pathway to the autonomic cell groups throughout the spinal cord, which also are known to receive serotonergic raphe spinal fibers[6].

Finally the VLPT, neurons of which give rise to the crossed pontospinal pathway[29,33] and which has been regarded as the pontine component of the rubrospinal system[13], seems to occupy a position between the rubrospinal and the raphe spinal system. Thus, it shows little somatotopic organization and also contains a high percentage of double-labeled neurons in the population projecting to the C5–C8 gray matter, i.e. in the same way as the neurons of the raphe spinal pathway. However, in contrast to the findings in the nucleus raphe magnus this percentage tend to show some decline when the NY injections are placed progressively more caudally in the cord. This suggests that the crossed pontospinal system displays a certain degree of specialization in that the bulk of the collaterals of the neurons projecting to C5–C8 tend to distribute to the thoracic and lumbosacral levels but not to the sacral cord. Further studies, however, are necessary to confirm this impression and to give clues to the functional distribution of this system, e.g. the control of movements.

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CHAPTER IV: DIFFERENCES IN COLLATERALIZATION OF THE DESCENDING SPINAL PATHWAYS FROM RED NUCLEUS AND OTHER BRAINSTEM CELL GROUPS IN CAT AND MONKEY

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Differences in Collateralization of the Descending Spinal Pathways from Red Nucleus and Other Brain Stem Cell Groups in Cat and Monkey

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INTRODUCTION

Classic anatomical studies using the Golgi technique (Cajal, 1952) demonstrated that many axons in the central nervous system give off collaterals along their trajectory through the brain. For a long time little attention has been paid anatomically to these collaterals, mainly because the available tracing techniques (Nauta and Gygax, 1954; Fink and Heimer, 1967; Cowan et al., 1972) did not make it possible to distinguish divergent axon collaterals from unbranched fibers. However, a few years ago electrophysiological studies have reintroduced the issue especially in respect to the descending pathways (Abzug et al., 1973, 1974; Illert et al., 1975; Peterson et al., 1975; Shinoda et al., 1977). Recently it has also become possible to study divergent axon collaterals anatomically by injecting two fluorescent tracers which, after being transported retrogradely through an axon and its collateral, label in different colors different features of the parent cell (Kuypers et al., 1980). Similar results may be obtained by using HRP and [^{3}H]apo-HRP (Hayes and Rustioni, 1979). However, this technique seems to produce relatively fewer double labeled neurons (cf., Hayes and Rustioni, 1981; Huisman et al., 1981). In the present study, which deals with the collateralization in several descending brain stem pathways in cat and monkey the former technique was therefore employed.

In a previous fluorescent double labeling study the degrees of collateralization of the rubrospinal, raphe spinal and crossed pontospinal pathways were compared in rat (Huisman et al., 1981). The results of this anatomical study in rat have much in common with the earlier electrophysiological findings (Peterson et al., 1975; Shinoda et al., 1977) in cat. However, a relatively larger number of rubrospinal neurons projecting to both the cervical and the lumbar cord have been demonstrated anatomically in rat than was found electrophysiologically in cat. This may reflect differences in the technique, but may also reflect interspecies differences in the degree of rubrospinal collateralization such that in rat a larger percentage of rubrospinal fibers distributes collaterals to different levels of the cord than in cat. The existence of such interspecies differences in degree of rubrospinal collateralization is suggested by the fact that injections of two fluorescent tracers at different spinal levels in opossum result in a relatively larger number of double labeled rubrospinal neurons than in rat (Huisman et al., 1981; Martin et al., 1982) (see Fig. 1). In order to detect a possible trend in the interspecies differences in degree of collateralization of the rubrospinal tract, in the present experiments the degree of collateralization of the rubrospinal tract was studied in cat and monkey and the findings were

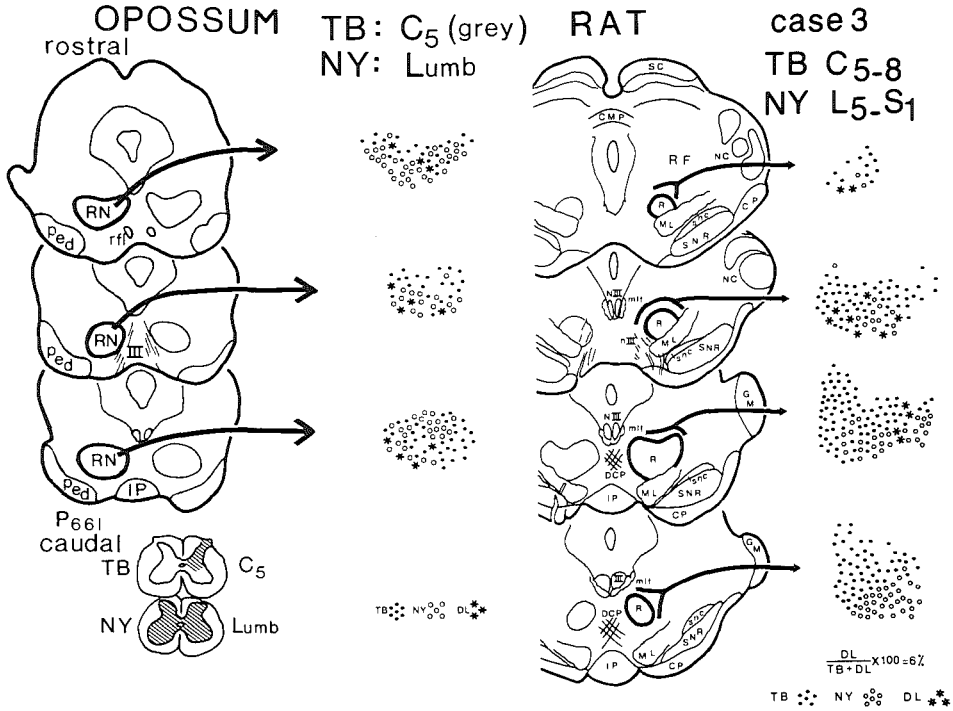


Fig. 1. Semidiagrammatic representation of the distributions of retrogradely labeled neurons in the red nucleus of the opossum (Martin, 1982) and of the rat (Huisman, 1981). True Blue (TB) was injected in the cervical grey matter in opossum at C5 and in the rat at C5–C8. Nuclear Yellow (NY) was injected both in opossum and rat in the lumbar cord. Note the difference in somatotopic organization and in the degree of collateralization between opossum and rat. Abbreviations: CMP, posterior commissure; CP, cerebral peduncle; DCP, decussation of superior cerebellar peduncles; GM, medial geniculate body; IP, interpeduncular nucleus; ML, medial lemniscus; mlf, medial longitudinal fasciculus; N III, oculomotor nucleus; n III, oculomotor nerve; NC, cochlear nuclei; ped, cerebral peduncle; R, red nucleus; RF, reticular formation; rfl, fasciculus retroflexus; SC, superior colliculus; snc, substantia nigra pars compacta; SNR, substantia nigra pars reticulata.

compared with those obtained earlier in rat (Huisman et al., 1981). Moreover, as in rat, the collateralization of the rubrospinal tract in cat and monkey was compared with that of the raphe spinal tract and of the crossed pontospinal tract. This was prompted by the fact that these two tracts, as the rubrospinal tract, project by way of the dorsolateral funiculus to the spinal dorsal grey (Nyberg-Hansen and Brodal, 1964; Petras, 1967; Edwards, 1972; Kuypers and Maisky, 1977; Basbaum and Fields, 1978; Basbaum et al., 1979; Holstege et al., 1979; Martin et al., 1979) but in rat (Huisman et al., 1981) display a much higher degree of collateralization than the rubrospinal tract. Therefore, if the collateralization of these two tracts in cat and monkey was of the same magnitude as in rat, the possible differences in the collateralization of the rubrospinal tract in these animals would be more convincing.

In the present experiments in cat and monkey the fluorescent tracers "Fast Blue" (FB) and "Nuclear Yellow" (NY) have been used since both are transported relatively effectively over long distances (Bentivoglio et al., 1980a,b; Kuypers et al., 1980), although FB more slowly than NY, and since they produce a fluorescent double labeling which can be observed at one excitation wavelength (i.e. 360 nm).

In cat and other species the red nucleus projects also to the ipsilateral inferior olive (Walberg, 1956; Edwards, 1972; Courville and Otabe, 1974; Martin et al., 1975, 1980; Strominger et al., 1979). Therefore, in two additional cats the relative number of rubrospinal neurons which distribute collaterals to the inferior olive was determined by means of the same technique.

The results of these experiments indicate that in cat and monkey as compared to rat a smaller percentage of the rubrocervical neurons distributes collaterals to more caudal levels. Further, it was found that in cat only very few rubrospinal neurons distribute collaterals to the inferior olive.

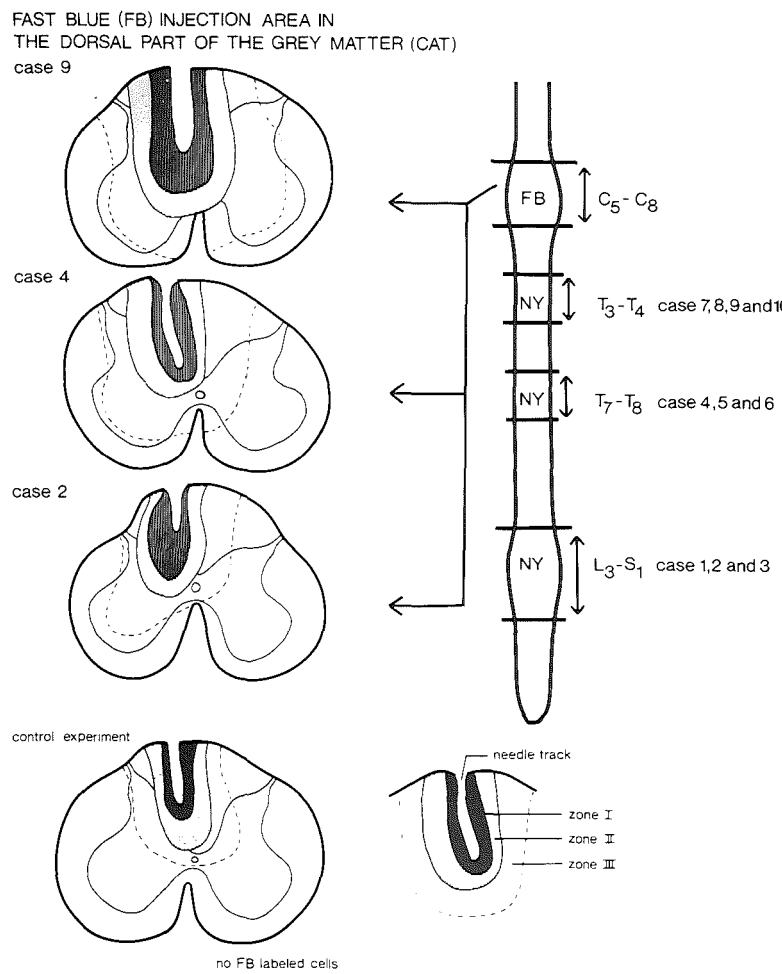


Fig. 2. Diagram of the injection areas in the 11 cats reported in this paper. Fast Blue (FB) was injected in 10 cats in the dorsal part of C5–C8 spinal grey matter and in 1 cat in the dorsal columns of C5–C8 spinal cord. For characteristics of zones, 1, 2 and 3, see text. The FB injection was combined with a Nuclear Yellow (NY) injection in cases 1, 2 and 3 at L3–S1, in cases 4, 5 and 6 at T7–T8 and in cases 7, 8, 9 and 10 at T3–T4.

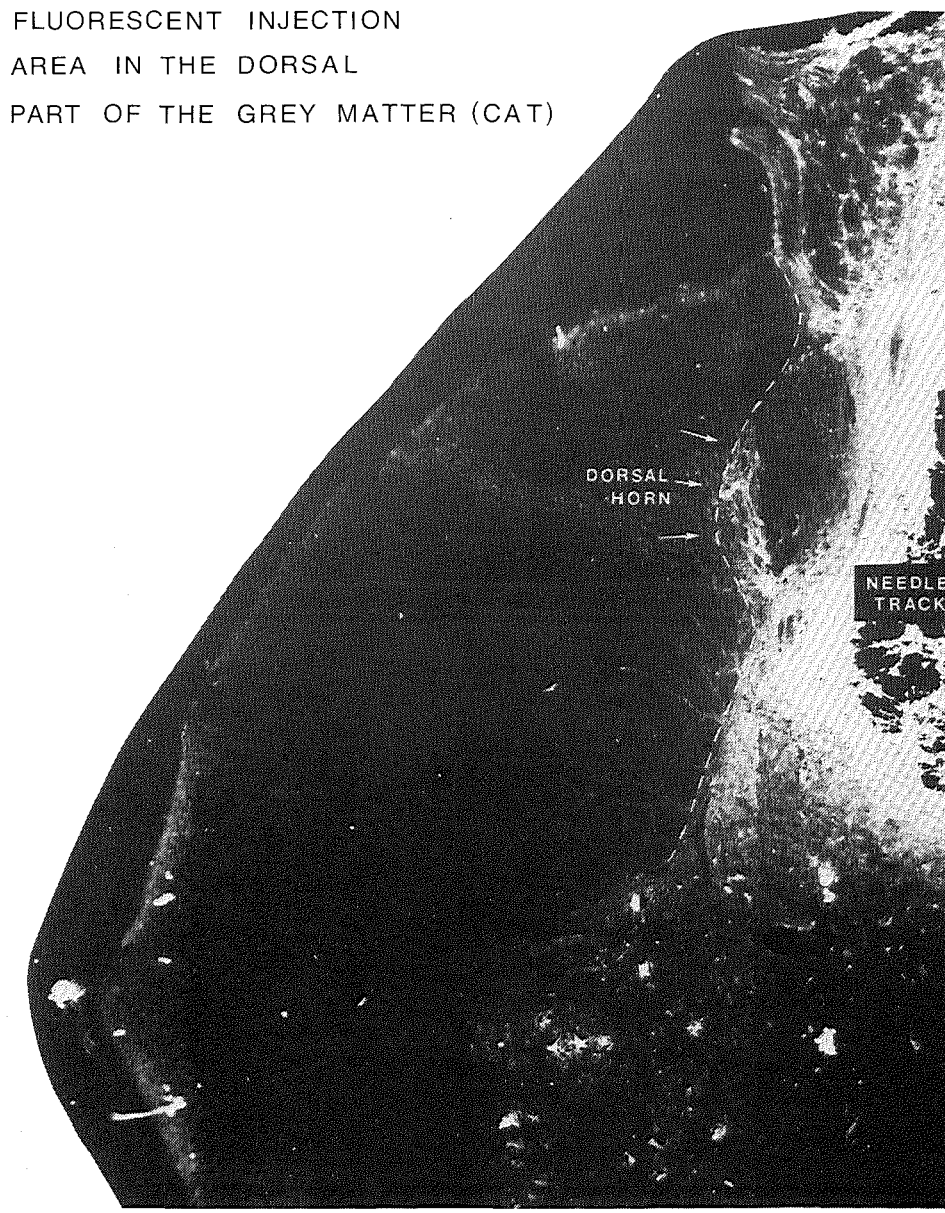


Fig. 3. Photomicrograph of a part of a cross-section between C5 and C8 with FB injection area in the dorsal part of the spinal grey matter of the cat. Note that zones I and II are restricted to the grey matter of the dorsal horn and intermediate zone.

MATERIALS AND METHODS

In 10 cats, anesthetized with Nembutal (6%), low cervical laminectomies were made. In each cat a rostrocaudal row of 7 glass micropipette injections of 3% "Fast Blue" (FB) (Bentivoglio et al., 1980a; Kuypers et al., 1980) were made into the dorsal horn and the dorsal part of the intermediate zone of C5, C6, C7 and C8 (see Fig. 3 and Table II). In one cat FB was injected in the C5–C8 dorsal columns (see Fig. 2). This case served as a control.

After 4 weeks the animals were reoperated. Under Nembutal, new laminectomies were made at more caudal levels (see Fig. 2) and 1% "Nuclear Yellow" (NY) either dissolved in water (Bentivoglio et al., 1980a,b; Kuypers et al., 1980) or in 2% dimethylsulfoxide (DMSO) was injected in the spinal white and grey matter in the different cats at different spinal levels (see Fig. 2 and Table II). DMSO was used because in the present experiments it was found that the admixture of DMSO in cat improves NY transport from damaged fibers, passing through the injected segments (Keefer, 1978). In the one cat, with FB injections in the dorsal columns, NY was injected at T7–T8.

In order to avoid migration of NY from the retrogradely labeled neurons relatively short survival times were used (Bentivoglio et al., 1980b). The appropriate survival times for labeling of red nucleus neurons at each of the various transport distances were determined empirically. They were found to display a more or less linear relationship with the NY transport distance (see Table I). The NY injections at L3 were therefore made 70 h prior to the perfusion of the animals, those at T8 : 58 h prior and those at T4 : 53 h prior (see Tables I and II). Using these survival times either no fluorescent glial nuclei or only a few dull fluorescent ones were present immediately around the single NY-labeled neurons as well as around the FB-NY double labeled ones. This indicates that minimal migration of NY from the retrogradely labeled neurons has occurred. Therefore the NY fluorescent neurons could with confidence be

TABLE I

In order to avoid migration of "Nuclear Yellow" (NY) out of the labeled neurons, the NY survival time should be restricted. The graph shows the appropriate NY survival times relative to the transport distance. The points which construct the line are found empirically in rubrospinal fibers in cat. Point 1 is found empirically in propriospinal fibers in cat. (1) The NY survival of T7–C6 in the cat, which distance is ± 8.5 cm, amounts to 30 h. (2) The NY survival time of C6 to the red nucleus in the cat, which distance is ± 10.5 cm, amounts to 46 h. (3) The NY survival time of T3 to the red nucleus in the cat, which distance is ± 15 cm, amounts to 53 h. (4) The NY survival time of T7 to the red nucleus in the cat, which distance is ± 19 cm, amounts to 58 h. (5) The NY survival time of L2 to the red nucleus in the cat, which distance is ± 28 cm, amounts to 69 h. Abbreviations: ps, propriospinal; rs, rubrospinal.

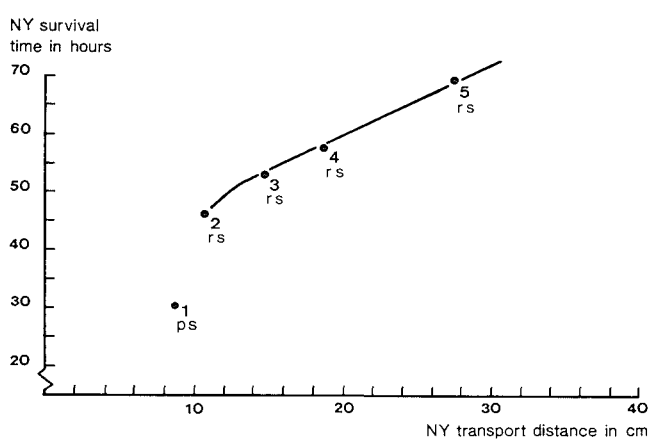


TABLE II

This table shows: (1) the amount in μl of Fast Blue 3 % (FB) and Nuclear Yellow 1 % (NY) used in the different cases; (2) the use of dymethylsulfoxide 2 % (DMSO); (3) the injection areas of FB and NY in the different cases; (4) the FB and NY survival times.

	μl FB (3 %)	*Injection area*	*Surv. time (weeks)*	*μl NY (1 %) use of DMSO (2 %)*	*Inj. area (hemiin-filtr.)*	*Surv. time (h)*
Case 1, cat	2.1	C5–C8 dorsal grey matter	4	3.6	L3 ipsilat. to FB inj.	70
Case 2, cat	2.1	C5–C8 dorsal grey matter	4	3.6	L3 ipsilat. to FB inj.	70
Case 3, cat	2.1	C5–C8 dorsal grey matter	4	3.6 in DMSO	L3–L6 ipsilat. to FB inj.	70
Case 4, cat	2.1	C5–C8 dorsal grey matter	4	3.6	T8 ipsilat. to FB inj.	58
Case 5, cat	2.1	C5–C8 dorsal grey matter	4	3.6	T8 ipsilat. to FB inj.	58
Case 6, cat	2.1	C5–C8 dorsal grey matter	4	3.6 in DMSO	T7–T8 ipsilat. to FB inj.	58
Case 7, cat	2.1	C5–C8 dorsal grey matter	4	3.6	T4–T5 ipsilat. to FB inj.	53
Case 8, cat	2.1	C5–C8 dorsal grey matter	4	3.6	T4–T5 ipsilat. to FB inj.	53
Case 9, cat	2.1	C5–C8 dorsal grey matter	4	3.6 in DMSO	T4 ipsilat. to FB inj.	53
Case 10, cat	2.1	C5–C8 dorsal grey matter	4	3.6	T3–T4 ipsilat. to FB inj.	53
Case 11, cat	2.8	inferior olive	2	3.6	C5–C8 contralat. to FB inj.	46
Case 12, cat	2.8	inferior olive	2	7.5 in DMSO	L3–L5 contralat. to FB inj.	46 after the C5–C8 inj.
				7.5 in DMSO	C5–C8 contralat. to FB inj.	
Case 13, monkey	2.4	C5–C8 dorsal grey matter	4	8.2 in DMSO	S1–S3 ipsilat. to FB inj.	70
Case 14, monkey	4.3	C5–C8 dorsal grey matter	4	8.2 in DMSO	L2–L5	72
Case 15, monkey	4.4	C5–C8 dorsal grey matter	4	12.8	T8–T9	60

TABLE III

This table shows: (1) the % formalin used at the perfusion; (2) the exact amount of single FB, single NY and FB–NY double labeled neurons in red nucleus of the different cases. In addition the single FB + FB–NY double labeled cells and the single NY + FB–NY double labeled neurons in red nucleus were calculated.

	Formaline perfusion (%)	*Single FB-labeled cells in red nucleus*	*Single NY-labeled cells in red nucleus*	*FB–NY cells in red nucleus*	*Single FB + FB–NY cells in red nucleus*	*Single NY + FB–NY cells in red nucleus*
Case 1, cat	10	1320	1622	28	1348	1650
Case 2, cat	10	1268	2792	30	1298	2822
Case 3, cat	30	2328	1896	93	2421	1989
Case 4, cat	30	1704	1428	156	1860	1584
Case 5, cat	30	1893	2415	213	2106	2628
Case 6, cat	30	1623	2268	207	1830	2475
Case 7, cat	30	1383	1005	231	1614	1236
Case 8, cat	30	1437	1146	312	1749	1458
Case 9, cat	30	1712	5294	426	2138	5720
Case 10, cat	10	786	3789	240	1026	4029
Case 11, cat	30		2442	0		2442
Case 12, cat	30		2886	1		2887
Case 13, monkey	30	1330	817	1	1331	818
Case 14, monkey	30	1236	1635	87	1323	1722
Case 15, monkey	30	1170	1524	120	1290	1644

regarded as genuinely retrogradely labeled. However, in all experiments always faint NY staining of glial nuclei occurred diffusely through the area of NY labeled neurons (see Fig. 9). This may have resulted from migration of NY out the rubrospinal axons.

In the two cats (cases 11 and 12), in which the rubrospinal collaterals to the inferior olive were studied, FB was injected in the latter structure through a hole drilled in the base of the skull and NY was injected contralaterally in the cervical spinal cord through a laminectomy (see Table II and Fig. 5).

All cats were sacrificed with an overdose of Nembutal (6%). They were transcardially perfused with 2 liters NaCl (2.7%) followed by 3 liters cacodylate-buffered (pH 7.2) formalin (10% or 30%, see Table III) followed by 2 liters cacodylate-buffered (pH 7.2) sucrose (8%).

In 3 monkeys (cases 13, 14 and 15) similar FB injections were made as in the 10 cats (cases 1–10), i.e., in the dorsal part of the C5–C8 spinal grey matter (see Figs. 4 and 13). After 4 weeks the animals were re-operated and NY either dissolved in water or dissolved in DMSO was injected in the spinal white and grey matter at different spinal levels caudal to T3 (see Table II). The animals were sacrificed at the appropriate NY survival times (see Table II) based on the relationship between transport distance and transport time found in the rubrospinal fibers in cat (see Table I). The monkeys were transcardially perfused with 2 liters NaCl (2.7%) followed by 3 liters cacodylate-buffered (pH 7.2) formalin (10% or 30%, see Table III), followed by 2 liters cacodylate-buffered (pH 7.2) sucrose (8%).

The brains and the injected segments of the cats and monkeys were cut transversally in frozen sections, 30 μm thick, on a freezing microtome. The sections, after being cut, were

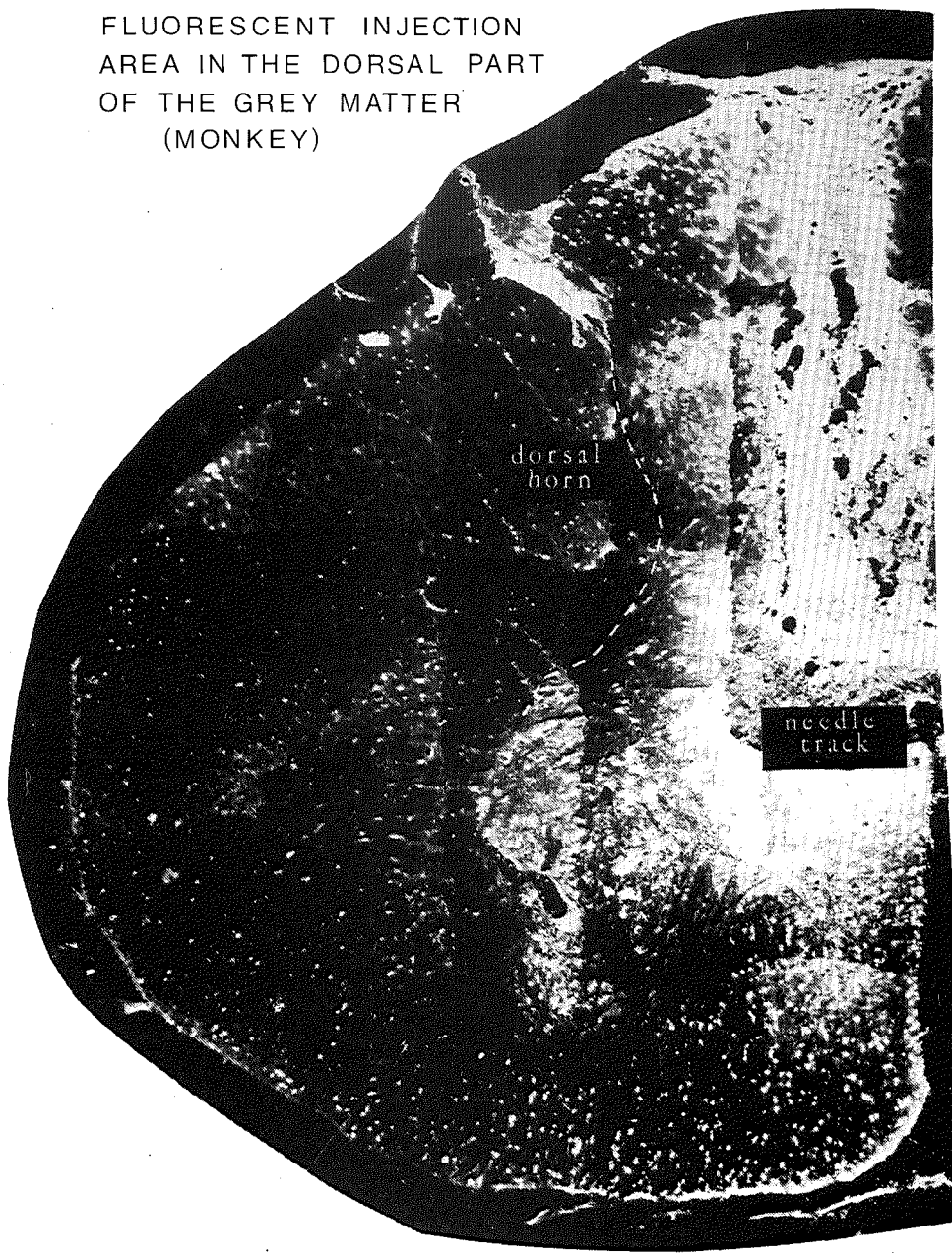


Fig. 4. Photomicrograph of a part of a cross-section between C5 and C8 with FB injection area in the spinal grey matter of the monkey. Note that zones I and II are restricted to the grey matter of the dorsal horn, the intermediate zone and in part of the ventral horn.

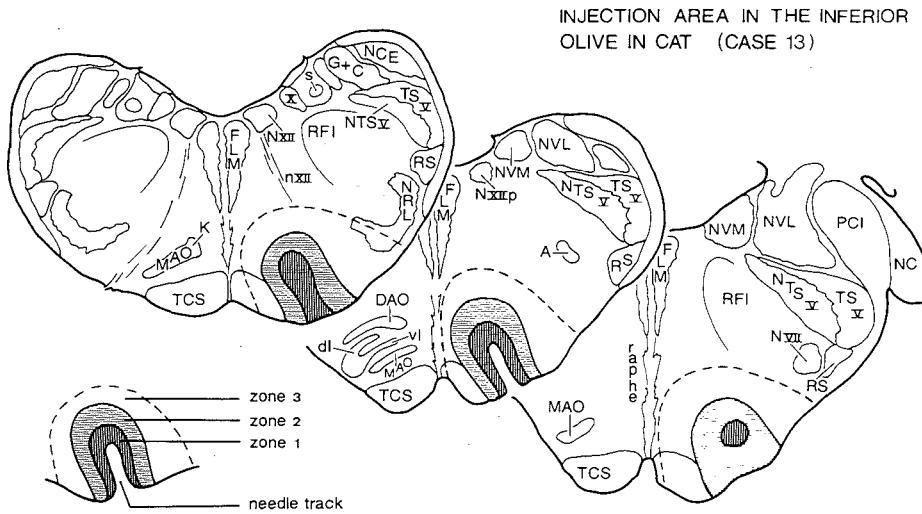


Fig. 5. Diagram of the injection area in inferior olive in cat. For characteristics of zones 1, 2 and 3, see text. Abbreviations: A, nucleus ambiguus; DAO, dorsal accessory olive; dl, dorsal lamella of principal olive; FLM, medial longitudinal fasciculus; G + C, nucleus gracilis and cuneatus; K, cap of Kooy; MAO, medial accessory olive; N VII, facial nucleus; N XII, hypoglossal nucleus; n XII, hypoglossal nerve; N XIIp, nucleus prepositus hypoglossi; NC, cochlear nucleus; NCE, external cuneate nucleus; NRL, lateral reticular nucleus; NTSV, nucleus of spinal V tract; NVL, lateral vestibular nucleus; NVM, medial vestibular nucleus; PCI, inferior cerebellar penduncle; RFl, lateral reticular formation; RS, rubrospinal tract; S, nucleus and tractus solitarius; TCS, corticospinal tract; TS V, spinal V tract; vl, ventral lamella of principal olive; X, dorsal motor nucleus of vagus.

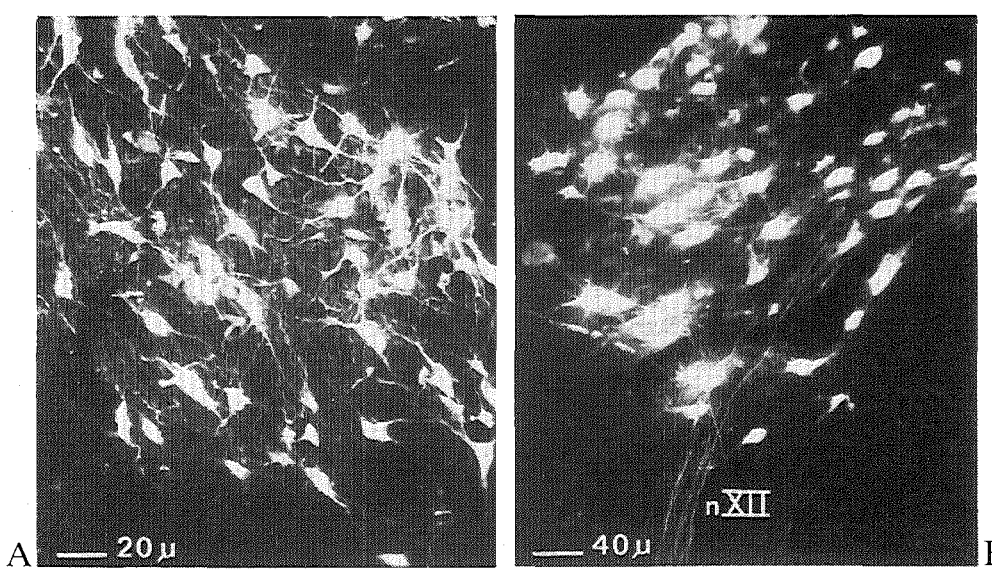


Fig. 6. A shows the FB-labeled neurons with their dendrites of the nucleus of Darkschewitsch after FB injections in the cat inferior olive. B shows the FB labeling of the hypoglossal neurons with their axons (nervus hypoglossus) after FB injections in the cat inferior olive, which also damaged the axons of the hypoglossal nerves, at their exit from the medulla oblongata. Note that after a perfusion with 30% formalin instead of 10% the FB labeling is very strong, and the proximal dendrites and axons are also labeled.

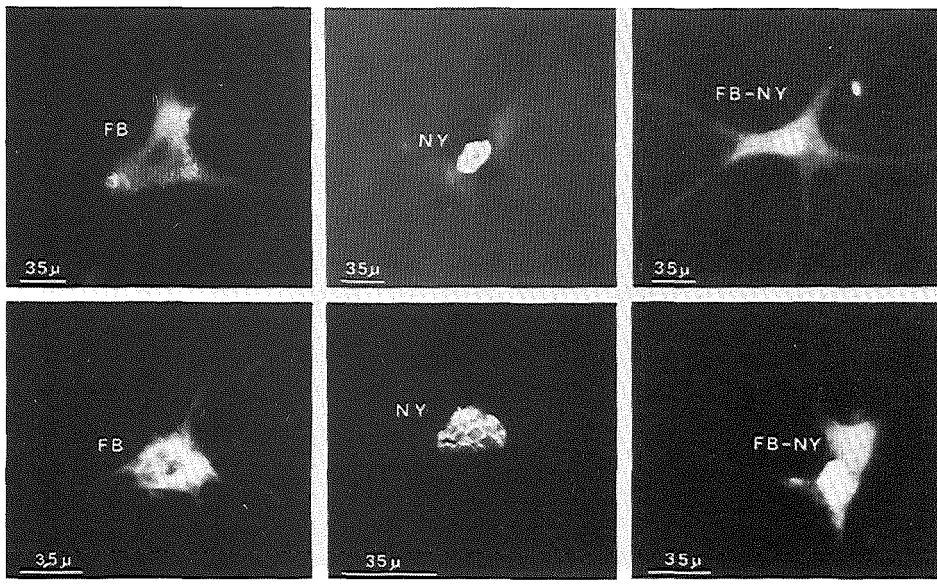


Fig. 7. Photomicrographs of single FB, single NY and FB–NY double labeled neurons in red nucleus (upper row) and the nucleus raphe magnus (bottom row) of the cat. The FB single labeled neurons show a fluorescent labeling of the cytoplasm with silver fluorescent granules and the NY single labeled ones a fluorescent nucleus and a clear fluorescent ring around the nucleolus. The FB–NY double labeled neurons display all features.

immediately mounted from distilled water and air dried at room temperature. In general, one out of every three sections was mounted. The material was studied with a Leitz Ploemopack fluorescence microscope equipped with a filter mirror system A (360 nm excitation wavelength). At this excitation wavelength single FB labeled neurons show a blue fluorescent labeling of the cytoplasm of cell body and proximal dendrites with silver fluorescent granules. Perfusion with 30% formalin produced a more intense FB fluorescence than with 10% formalin and also resulted in labeling of axons and proximal dendrites of the labeled cells (see Fig. 6). Single NY-labeled neurons, after short survival times (Bentivoglio et al., 1980b), show mainly a golden yellow fluorescence of the nucleus with a clear ring around the nucleolus. Double labeled neurons show all mentioned features (see Figs. 7 and 15) at the same 360 nm excitation wavelength (Kuypers et al., 1980).

RESULTS

(A) Injection areas

The FB injection area around the needle tracks in the spinal cord and inferior olive in cat and monkey showed 3 concentric zones (Bharos et al., 1981). Zone I, surrounding the needle track, was brilliantly white-blue fluorescent and contained few cellular elements but many orange fluorescent granules. In cresyl violet sections, it represented a pale area with a few neurons or glial cells. Zone II was characterized by a dense accumulation of blue fluorescent

glial cells with a blue fluorescent nucleus. In cresyl violet sections this zone was darkly stained because of many glial nuclei. Zone III contained some fluorescent glial nuclei and neurons as well as blue fluorescent fibers. Towards its periphery the fluorescent glial nuclei progressively diminished in number and zone III thus faded into an area of normal tissue with little fluorescence.

FB transport occurs from zones I and II, but not from zone III, since in the control cat with FB injections in the dorsal columns in which only zone III involved the rubrospinal termination area (see Fig. 2) no FB-labeled cells occurred in red nucleus. In the cases (cats 1–10 and the 3 monkeys) in which FB was injected in the C5–C8 grey matter, zone II extended laterally up to the border of the grey matter and ventrally into the ventral horn (Figs. 2, 3, 4 and 13). Rostrocaudally it extended up to the rostral border of C5 and down to the caudal border of C8. No FB fluorescence occurred in those parts of the dorsolateral funiculus containing the rubrospinal, raphe spinal and crossed pontospinal tracts (Nyberg-Hansen and Brodal, 1964; Petras, 1967; Edwards, 1972; Kuypers and Maisky, 1977; Basbaum and Fields, 1978, 1979; Holstege et al., 1979; Martin et al., 1979). In the two cats (cats 11 and 12), with FB injections in the inferior olive, zone II extended from the medial to the lateral border of the inferior olive. It extended dorsally 0.5 mm beyond its dorsal border (see Fig. 5) and extended rostrocaudally throughout the length of the inferior olive.

The NY injection areas also displayed 3 concentric zones (Bharos et al., 1981). Zone I was very narrow and contained yellow tissue fluorescence and a dense accumulation of bright yellow-white glial nuclei. Zone II was much wider than the first. It also displayed yellow tissue fluorescence but contained fewer fluorescent glial nuclei. Zone III contained only dull fluorescent glial nuclei. The zones I and II involved both the grey and white matter of the injected half of the cord, while zone III extended into the contralateral half. Rostrocaudally, zones I and II involved the injected segments, while zone III extended into the rostrally and caudally adjacent ones. However, retrograde transport over long distances does not take place from zone III since no NY retrograde labeling was present in the red nucleus contralateral to the non-injected half of the spinal grey which was involved by zone III only.

(B) Measures taken to increase the number of labeled neurons (Table III)

From some of the findings in the present study the impression was gained that in cat NY is much less effectively transported from damaged axons than in rat (Huisman et al., 1981). In order to determine whether this transport in cat could be improved by the admixture of DMSO: in one cat 1 % NY dissolved in water was injected in the C5–C8 dorsolateral funiculus while in another cat 1 % NY dissolved in 2 % DMSO (Keefer, 1978) was injected. Only in the latter cat a substantial number of NY-labeled neurons were present contralaterally in the ventrolateral part of the red nucleus, which neurons project to the lumbosacral cord (Pompeiano and Brodal, 1957; Nyberg-Hansen and Brodal, 1964). The same was observed in other animals of the present study in which NY dissolved in DMSO was injected (see number of NY-labeled neurons in Table III). It was therefore concluded that in cat an effective NY transport from damaged fibers, which pass through the injection area, only occurs when NY is dissolved in DMSO*.

* The facilitatory influence of DMSO on this transport probably results from the fact that it increases the permeability of the membranes. In this respect it is of interest to recall that during bathing the intact vagus nerve bisbenzimide (Bb) remained in solution, while True Blue (TB) adhered directly to the sheath of the nerve and resulted in retrograde transport (Sawchenko and Swanson, 1981).

In order to determine whether the FB fluorescence could be improved by a stronger fixation a 30% formalin perfusion was used in cat and monkey instead of 10%. This measure increased the intensity of FB fluorescence and thus also increased the total number of cells which could be recognized as FB-labeled (see numbers of FB-labeled neurons in Table III).

(C) Red nucleus in cat and monkey

Cytoarchitecture

The red nucleus in cat and monkey may be subdivided into a caudal (magnocellular) part and a rostral part. The magnocellular part in cat occupies the caudal 2/3 of the nucleus but in monkey occupies the caudal 1/2. In both species the caudal portion of the magnocellular part of the red nucleus is round in cross-section and contains large neurons which are dispersed between the tegmental fiber bundles. The rostral portion of the magnocellular red nucleus in monkey is also round in cross-section but contains mainly medium sized neurons. In cat this portion of the magnocellular part as seen in cross-section has the shape of a flattened disk and contains both large and small neurons. According to earlier retrograde degeneration findings these various types of neurons in the magnocellular part of the red nucleus in cat and monkey all give rise to rubrospinal fibers (Pompeiano and Brodal, 1957; Poirier and Bouvier, 1966; Kuypers and Lawrence, 1967).

The rostral part of the red nucleus in monkey is rather sharply delineated against the surrounding reticular formation and contains relatively small neurons. Therefore, as in other higher primates, this part of the red nucleus is called the parvicellular part. In the dorsal portion of this parvicellular part the cells form a loose network but in the ventral parts they are rather densely packed. The parvicellular part in monkey extends rostrally close to the mesodiencephalic junction, i.e., just beyond the level where the fasciculus retroflexus (FR) traverses in full length the mesencephalic tegmentum. At these levels the parvicellular part is mainly located lateral to the FR. In monkey, a small group of medium sized multipolar neurons extends from the caudal part of the magnocellular part rostrally over a short distance along the lateral aspect of the parvicellular part, up to the level where the cross-section through the fasciculus retroflexus (FR) is located immediately ventral to the red nucleus.

The rostral part of the red nucleus in cat is also situated lateral to the FR. However, in the cat this part of the nucleus is not truly parvicellular as in monkey but consists of a loose network of small multipolar neurons which is difficult to delineate from the surrounding reticular formation. Yet, it seems reasonable to regard this portion of the red nucleus in cat as the counterpart of the parvicellular part in monkey since both structures give rise to the ipsilaterally descending rubro-olivary fibers (Walberg, 1956; Edwards, 1972; Courville and Otabe, 1974; Strominger et al., 1979; Condé and Condé, 1982).

Rubrospinal neurons in cat and monkey

In all 10 cats and in the 3 monkeys a population of single FB-labeled and single NY-labeled neurons was present in the caudal portion of the magnocellular red nucleus. In both groups of animals the single FB-labeled neurons were concentrated dorsomedially while the single NY-labeled ones were concentrated ventrolaterally (see Figs. 8, 9 and 13). In the rostral portion of the magnocellular part of the nucleus in monkey this arrangement was roughly maintained but in the rostral portion of the magnocellular red nucleus in cat the two populations of single FB- and single NY-labeled neurons which comprised both large and small cells became interdigitated to some degree as observed also in rat (Huisman et al., 1981). It was quite striking to note that the population of labeled rubrospinal neurons in monkey was not

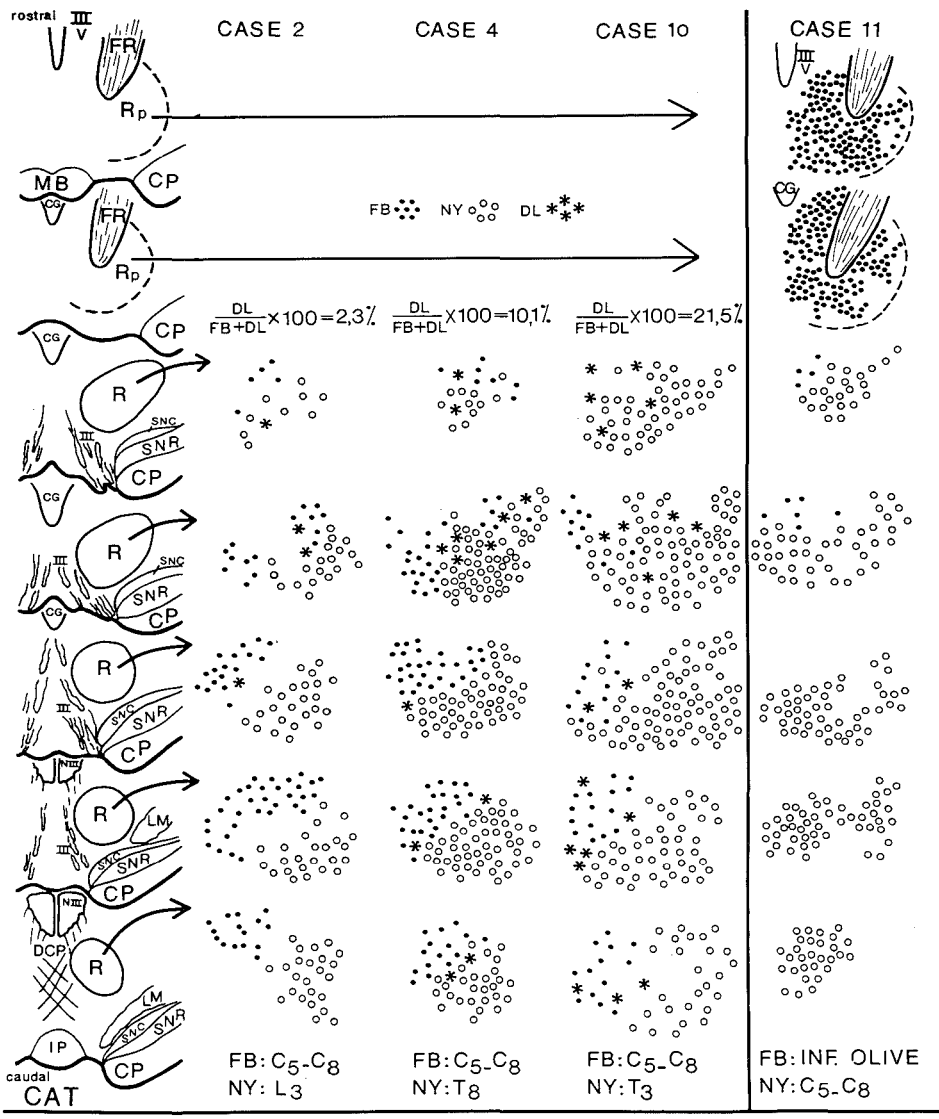


Fig. 8. Semidiagrammatic representation of the distributions of retrogradely labeled neurons in the red nucleus of the cat in case 2: FB in C5–C8 and NY in L3; case 4: FB in C5–C8 and NY in T8; case 10: FB in C5–C8 and NY in T3; case 11: FB in the ipsilateral inferior olive and NY in the contralateral C5–C8 spinal cord. Abbreviations: CG, central grey; CP, cerebral peduncle; DCP, decussation of superior cerebellar penduncles; IP, interpeduncular nucleus; LM, medial lemniscus; N III, oculomotor nucleus; MB, mammillary bodies; III, oculomotor nerve; R, red nucleus; Rp, parvicellular part of red nucleus; SNC, substantia nigra pars compacta; SNR, substantia nigra pars reticulata; III V, third ventricle.

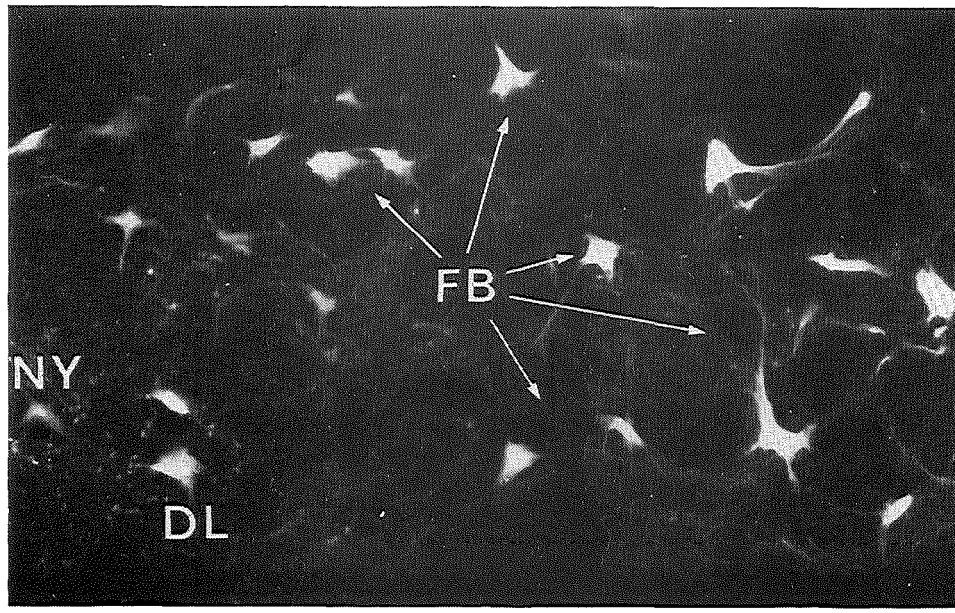


Fig. 9. Photomicrograph of dorsomedial part of caudal red nucleus in cat, labeled with FB from C5–C8 contralateral spinal cord, which part passes into the ventrolateral part, labeled with NY from T8–T9 contralateral spinal cord. Note the faint staining of glial nuclei diffuse through the ventrolateral part and the FB–NY double labeled neuron located at the transition of the dorsomedial FB-labeled part to the ventrolateral NY-labeled part.

restricted to the magnocellular part of the red nucleus but extended rostrally as a thin shell alongside the lateral aspect of the parvicellular part up to the level where the cross-section of the fasciculus retroflexus (FR) is located immediately ventral to the nucleus. This is in keeping with the retrograde HRP findings of Kneisley et al. (1978) as inferred from their illustrations. In this lateral shell of rubrospinal neurons both single FB- and single NY-labeled neurons were present but the NY-labeled rubrospinal neurons were much more numerous than the FB-labeled ones and the latter were located more dorsally than the former (see Fig. 13). This is in keeping with the HRP findings (Kneisley et al., 1978) that the rubrolumbar neurons in monkey extend more rostrally than the rubrocervical ones.

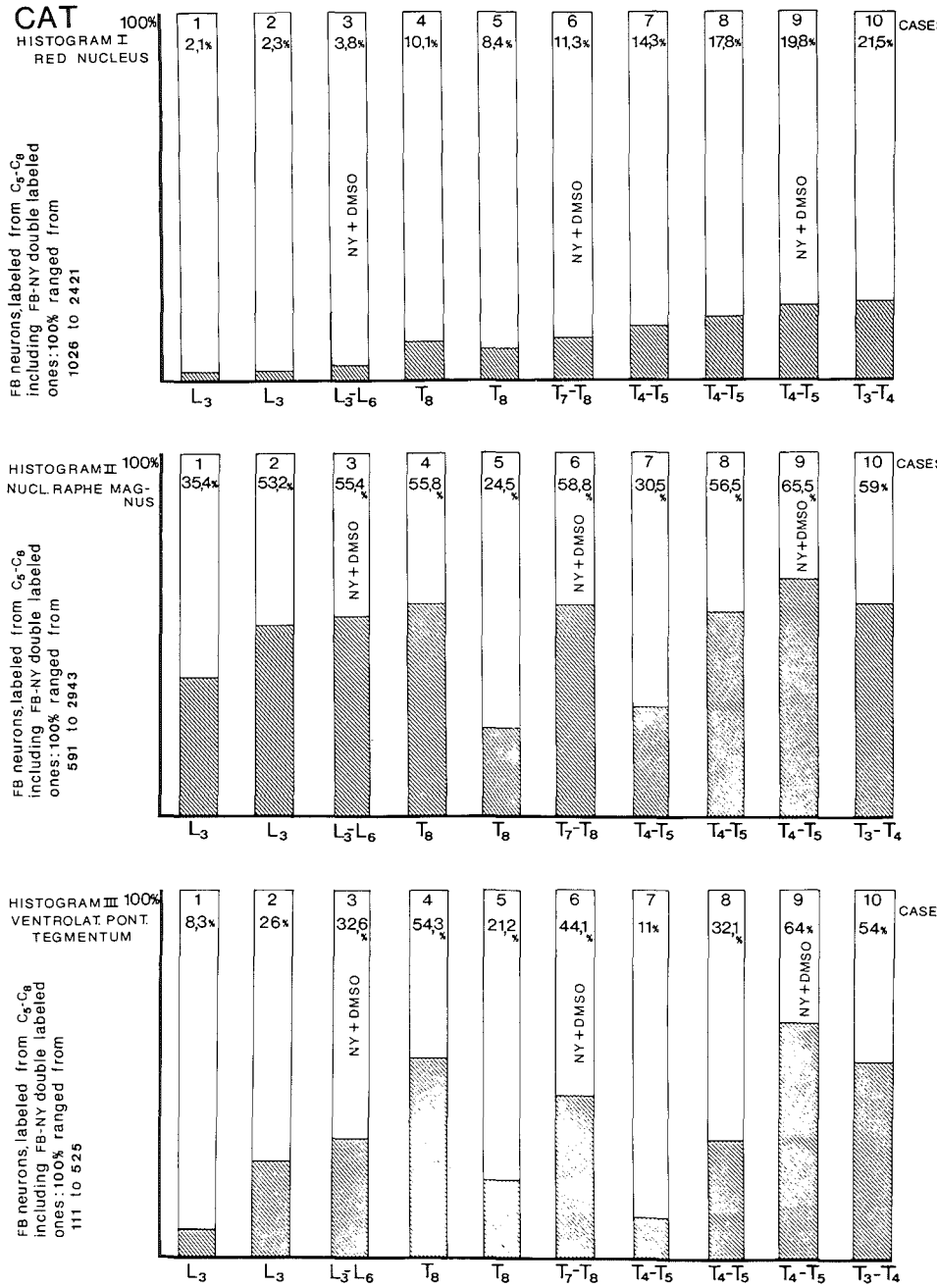
In the cats and monkeys double labeled neurons were also present in the red nucleus. In all cases (cats and monkeys) some FB–NY double labeled neurons were present in the caudal portion of the magnocellular part of the red nucleus, i.e., between the dorsomedially located FB-labeled neurons and the ventrolaterally located NY-labeled ones. However, as in rat (Huisman et al., 1981), the majority of the double labeled neurons were observed in the rostral portion of the magnocellular part of the nucleus, where they were intermixed with the single FB- and single NY-labeled ones. In monkey double labeled neurons were also observed in the lateral shell alongside the parvicellular part, where they were in general situated dorsally.

When comparing the findings in cats and monkeys it appeared that when the NY injections were made progressively more rostrally in the spinal cord starting at the sacral levels the FB–NY double labeled neurons in red nucleus increased in number. In order to compare the present findings in cat and monkey with those in rat (Huisman et al., 1981) and with earlier electrophysiological findings in cat (Shinoda et al., 1977) the numbers of single FB-labeled

TABLE IV

The percentages $\frac{\text{FB–NY double labeled neurons}}{\text{FB single} + \text{FB–NY double labeled neurons}} \times 100\%$

Histogram 1: contralateral red nucleus of the cat; histogram 2: ipsilateral nucleus raphe magnus of the cat; histogram 3: contralateral ventrolateral pontine tegmentum of the cat.



neurons, of single NY-labeled neurons and of FB–NY double ones were counted in each section. From the total number of these neurons the percentages of FB-labeled neurons which were FB–NY double labeled were computed according to the following formula:

$$\frac{\text{FB–NY double labeled neurons}}{\text{single FB} + \text{FB–NY double labeled neurons}} \times 100\%.$$

Percentages of double labeled neurons in cat

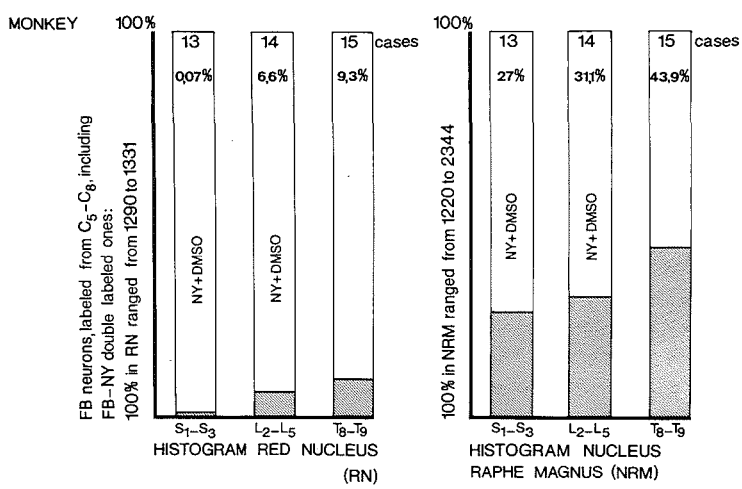
In each of the 3 groups of cats injected at L3, T7–T8 and T4, one case was injected with 1% NY dissolved in 2% DMSO (case 3 at L3, case 6 at T7–T8 and case 9 at T4) and was also perfused with 30% formalin (see Tables II and III). In these 3 cases the largest number of FB- and NY-labeled neurons was obtained. In the 3 cats (cases 1, 2 and 3) with NY injections at L3: 2.1%, 2.3% and 3.8% of the FB-labeled neurons in the red nucleus were FB–NY double labeled. In the 3 cats (cases 4, 5 and 6) with NY injections at T7–T8: 10.1%, 8.4% and 11.3% of the FB-labeled neurons were double labeled. In the 3 cats (cases 7, 8 and 9) with NY injections at T4–T5: 14.3%, 17.8% and 19.8% of the FB-labeled neurons were double labeled and in the one cat (case 10) with NY injections at T3–T4: 21.5% of the FB-labeled neurons were double labeled (see Table IV, histogram 1).

Percentages of double labeled neurons in monkey

In the monkeys the following percentages were found: in case 13 with NY injections at S1–S3 0.07% of the FB-labeled rubrospinal neurons were found to be FB–NY double labeled, in case 14 with NY injections at L2–L5 6.6% were double labeled and in case 15 with NY injections at T8–T9 9.3% (see Table V, left histogram).

TABLE V

Histogram of the percentages $\frac{\text{FB–NY double labeled neurons}}{\text{FB single} + \text{FB–NY double labeled neurons}} \times 100\%$ in monkey red nucleus (left histogram) and in monkey nucleus raphe magnus (right histogram).



Percentages of double labeled neurons in cat and monkey compared

The findings in this entire group of cats and monkeys thus imply that some of the red nucleus neurons, which project to the C5–C8 spinal grey matter, also distribute branches to more caudal parts of the spinal cord. They further indicate that in cat a relatively high proportion of these neurons gives rise to branches which terminate in or pass through the T4–T5 spinal segments (± 20%); a smaller proportion gives branches which terminate in or pass through T7–T8 (± 10%), and only a very few neurons give branches which terminate in or pass through the lumbosacral cord (± 3%). The arrangement in monkey seems to be similar to that in cat, such that a small percentage of rubrocervical neurons gives rise to collaterals which terminate in or pass through T8–T9 (9.3%), an even smaller percentage gives rise to collaterals which terminate in or pass through L2–L5 (6.6%) while extremely few rubrocervical neurons (0.07%) give rise to collaterals to the sacral cord.

Rubro-olivary neurons in cat

In the two cats (cases 11 and 12) with FB injections in inferior olive and NY injections contralaterally in spinal cord the following findings were obtained. In case 11 in which NY dissolved in water was injected in the cervical cord (C5–C8) the population of single NY labeled neurons was located in the dorsomedial part of the red nucleus. In case 12 in which NY dissolved in DMSO was injected in both the cervical (C5–C8) and the lumbar cord (L3–L5) the single NY-labeled population occupied both the dorsomedial and the ventrolateral parts of the red nucleus. It was therefore concluded that in the former case (11) there was only NY transport from the C5–C8 spinal grey matter, while in the latter case (12) there was NY transport from the C5–C8 and the L3–L5 spinal grey matter and probably also from damaged fibers passing through the injected segments. Both cases were perfused with 30% formalin which resulted in a high intensity of the FB fluorescence in retrogradely FB-labeled cells (see Figs. 10 and 11).

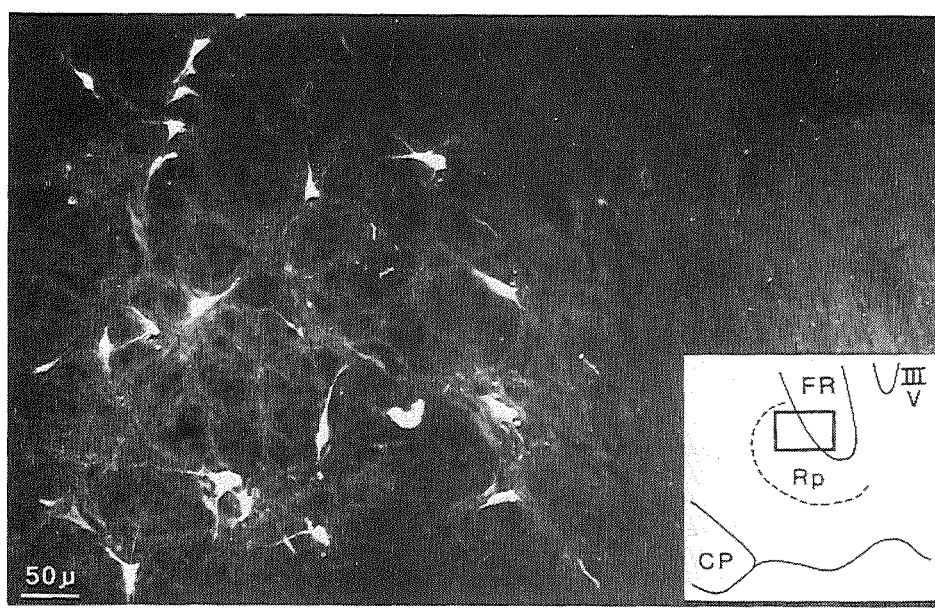


Fig. 10. Photomicrograph of the single FB-labeled neurons in the parvicellular red nucleus of the cat, immediately lateral to the fasciculus retroflexus (FR). Abbreviations: CP, cerebral peduncle; FR, fasciculus retroflexus; Rp, parvicellular part of the red nucleus; III V, third ventricle.

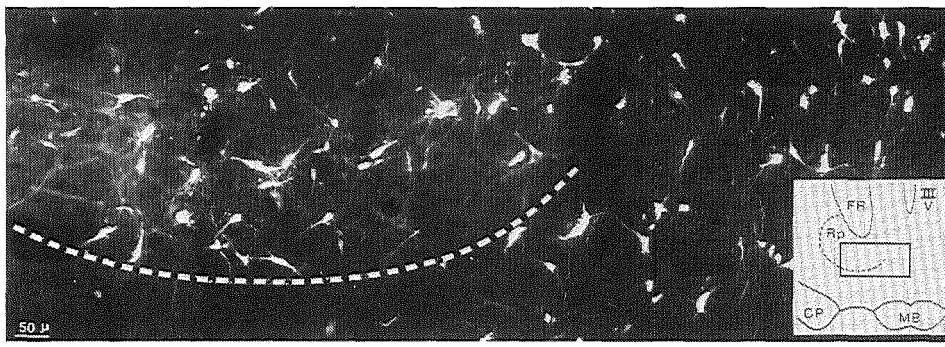


Fig. 11. Photomicrograph of the single FB-labeled neurons in the parvicellular red nucleus of the cat, ventrally to the fasciculus retroflexus (FR). Abbreviations: CP, cerebral peduncle; FR, fasciculus retroflexus; MB, mammillary bodies; Rp, parvicellular part of the red nucleus; III V, third ventricle.

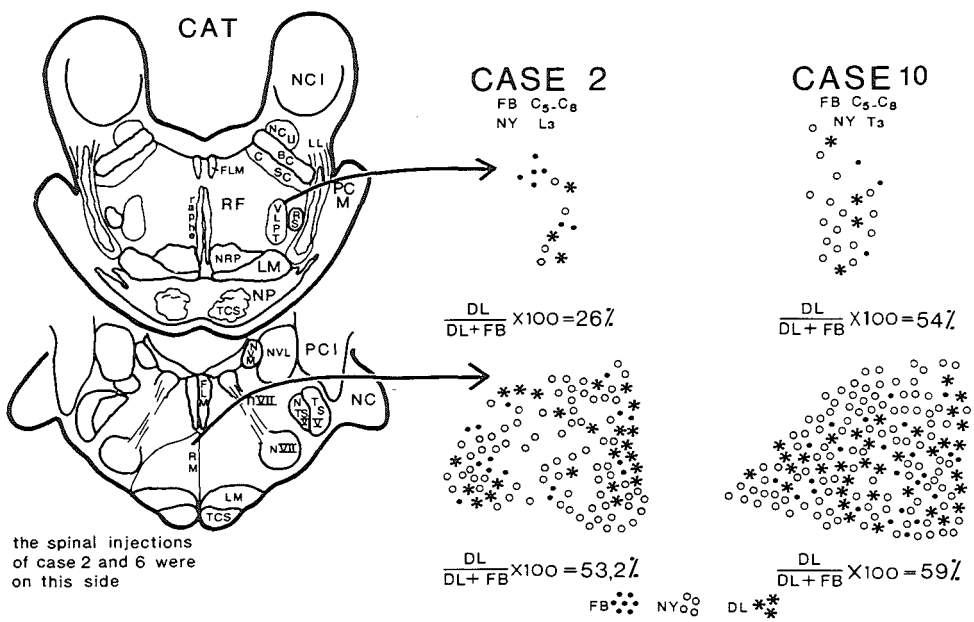


Fig. 12. Semidiagrammatic representation of the distributions of retrogradely labeled neurons in the ventrolateral pontine tegmentum (VLPT) and the nucleus raphe magnus (RM) after spinal injections in cat. In case 2: Fast Blue (FB) in C5–C8 and Nuclear Yellow (NY) in L3 and in case 10 FB in C5–C8 and NY in T3. Note the relatively high number of double labeled neurons especially in RM, even in case 2 with NY injected in L3. Abbreviations: BC, brachium conjunctivum; C, nucleus coeruleus; FLM, medial longitudinal fasciculus: LL, lateral lemniscus; LM, medial lemniscus; NC, cochlear nucleus; NCI, nucleus of inferior colliculus; NCu, cuneate nucleus; NP, pontine nuclei; NRP, reticular nucleus of pontine tegmentum; NTS V, nucleus of spinal V tract; NVL, lateral vestibular nucleus; NVM, medial vestibular nucleus; N VII, facial nucleus; n VII, facial nerve; PCI, inferior cerebellar peduncle; PCM, medial cerebellar peduncle; RF, reticular formation; RM, nucleus raphe magnus; RS, rubrospinal tract; SC, nucleus subcoeruleus; TSC, corticospinal tract; TSV, spinal V tract; VLPT, ventrolateral pontine tegmentum.

In the caudal one-third of the red nucleus in both cases only single NY-labeled neurons occurred, but many FB-labeled neurons were present in the central grey at these levels. In the middle one-third a large population of NY-labeled neurons was present but some single FB-labeled cells occurred, i.e., in the most dorsomedial part of the nucleus. This group of red nucleus neurons was continuous with a group of single FB-labeled cells in the dorsomedially adjoining interstitial nucleus of Cajal (INC) and many FB-labeled neurons were also present in the nucleus of Darkschewitsch (see Fig. 6A) (cf., Henkel et al., 1975; Brown et al., 1977; Cintas et al., 1980; Martin et al., 1980; Saint-Cyt and Courville, 1980). Finally in the rostral one-third of the red nucleus only single FB-labeled neurons were present, which were located medial, ventral and lateral to the fasciculus retroflexus (see Figs. 8, 10 and 11). This

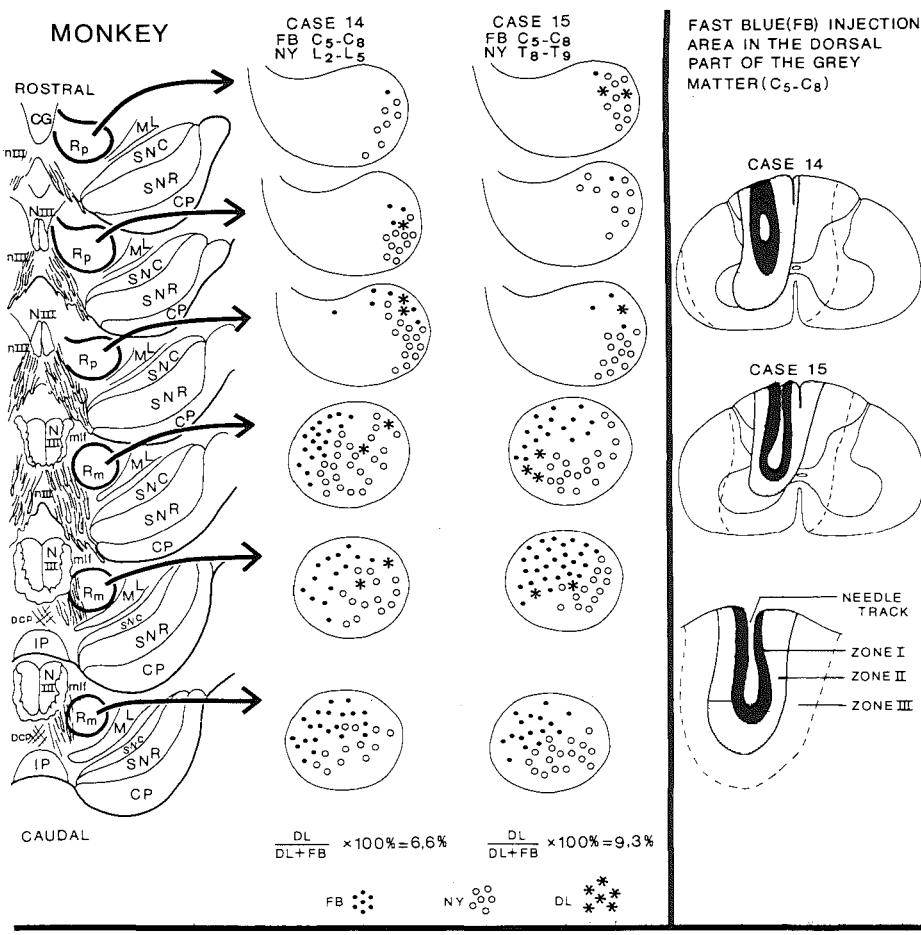


Fig. 13. Semidiagrammatic representation of the distributions of retrogradely labeled neurons in the red nucleus after spinal injections in monkey; in case 14: Fast Blue (FB) in C5–C8 and Nuclear Yellow (NY) in L2–L5 and in case 15: FB in C5–C8 and NY in T8–T9. Note the rostral extension of rubrospinal neurons in the lateral part of red nucleus, particularly the neurons which project to lower levels in the spinal cord. Abbreviations: G, central grey; CP, cerebral peduncle; DCP, decussation of superior cerebellar peduncles; IP, interpeduncular nucleus; ML, medial lemniscus; mlf, medial longitudinal fasciculus; N III oculomotor nucleus; n III, oculomotor nerve; Rm, red nucleus, magnocellular part; Rp, red nucleus, parvicellular part; SNC, substantia nigra, pars compacta; SNR, substantia nigra, pars reticulata.

population of FB-labeled neurons, which gradually tapered down at the level of the mammillary bodies, was continuous dorsomedially with a population of single FB-labeled cells in the nucleus of Darkschewitsch. The group of small FB-labeled cells in the rostral part of the red nucleus could be effectively delineated on the lateral and ventral sides and in part also on the medial side (see Figs. 10 and 11). It resembled in its configuration the parvicellular part of the red nucleus as observed in monkey (Kuypers and Lawrence, 1967).

In these two cats hardly any double labeled neurons were observed. Thus, in case 11 no FB–NY double labeled cells occurred and in case 12 only one FB–NY double labeled neuron was present, at the rostral border of the single NY-labeled population. These findings indicate that the rubro-olivary fibers in cat are derived virtually only from neurons in the rostral part of the red nucleus and that extremely few rubrospinal neurons distribute collaterals to the inferior olive (cf. Anderson, 1971).

(D) Nucleus raphe magnus (NRM) and ventrolateral pontine tegmentum (VLPT) in cat and monkey

Nucleus raphe magnus (NRM)

The NRM distributes fibers to the dorsal part of the spinal grey matter including the dorsal horn, by way of the dorsolateral funiculus (Basbaum et al., 1978; Leichnetz et al., 1978; Basbaum and Fields, 1979; Tohyama et al., 1979a,b; Goode et al., 1980; Martin et al., 1981a). However, the retrogradely labeled neurons in the area of the NRM after HRP injections in the dorsolateral funiculus (Leichnetz et al., 1978; Basbaum and Fields, 1979; Tohyama et al., 1979a) are not restricted to the NRM but extend laterally through the ventral part of the reticular formation dorsal to the pyramidal tract. In the present study the NRM projecting to the spinal dorsal grey was therefore defined according to the distribution of the retrogradely labeled neurons. This area corresponds to the distribution area of serotonergic neurons in rat (Dahlström and Fuxe, 1964, 1965), cat (Persson et al., 1978; Wiklund et al., 1982) and monkey (Hubbard and DiCarlo, 1974; Schofield and Everitt, 1981) at this level, because they extend laterally from the nucleus raphe as defined by Taber (1960, 1961) into the nucleus gigantocellularis lateralis, i.e., dorsal and lateral to the pyramidal tract.

Ventrolateral pontine tegmentum (VLPT)

Retrograde HRP studies showed that a group of neurons in the ventrolateral pontine tegmentum adjoining the area of the rubrospinal tract (TRS) (Kuypers and Maisky, 1975; Leichnetz et al., 1978; Basbaum and Fields, 1979) give rise to mainly crossed pontospinal fibers which descend in the dorsolateral funiculus throughout the spinal cord (Kuypers and Maisky, 1977; Holstege et al., 1979; Tohyama et al., 1979). These fibers seem to correspond to the crossed pontospinal tract of Busch (1964) and the lateral reticulospinal tract of Papez (1926). Anterograde labeled amino acid transport studies (Holstege et al., 1979; Martin et al., 1979) showed that this crossed descending pathway terminates in roughly the same portion of the spinal grey as the rubrospinal tract, i.e., in the dorsal part of the spinal intermediate zone, but also terminates in the spinal dorsal horn.

Retrograde labeling in nucleus raphe magnus (NRM) and ventrolateral pontine tegmentum (VLPT)

(a) Cat. In the 10 cats with FB and NY spinal injections the single FB, single NY and the FB–NY double labeled neurons in the NRM and the VLPT did not display any topographical distribution but were randomly intermixed with one another (see Fig. 12). This is in keeping

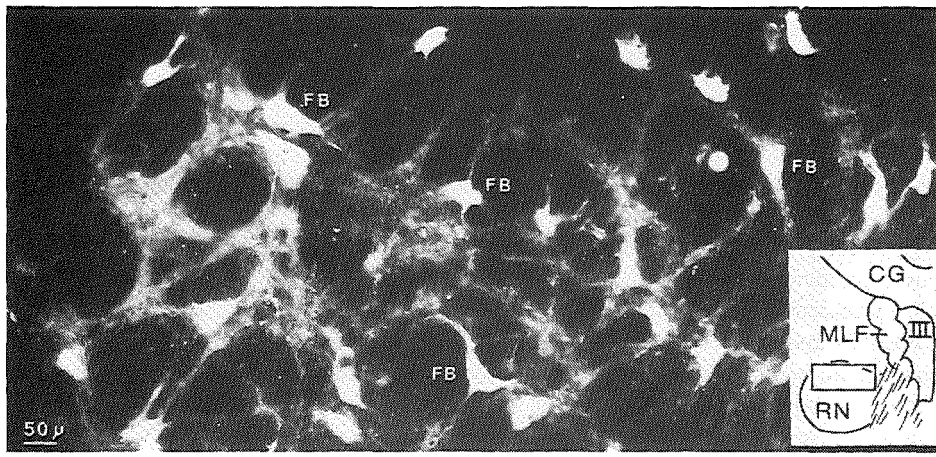


Fig. 14. Photomicrograph of dorsomedial part of caudal red nucleus in monkey, labeled with FB from C5–C8 contralateral spinal cord. Note the reticulated structure, in which the single FB-labeled neurons are arranged.

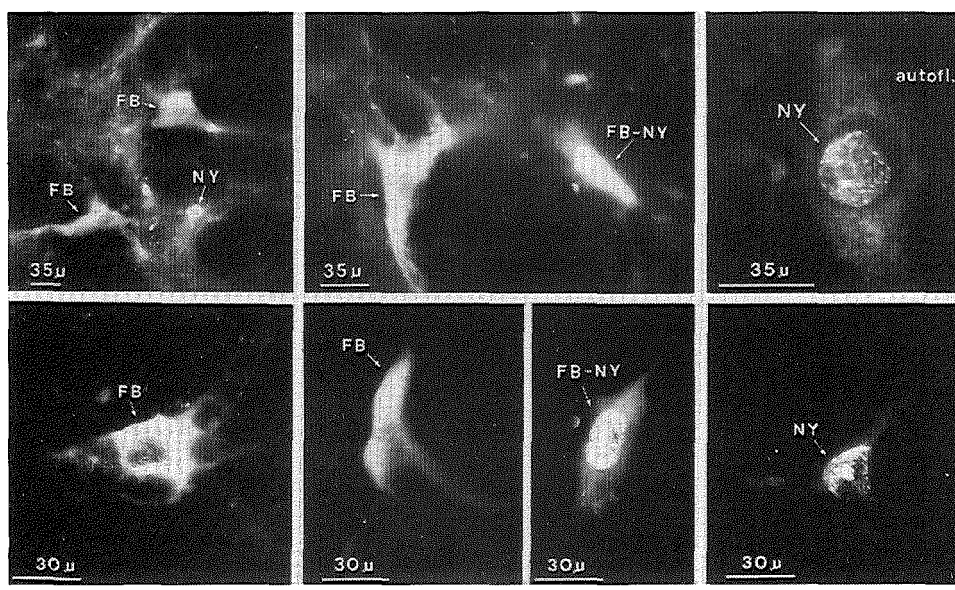


Fig. 15. Photomicrographs of single FB, single NY and FB–NY double labeled neurons in red nucleus (upper row) and nucleus raphe magnus (bottom row) of the monkey. The single FB-labeled neurons show a fluorescent labeling of the cytoplasm with silver fluorescent granules and the single NY-labeled ones a fluorescent nucleus and a clear ring around the nucleolus. The FB–NY double labeled neurons display all features.

with the anatomical findings in rat (Huisman et al., 1981) and opossum (Martin et al., 1981a,b). In the different experiments in cat the total number of neurons which were FB labeled from C5–C8 in the ipsilateral NRM ranged from 591 to 2943. In the contralateral VLPT the number ranged from 111 to 525. The percentages of the FB-labeled neurons in these nuclei which were FB–NY double labeled after injections at different spinal levels were

entirely different from those obtained in red nucleus (see histograms 1, 2 and 3 in Table IV). Thus in all cases a large percentage of the FB-labeled neurons in NRM and VLPT was double labeled. This was particularly striking in the NRM, as observed also in rat (Huisman et al., 1981) and opossum (Martin et al., 1981b). Moreover, the percentages of the FB–NY double labeled neurons in the NRM were always of roughly the same magnitude, varying from 55 % to 60 %, no matter at which spinal level NY was injected. Only in cases 5 and 7, they were lower: 24.5 % and 30.5 %, respectively, in which cases the percentages of FB–NY double labeled neurons in the VLPT were also lower than in the other cases (Table IV).

The findings in the red nucleus, the nucleus raphe magnus and the ventrolateral pontine tegmentum therefore suggest that in red nucleus to a large extent different groups of neurons project preferentially to different levels of the spinal cord and distribute only few collaterals to the other levels, while a large portion of NRM neurons as well as of VLPT ones distribute collaterals throughout the length of the spinal cord. Since always roughly the same percentage of double labeled neurons occurred in NRM and VLPT independent of the level of NY injections, it seems likely that many NRM and VLPT neurons give rise to long descending fibers proceeding throughout the length of the spinal cord giving off collaterals to the spinal grey at many different levels.

(b) Monkey. The distribution of the labeled cells in NRM and VLPT in monkey was the same as observed in cat (see Fig. 16). The numbers of single FB-labeled neurons including

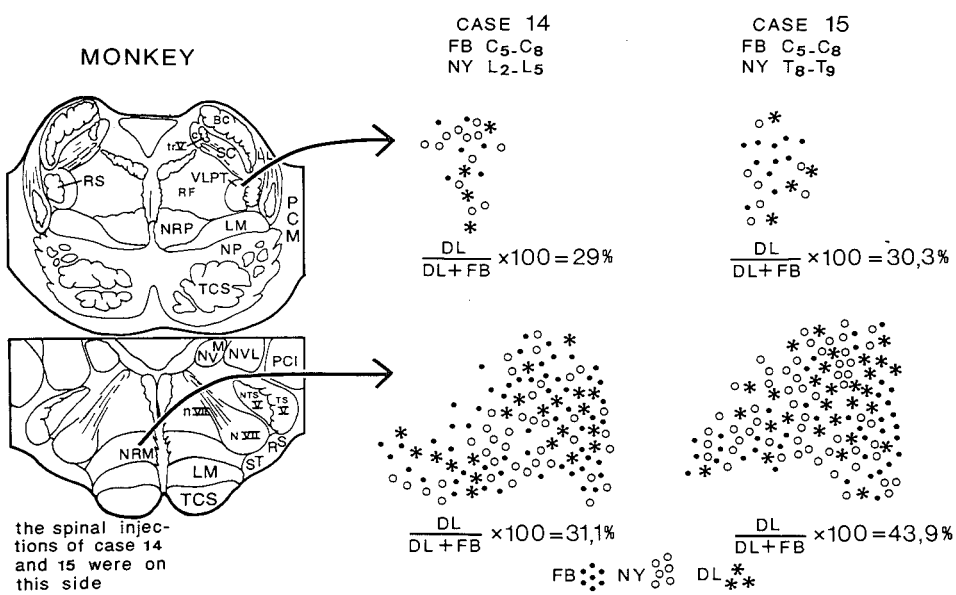


Fig. 16. Semidiagrammatic representation of the distributions of retrogradely labeled neurons in the ventrolateral pontine tegmentum (VLPT) and the nucleus raphe magnus (NRM) after spinal injections in monkey. In case 14: Fast Blue (FB) in C5–C8 and Nuclear Yellow (NY) in L2–L5 and in case 15 FB in C5–C8 and NY in T8–T9. Note the relatively high number of double labeled neurons, especially in NRM, even in case 14 with NY injected in L2–L5. Abbreviations: BC, brachium conjunctivum; C, nucleus coeruleus; LL, lateral lemniscus; LM, medial lemniscus; N VIII, facial nucleus; n VII, facial nerve; NP, pontine nuclei; NRM, nucleus raphe magnus; NRP, reticular nucleus of pontine tegmentum; NTS V, nucleus of spinal V tract; NVL, lateral vestibular nucleus; NVM, medial vestibular nucleus; PCI, inferior cerebellar peduncle; PCM, medial cerebellar peduncle; RF, reticular formation; RS, rubrospinal tract; SC, nucleus subcoeruleus; ST, spinothalamic tract; TCS, corticospinal tract; tr V, tract of mesencephalic V nucleus; TS V, spinal V tract; VLPT, ventrolateral pontine tegmentum.

FB–NY double labeled ones in the ipsilateral NRM ranged from 1220 to 2344. In the contralateral VLPT these numbers were 321 in case 14 and 525 in case 15. In case 13 the number of such labeled neurons in the contralateral VLPT was very low, therefore no percentages were computed in this case. The percentages of FB-labeled neurons which were FB–NY double labeled in NRM were 27 % (case 13), 31.1 % (case 14) and 43.9 % (case 15) (see Table V). In VLPT these percentages were 29 % (case 14) and 30.3 % (case 15). Therefore in the NRM, VLPT and the red nucleus in the monkeys the same differences in the percentages of the double labeled neurons were observed as in the cats (compare the histograms in Tables IV and V). This indicates that also in monkey different groups of RN neurons to a large extent project preferentially to certain levels of the spinal cord and distribute only few collaterals to the other levels, while many of the NRM and VLPT neurons distribute collaterals throughout the length of the spinal cord.

DISCUSSION

The present study tried to elucidate in cat and monkey differences in the degree of collateralization in the spinal cord between 3 brain stem pathways, i.e., the rubrospinal tract, the rostral medullary raphe spinal tract and the crossed pontospinal tract, all of which descend through the dorsolateral funiculus and terminate in the dorsal part of the spinal grey (Nyberg-Hansen and Brodal, 1964; Petras, 1967; Edwards, 1972; Basbaum and Fields, 1978, 1979; Holstege et al., 1979; Martin et al., 1979). On the basis of the present findings the degree of collateralization of the rubrospinal tract in cat and monkey could also be compared with that in other species. This latter comparison appeared to be of interest because earlier findings suggested that the degree of collateralization of the rubrospinal tract differs in different animals (Huisman et al., 1981; Martin et al., 1982).

Technique

The multiple retrograde fluorescent tracer technique (Kuypers et al., 1980) and the HRP–[^{3}H]apo-HRP technique (Hayes and Rustioni, 1979, 1981) seem to be most suitable for demonstrating the existence of collaterals in brain pathways. However, a comparison of the results obtained by means of these two techniques, e.g., in respect to the raphe spinal projections (cf., Hayes and Rustioni, 1981; Huisman et al., 1981), suggested that the retrograde fluorescent tracer technique produces a larger proportion of double labeled neurons. Therefore this method was chosen. This choice was reinforced by the fact that in the present study a larger number of double labeled neurons were found in raphe and red nucleus in cat than seems to have been obtained by means of the HRP–[^{3}H]apo-HRP method (Hayes and Rustioni, 1981) in the same animal. This difference may be explained by the fact that in the autoradiographic material silver grains will only be elicited in the emulsion by sources of radioactivity which are located in the upper 3 μm layer of the 40 μm sections (Sidman, 1970). As a consequence, even under optimal circumstances, only a small percentage of the total population of HRP-labeled neurons observed in each 40 μm section can be double labeled. The fluorescent tracers are also more sensitive than HRP, as suggested by the findings of Sawchenko and Swanson (1981) that the fluorescent tracers label approximately twice as many neurons than the HRP–TMB technique (Mesulam and Rosene, 1979). This is supported by a comparison of the present findings with those obtained by Condé and Condé (personal communications) which shows that after Fast Blue (FB) injections in inferior olive the number

of labeled rubro-olivary neurons at a given level in the red nucleus was significantly larger than obtained with the HRP–BDHC technique (Mesulam, 1976; Condé and Condé, 1982) at approximately the same level (i.e., in a 30 μm section about 80 FB-labeled neurons and in a 60 μm section about 40 HRP-labeled neurons).

In order to obtain reliable quantitative data concerning single FB- and NY-labeled neurons and FB–NY double labeled neurons several requirements had to be fulfilled. First the FB injections in C5–C8 had to be restricted to the grey matter and must not have involved the fiber bundles of the 3 tracts in the dorsolateral funiculus. Under the present circumstances with 4 weeks survival time FB was only transported from zones I and II (see Results). Therefore only those cases have been selected for study (10 cats and 3 monkeys) in which zones I and II were restricted to the grey matter and did not involve the dorsolateral funiculus. The remaining cases (10 cats) were discarded. In order to obtain reliable quantitative data it was also necessary to label retrogradely a very large number of neurons. For this purpose two measures were taken. First perfusion with 30% formalin instead of with 10% formalin was used, because the former results in a much more intense FB labeling than the latter. As a consequence a much larger number of neurons could be recognized as FB labeled. Moreover, in the more recent cases a NY–DMSO mixture was injected in cat since this mixture in cat strongly increased the number of neurons which were retrogradely labeled from damaged axons passing through the injected segments (see Results).

Earlier electrophysiological studies have dealt with the collaterals of descending pathways. Shinoda et al. (1977) reported the number of red nucleus neurons which could be antidromically invaded from C3–C8 grey matter and the percentage of these neurons which could be antidromically invaded by stimulation of the fibers passing through different more caudal segments of the cord. In order to facilitate a comparison between our anatomical data and these physiological data the anatomical experiments were set up in a comparable manner. Thus the number of FB-labeled neurons which project to the C5–C8 dorsal grey matter was counted and the percentages of these neurons which were also labeled with NY were computed. This procedure also allowed a comparison between the present findings in cat and monkey and those obtained earlier in rat (Huisman et al., 1981).

Somatotopic organization of red nucleus

In *cat* the injections of the two tracers in C5–C8 and in more caudal spinal segments in essence labeled two different populations of rubrospinal neurons. The distribution of the total population of these retrogradely labeled rubrospinal neurons confirmed previous findings (Pompeiano and Brodal, 1957; Hayes and Rustioni, 1981) and showed that the rubrospinal neurons are mainly present in the caudal two-thirds of the nucleus. The findings in the experiments with FB injections in the inferior olive and NY injections in the cervical cord further showed that in cat the red nucleus neurons, which distribute fibers to the ipsilateral inferior olive (Walberg, 1956; Courville and Otabe, 1974; Martin et al., 1975, 1980; Strominger et al., 1979), represent a separate population which is largely restricted to the rostral part of the nucleus (see Fig. 8). This is in keeping with other HRP findings (Saint-Cyr and Courville, 1980; Condé and Condé, 1982).

The distribution of the single FB- and single NY-labeled rubrospinal neurons confirmed the somatotopic organization of this nucleus as demonstrated earlier by means of other techniques (Pompeiano and Brodal, 1957; Tsukahara et al., 1967; Gwyn, 1971; Hayes and Rustioni, 1981). Thus, in the caudal half of the magnocellular part the rubrocervical, the rubrothoracic and the rubrolumbar neurons are located in the dorsomedial, intermediate and ventrolateral

part of the nucleus, respectively. On the other hand in the rostral half of the magnocellular part these neurons become intermixed (see Fig. 8).

In *monkey* retrograde degeneration studies (Poirier and Bouvier, 1966; Kuypers and Lawrence, 1967) showed that the rubrospinal neurons are largely restricted to the magnocellular part. However, the present findings showed that the population of rubrospinal neurons extends further rostrally and continues along the lateral aspect of the parvicellular part (see Fig. 13) up to the level, where the cross-section through the fasciculus retroflexus is located ventral to the nucleus. This seems in keeping with the retrograde HRP finding of Kneisley et al. (1978) as inferred from their illustrations. The location of these rubrospinal neurons appears to correspond with that of neurons with coarse Nissl bodies (Miller and Strominger, 1973). This supports the idea that rubrospinal neurons are characterized by their internal configuration more or less independent of the size of the neurons (King et al., 1971).

The present findings support the earlier reported somatotopic organization in the caudal magnocellular part of the monkey red nucleus (Kneisley et al., 1978). They also indicate that in the rostral extension of the population of rubrospinal neurons along the lateral aspect of the parvicellular part the rubrocervical neurons are located dorsal to the rubrolumbar ones and that the latter continue more rostrally than the former (see Fig. 13).

Collateralization in rubrospinal tract

The percentages of the rubrocervical neurons in cat projecting to C5–C8 which also distribute collaterals to more caudal levels, as computed from the present anatomical findings, are of the same order of magnitude as those inferred from the earlier electrophysiological findings in this animal. Thus according to the electrophysiology (Shinoda et al., 1977) 5% of the rubrocervical neurons projecting to C3–C8 distribute collaterals to segments caudal to L1, and 50% distribute collaterals to segments caudal to T2. According to the anatomical findings about 3% of the rubrocervical neurons projecting to C5–C8 distribute collaterals to the segments caudal to L2 and about 20% distribute collaterals to the segments caudal to T3 (see Table IV). In monkey a similar arrangement was found anatomically: 9.3% of the rubrocervical neurons projecting to C5–C8 were found to distribute collaterals to the segments caudal to T8 and 6.6% to the segments caudal to L1 (see Table V).

The slight differences between the percentages obtained by means of the electrophysiological and the anatomical techniques may be due to the fact that in the two types of experiments slightly different cervical segments were studied and slightly different thoracic and lumbar segments were stimulated and injected respectively. The differences may also be due to differences in the rostrocaudal extent of the nucleus, explored in the two types of experiments.

Rubrospinal collateralization in different animals

A comparison of the present anatomical findings in cat and monkey with those obtained in rat (Huisman et al., 1981) indicates that the rubrospinal system in rat displays a higher degree of collateralization than in cat and monkey. Thus in rat 20% of the rubrocervical neurons projecting to C5–C8 distribute collaterals to segments caudal to L1 versus only about 3% in cat and about 6% in monkey. Further, in rat about 35% of the rubrocervical neurons distribute collaterals to segments caudal to T7 versus about 10% in cat and monkey. The rubrospinal tract in cat and monkey therefore appears to be more focussed than in rat such that in the former species a larger proportion of the rubrospinal neurons distributed fibers to a restricted part of the spinal cord than in the latter. This is further emphasized by the fact that the high degrees of collateralization of the raphe spinal and the crossed pontospinal tract are of the same order of magnitude in the 3 species (see below).

The comparison of the anatomical findings in opossum and rat (Huisman et al., 1981; Martin et al., 1982) strongly suggests that the rubrospinal tract in opossum shows an even higher degree of collateralization than in rat. This would imply that the rubrospinal tract in rat, in turn, is more focussed than in opossum. The fact that the red nucleus in opossum displays a much less rigid somatotopic organization than in rat (Martin et al., 1974; Huisman et al., 1981) suggests that with an increase in collateralization the somatotopic organization becomes less sharply defined. If this is correct the absence of any somatotopic organization in the red nucleus in pigeon (Wild et al., 1979) and in reptiles (ten Donkelaar and de Boer-van Huizen, 1978) would imply that in these species the rubrospinal tract displays an even more pronounced collateralization than in opossum. In snakes this tract seems to be lacking (ten Donkelaar, 1976a,b). It therefore appears that the rubrospinal tract, as a highly focussed fiber brain stem system, emerges only gradually during phylogeny. It would be of interest to determine whether in respect to the rubrospinal collateralization the ontogeny mimics the phylogeny, such that e.g. in newborn kittens a higher degree of collateralization occurs than in adult cat. This question is prompted by the fact that in other structures such an ontogenetic decrease in collateralization has been established (Innocenti, 1981; O'Leary et al., 1981).

In respect to the phylogenetic decrease in rubrospinal collateralization, it is of interest to recall that in cat and monkey (Kuypers, 1964; Lawrence and Kuypers, 1968; Smith, 1970; Gorska and Sybirska, 1978; Kohlerman et al., 1980) this tract together with the corticospinal tract contributes to the capacity to execute relatively independent movements of the distal parts of the limbs. This is probably based on the fact that in these animals a relatively limited degree of collateralization occurs such that many of the rubrospinal neurons project to very restricted parts of the spinal cord. The suggested decrease in collateralization of this tract from reptiles to pigeon, opossum, rat, cat and monkey makes it likely that the capacity of the rubrospinal tract to provide the facility for the execution of relatively independent movements of the individual extremities also gradually emerges during phylogeny. This notion is strengthened by the fact that such a capacity is clearly present in monkey and cat, in that sense that it seems to be lacking in reptiles.

Collateralization in the three tracts compared

The relatively limited degree of collateralization of the rubrospinal tract is emphasized by the contrast between the number of double labeled neurons in red nucleus and in nucleus raphe magnus (NRM) at the level of the facial nucleus. The NRM in cat and monkey contained a relatively small population of FB and NY single labeled neurons which project to the cervical and the more caudal segments respectively, but contained a relatively large population of double labeled neurons, i.e., 55–60% of the raphe cervical neurons in cat and about 40% in monkey. These high percentages occurred more or less independent of the level of the caudal NY injection (see Tables IV and V). Moreover, no clear-cut somatotopic organization in the distribution of these 3 types of neurons was observed. These findings in cat and monkey are in keeping with those in rat (Huisman et al., 1981) in which about 40% of the raphe cervical neurons were always double labeled and with those in opossum (Martin et al., 1981a,b). The raphe spinal system therefore resembles the reticulospinal system since according to an electrophysiological study (Peterson et al., 1975), 66% of the reticulocervical neurons distribute collaterals to the segments caudal to L1. The anatomical findings suggest that a large percentage of the raphe spinal neurons in NRM distribute collaterals throughout the length of the spinal cord, but that the remainder project to restricted parts of the cord. This would imply that the raphe spinal system comprises both a focussed component and a diffuse component.

In rat, cat and monkey many NRM neurons contain serotonin (Dahlström and Fuxe, 1964,

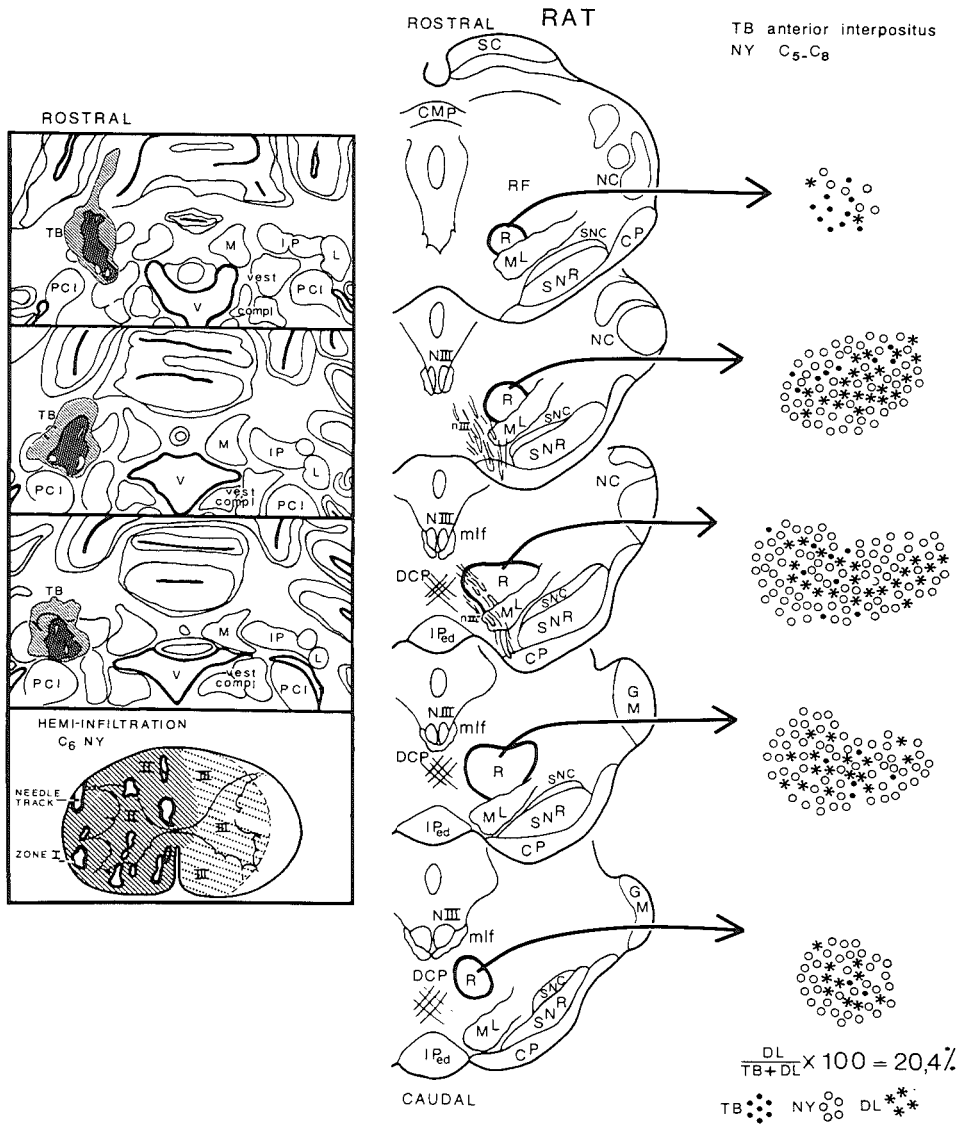


Fig. 17. Semidiagrammatic representation of the distributions of retrogradely labeled neurons in the red nucleus in rat after True Blue (TB) injections in the interpositus nucleus and Nuclear Yellow (NY) injections in the spinal cord, ipsilateral to the interpositus injection. Note that almost all TB-labeled rubrocerebellar neurons are double labeled with NY from the spinal cord. Abbreviations: CMP, posterior commissure; CP, cerebral peduncle; DCP, decussation of superior cerebellar peduncles; GM, medial geniculate body; IP, nucleus interpositus; IPed, interpeduncular nucleus; L, lateral nucleus of the cerebellum; M, medial nucleus of the cerebellum; ML, medial lemniscus; mlf, medial longitudinal fasciculus; NC, cochlear nuclei; N III, oculomotor nucleus; n III, oculomotor nerve; R, red nucleus; RF, reticular formation; SC, superior colliculus; SNC, substantia nigra, pars compacta; SNR, substantia nigra, pars reticulata; V, fourth ventricle; vest, compl., vestibular complex.

1965; Hubbard and DiCarlo, 1974; Persson et al., 1978; Schofield and Everitt, 1981; Wiklund et al., 1982) and in rat about 45% of the raphe spinal neurons contain serotonin (Bowker et al., 1981). It would therefore be of interest to determine whether either the focussed or the diffuse component of the raphe spinal system would be preferentially derived from serotonergic neurons. Raphe spinal neurons also contain substance P and enkephalin (Hökfelt et al., 1979; Bowker et al., 1981) probably in some neurons in coexistence with serotonin (Hökfelt et al., 1977, 1978; Bowker et al., 1981). If the serotonergic neurons in the NRM give rise either to the focussed or the diffuse component of the raphe spinal system one of these two components would in particular subserve the pain modulating function of the raphe spinal pathway (Oliveras et al., 1975, 1977; Basbaum et al., 1976a,b, 1977; Beall et al., 1976; Fields et al., 1977; Guilbaud et al., 1977; Basbaum and Fields, 1978, 1979; Fields and Basbaum, 1978; Giesler et al., 1981) since this function is presumably provided by serotonergic raphe spinal neurons (Dahlström and Fuxe, 1965; Proudfit and Anderson, 1973, 1974; Proudfit et al., 1980). However, the focussed or the diffuse component may also represent the serotonergic raphe spinal fibers to the autonomic cell groups throughout the spinal cord (Dahlström and Fuxe, 1965; Basbaum et al., 1978; Steinbusch, 1981).

The crossed pontospinal tract from VLPT neurons

The crossed pontospinal tract from VLPT neurons in cat and monkey (Kuypers and Maisky, 1975) seems to occupy a position between the rubrospinal and the raphe spinal system as also observed in rat. Thus, it shows little somatotopic organization and a relatively high degree of collateralization (see Figs. 12 and 16).

Rubro-olivary connections

The rubro-olivary neurons in cat were found in the rostral portion of the red nucleus which is in keeping with the HRP findings (Saint-Cyr and Courville, 1980; Condé and Condé, 1982). This part probably corresponds with the pars parvicellularis in monkey (Kuypers and Lawrence, 1967). Moreover, from the present findings it may be concluded that the rubrospinal neurons and the rubro-olivary ones constitute two separate populations as suggested also by HRP findings (Condé and Condé, 1980, 1982) and that rubrospinal neurons distribute virtually no collaterals to the inferior olive (see Fig. 8).

Rubrocerebellar connections

In view of the above discussed rubrospinal and rubro-olivary connections it is of interest to recall that red nucleus neurons also distribute fibers to the crossed cerebellar interpositus nucleus (Courville and Brodal, 1966). Recently another series of double labeling experiments in rat was initiated in order to determine whether the rubrocerebellar fibers represent collaterals of rubrospinal neurons (Huisman, Kuypers, Condé and Keizer, in preparation). In this study "True Blue" and "Nuclear Yellow" were used as retrograde tracers. Preliminary double labeling findings in this study show that at least 25% of rubrospinal neurons in rat distribute collaterals to the cerebellar interpositus nucleus (see Fig. 17). The existence of these collaterals was suggested earlier by retrograde degeneration findings in cat (Brodal and Gogstad, 1954). Electrophysiological studies in cat showed that all rubrocerebellar fibers are collaterals of rubrospinal fibers (Anderson, 1971). This is in agreement with our preliminary anatomical findings that in rat almost all red nucleus neurons labeled from the cerebellum were also labeled from the spinal cord.

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CHAPTER V: COLLATERALS OF RUBROSPINAL NEURONS TO THE CEREBELLUM IN RAT. A RETROGRADE FLUORESCENT DOUBLE LABELING STUDY

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Brain Research: in press

Key words: red nucleus - cerebellar interpositus nucleus - multiple fluorescent tracers - collateralization.

Summary

In a previous study the collateralization of the rubrospinal tract in the spinal cord of rat, cat and monkey was studied by means of the fluorescent retrograde double labeling technique. In the present study the existence of rubrospinal collaterals to the cerebellar interpositus nucleus (NI) has been studied using the same technique. In rat 'True Blue' (TB) was injected in the cerebellar NI and 'Nuclear Yellow' (NY) was injected ipsilaterally in white and grey matter of C5-C8 spinal segments. In some cases a new fluorescent retrograde tracer was used instead of NY, i.e. 'Diamidino Yellow' (DY) which produces retrograde labeling similar to NY but which migrates only very slowly out of the retrogradely labeled neurons. In these experiments only very few single TB labeled rubrocerebellar neurons occurred, but many (± 90%) of the TB fluorescent rubrocerebellar neurons were TB-NY or TB-DY double-labeled from the spinal cord. At least 37% of the NY and DY fluorescent rubrospinal neurons were NY-TB and DY-TB double labeled from the cerebellum. These findings indicate that, in rat, almost all rubrocerebellar fibers represent collaterals of rubrospinal neurons, and that at least 37% of the rubrospinal neurons give rise to such cerebellar collaterals.

Introduction

Anatomical studies demonstrate that the red nucleus projects not only to the lower brain stem and the spinal cord, but also to the cerebellum.[6,12] Earlier anatomical findings[6] supported by some electrophysiological data[1] suggested that the rubrocerebellar fibers represent collaterals of rubrospinal neurons. In the present study an attempt has been made to clarify this point with the aid of the multiple fluorescent retrograde labeling technique.[27]

Classic anatomical studies using the Golgi technique[8] demonstrated that many axons in the central nervous system give off collaterals along their trajectory through the brain. For a long time little attention has been paid anatomically to these collaterals, mainly because the available anterograde degeneration and transport techniques[31,16,28,13] did not make it possible to distinguish divergent axon collaterals from unbranched fibers. Recently it has become possible to study divergent axon collaterals anatomically, either by using two retrograde fluorescent tracers, which for example label different features of the parent cell,[27] or by using HRP and ^{3}H-apo-HRP.[20] However, it seems that with the latter technique fewer double labeled neurons are demonstrated than with the former.[22,23,21] Therefore the multiple fluorescent

	TB (2%) injections in cerebellum	TB survival times till perfusion	NY (1%) or DY (2%) injections in C5-C8 spinal segments	NY and DY survival times till perfusion
case 1	1,2 ul 2% TB	7 days	2 ul 1% NY	24 hours
case 2	1,2 ul 2% TB	7 days	2 ul 1% NY	24 hours
case 3	2,4 ul 2% TB	7 days	2 ul 2% DY	7 days
case 4	1,6 ul 2% TB	7 days	2 ul 2% DY	7 days
case 5	1,2 ul 2% TB	7 days	2 ul 1% NY	24 hours
case 6	1,6 ul 2% TB	7 days	2 ul 2% DY	7 days
case 7	1,2 ul 2% TB	7 days	2 ul 1% NY	24 hours
case 8	1,2 ul 2% TB	7 days	2 ul 2% DY	7 days

Table I shows: the amount of 2% TB injected in the cerebellum (column 1), the TB survival times (column 2), the amount of 1% NY and 2% DY, injected in the spinal cord (column 3) and the NY and DY survival times (column 4).

retrograde tracing technique was used in the present study. In rats, 'True Blue' (TB)[2] was injected in the cerebellar interpositus nucleus and 'Nuclear Yellow' (NY)[3,4] was injected ipsilaterally in the spinal cord. In frozen section material, TB produces a blue fluorescent retrograde labeling of neuronal cytoplasm, while NY (after short survival time) produces mainly a yellow fluorescence of the nucleus. On the basis of earlier findings, double retrogradely labeled neurons in the red nucleus which are labeled from the cerebellum as well as from the spinal cord would display a blue fluorescent cytoplasm and a yellow fluorescent nucleus when viewed at 360 nm excitation wavelength.[27] In some cases of the present study a new fluorescent retrograde tracer was used instead of NY. This new tracer, which will be described separately, is a diamidino compound: 'Diamidino Yellow' (DY).[24] It produces a retrograde neuronal labeling somewhat similar to that obtained with NY, but it migrates more slowly out of the retrogradely labeled neurons.

In the present experiments, the distribution of the single and double labeled neurons in the contralateral red nucleus was studied, after injections of the two tracers in the cerebellum and the spinal cord, respectively. Moreover, the neurons labeled from the spinal cord were counted and the percentage of these neurons which were double labeled from the cerebellum was computed. Thus the proportion of rubrospinal neurons which distribute collaterals to the cerebellum could be approximated.

In earlier fluorescent double labeling experiments[22,23] the collateralization of the rubrospinal and raphespinal fibers in the spinal cord was studied in rat, cat and monkey. The fluorescent retrograde labeling findings regarding the rubrospinal collateralization to the cerebellum in rat were compared with the earlier findings regarding the collateralization of the rubrospinal fibers in the spinal cord.

Materials and Methods

Injections

In 29 rats anaesthetized with Nembutal (intrahepatic, 6%), 3 to 6 glass micropipette injections of 0.4 µl 2% 'True Blue' (TB)[2] were made (1.2 - 2.4 µl in total) in the anterior two thirds of the cerebellar interpositus nucleus (NI) (Table I). In one of the rats no other injections were made. This animal was sacrificed after 7 days survival time and served as a control. In some of the rats, immediately after the cerebellar TB injections, laminectomies were made at C5-C8 and in each of these rats a spinal hemiinfiltration (i.e.

multiple penetrations in one half of the spinal cord) was made with 2 µl 2% 'Diamidino Yellow' (DY)[24] ipsilateral to the interpositus injections. These animals survived for one week. The other rats, after they had survived the TB injections for 6 days, were reoperated under Nembutal anaesthesia. In these animals laminectomies were made at C5-C8 and in each of these rats a hemi-infiltration was made with 2 µl 1% 'Nuclear Yellow' (NY)[3,4,27] dissolved in 2% dimethylsulfoxide (DMSO) (Table I). DMSO was used because it improves NY transport from damaged fibers passing through the injected segments.[23] These animals were sacrificed 24 hours after the NY injections. Thus, in these cases a 7 days TB survival time and a 24 hours NY survival time was used (see Table I).

The short NY survival time was necessary in order to prevent in vivo migration of NY from the retrogradely labeled neurons in red nucleus.[4] The NY and DY spinal hemiinfiltrations were made by means of multiple micropipette penetrations to damage a large number of passing fibers. This was in order to obtain NY and DY transport by fibers passing through the injected segments and damaged by the penetrations, as well as by fibers terminating there.

Of the 29 rats only 9 were used because in the others the TB injections were either located too medially and the needle had penetrated the fourth ventricle or the injection areas extended too deeply and involved the brain-stem including the area of the rubrospinal tract. In 4 of the 9 rats the TB cerebellar injections were centered in the anterior interpositus nucleus (NIA) (cases 1,2,3 and 4). Two of these cases (1 and 2) had a spinal hemiinfiltration with NY and the other two cases (3 and 4) had a spinal hemiinfiltration with DY (Table I). In the one control rat with only the TB injection in the cerebellum, the injection area was also centered in the NIA. In the remaining 4 rats (cases 5,6,7 and 8) TB cerebellar injections were centered outside the NI. In case 5 it was located just medial and rostral to the nucleus, in cases 6 and 7 dorsal to it and in case 8 dorsal and medial to it. Cases 5 and 7 had a spinal hemiinfiltration with NY while cases 6 and 8 had a spinal hemi-infiltration with DY. (Table I)

Perfusion, Fixation

At the end of the survival period the animals were deeply anaesthetized with Nembutal and were perfused transcardially with 1.00 l. Na Cl (1.5%) followed by 1.00 l. cacodylate buffered formaline (10%, pH 7,2). The brains and the injected segments were dissected and stored overnight in cacodylate buffered sucrose (30%, pH 7,2) at 4°C. They were cut transversally in frozen sections, 30 µm thick, on a freezing microtome. The injected segments, before

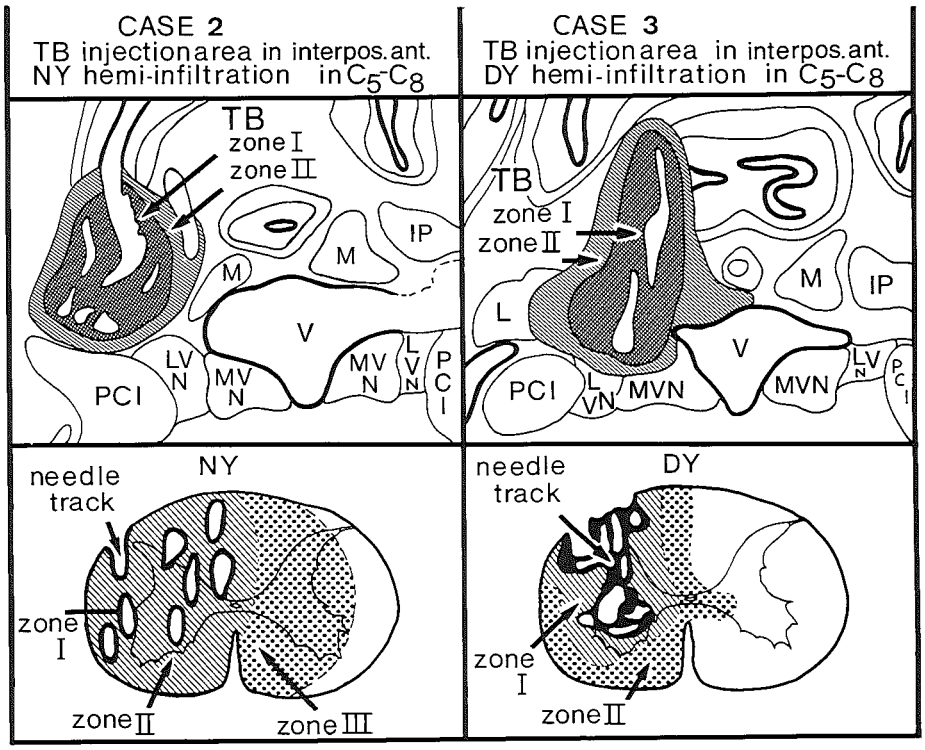

Fig. 1. *Upper row:* Diagrams of TB injection areas centered in anterior interpositus nucleus in case 2 (left diagram) and case 3 (right diagram). For characteristics of zones I and II see text. *Abbreviations:* IP, interpositus nucleus; L, lateral cerebellar nucleus; LVN, lateral vestibular nucleus; M, medial cerebellar nucleus; MVN, medial vestibular nucleus; PCI, inferior cerebellar peduncle; V, fourth ventricle. *Bottom row:* Diagrams of the NY hemiinfiltration at C5-C8 in case 2 (left diagram) and of DY hemiinfiltration at C5-C8 in case 3 (right diagram). For characteristics of zones I, II and III see text. Note: after injecting the same quantity of 2% DY and 1% NY the DY injection area is more restricted than the NY injection area.

being cut, were coated with carboxymethyl-cellulose in order to keep the sections containing the needle tracks intact. After being cut, the sections through the brain stem and spinal cord were immediately mounted from distilled water and air dried at room temperature.[4] In general every fourth brain stem section was studied in detail.

Data analysis

The material was studied with a Leitz Ploemopack fluorescence microscope

equipped with a filter mirror system A providing excitation light of 360 nm wavelength. The fluorescent injection areas were outlined and the distributions of the single TB- and single NY or DY-labeled neurons and of the TB-NY or TB-DY double labeled ones in the contralateral red nucleus (RN) were charted with the aid of an X-Y plotter connected with transducers attached to the microscope stage. The sections through the cerebellum were counterstained with cresyl violet and the relationship between the injection areas and the cerebellar nuclei was determined.

At 360 nm excitation wavelength the single TB labeled neurons showed a deep blue fluorescent cytoplasm and a blue fluorescent nucleolus but no labeling of the nucleus, which is in keeping with earlier observations.[2] The single NY labeled rubrospinal neurons, in the present cases showed mainly a golden yellow granular fluorescence of the nucleus with a clear fluorescent ring around the nucleolus as described earlier.[3] At this same excitation wavelength, single DY labeled neurons showed a rather diffuse golden yellow fluorescent nucleus, which appeared smaller than the NY labeled ones.[24] The fluorescent ring around the nucleolus in the DY labeled nuclei was less pronounced than in the NY labeled ones. In addition, the cytoplasm of both the cell body and proximal dendrites of the single DY labeled neurons frequently showed some diffuse yellow fluorescence with golden yellow fluorescent granules. Many neurons in the red nucleus showed a blue TB fluorescent cytoplasm and a yellow NY fluorescent nucleus at the same 360 nm excitation wavelength. These neurons were therefore regarded as TB-NY double labeled.[27] TB-DY double labeled neurons showed a rather diffuse golden DY fluorescent nucleus with a slightly fluorescent ring around the nucleolus and a blue TB fluorescent cytoplasm. In some cases the blue TB fluorescent cytoplasm showed some slight admixture of yellow DY fluorescence and contained some golden yellow fluorescent granules.[24]

Results

A) Injection areas

Cerebellum

The TB fluorescent injection areas in the cerebellum consisted of two concentric fluorescent zones. Zone I, immediately surrounding the needle track, contained a bright blue fluorescent cellular debris and a dense accumulation of fluorescent glial nuclei, but otherwise had lost its normal histological texture. Zone II contained much fewer fluorescent glial nuclei, which decreased in number and brightness towards the periphery of this zone. In the periphery

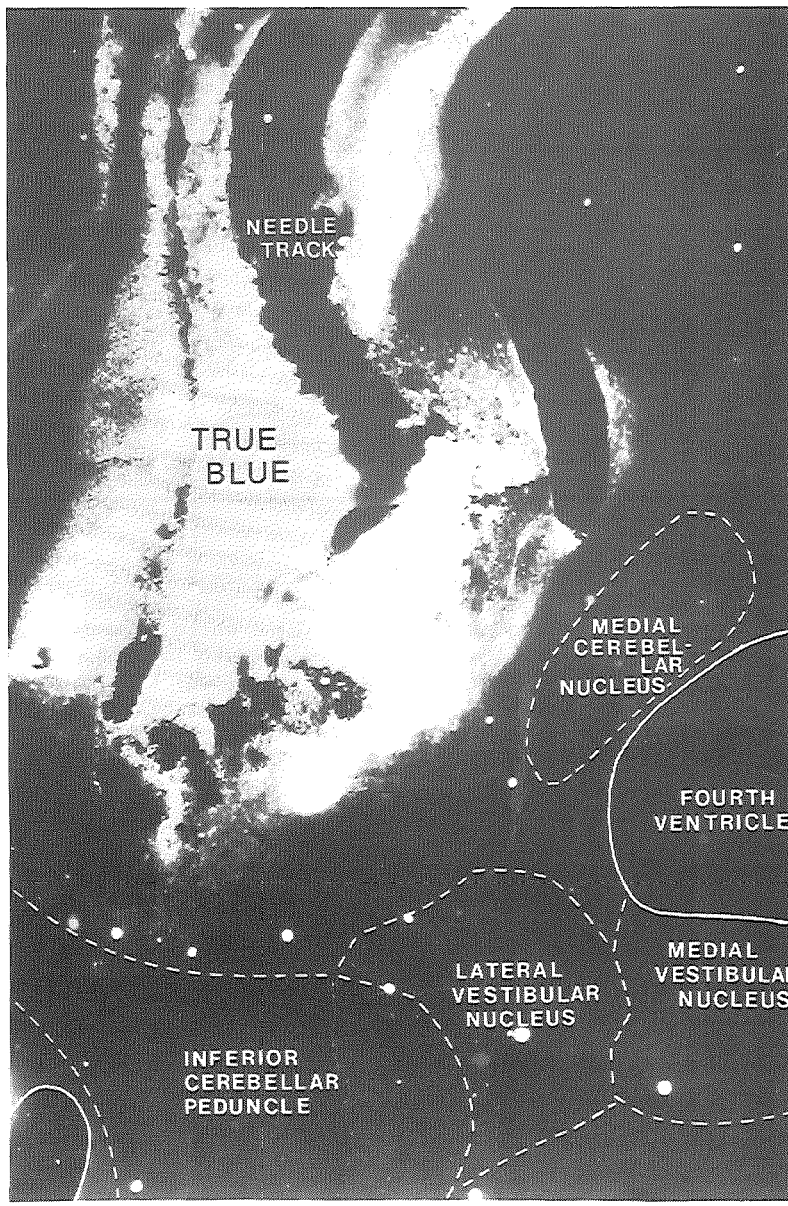

Fig. 2. Composite photomicrograph of a part of a cross section through cerebellum at the level of the anterior interpositus nucleus (NIA) with TB injection area centered in the (NIA)(case 2). Note: no fluorescence occurs in inferior cerebellar peduncle or in lateral vestibular nucleus and no ependymal cells of the fourth ventricle are fluorescent. For location of TB zones I and II see left diagram Fig. 1.

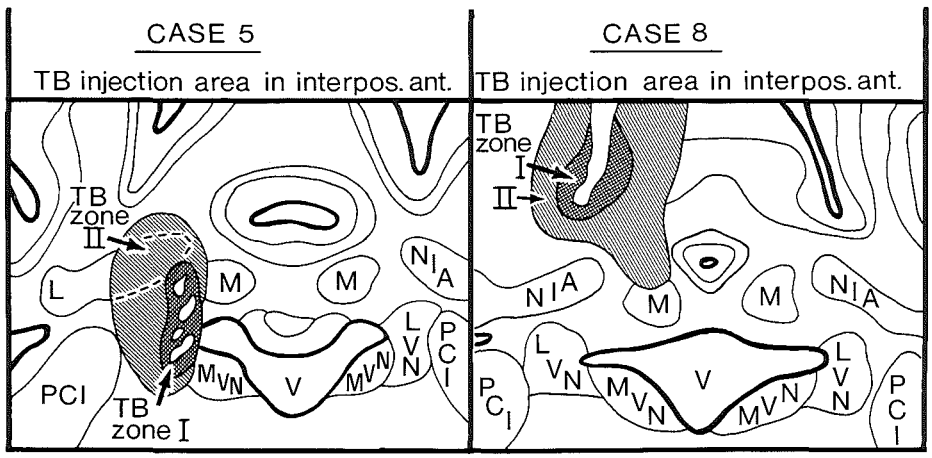

Fig. 3. Diagrams of the TB injection areas in case 5 (left diagram) and in case 6 (right diagram). Note: in case 5 only TB zone II involves the anterior interpositus nucleus (NIA) while in case 8 both TB zones I and II are located dorsomedially to the NIA. *Abbreviations:* L, lateral cerebellar nucleus; LVN, lateral vestibular nucleus; M, medial cerebellar nucleus; MVN, medial vestibular nucleus; NIA, anterior interpositus nucleus; PCI, inferior cerebellar peduncle; V, fourth ventricle.

of this zone blue fluorescent neurons and fibers also occurred. Zone II gradually faded into the surrounding normal tissue with little or no fluorescence.

In cases 1, 2, 3 and 4 as well as in the control animal the TB zone was centered in the rostral part of the NI, referred as NIA (Figs. 1 and 2) with some involvement of the caudal part (NIP). The involvement of the deep cerebellar nuclei by the injection areas was studied in cresyl violet sections, in which the nuclei were defined in keeping with the descriptions of Korneliussen[26] and of Courville and Brodal.[12] In case 1 zone II extended ventrally only into the most dorsal portion of the vestibular complex, but spared the inferior cerebellar peduncle. In case 2 zone II spared also the vestibular complex (Figs 1 and 2). In case 3, zone I extended medially up to the border of the fourth ventricle and zone II extended ventrally only into the dorsal portion of the vestibular complex (Fig. 1). In case 4, zone II extended ventrally both into the vestibular complex and into the inferior cerebellar peduncle. In this case fluorescent fibers could be traced caudally through the inferior cerebellar peduncle lateral to the descending tract of the spinal trigeminal nerve. However, no blue fluorescent fibers were present in the area of the rubrospinal

tract. The ependymal cells lining the fourth ventricle were not fluorescent except in case 3 in which fluorescent ependymal cells were present but only in the area where zone II extended up to the ventricle (Fig. 1).

In the cases 5, 6, 7 and 8 zone I did not involve the NI (Fig. 3). In case 5 it occupied an area medio-rostral to the NI in cases 6 and 7 an area dorsal to it and in case 8 an area dorsal and medial to it (Fig. 3). In cases 5 and 6 zone II involved a major part of the NI (Fig. 3). On the other hand, in case 7 zone II extended just up to the dorsal border of the NI and in case 8 up to its dorsal and medial border (Fig. 3).

Spinal cord

The NY injection area in the C5-C8 spinal segments (case 1, 2, 5 and 7) displayed 3 concentric zones (Fig. 1). Zone I was very narrow and showed yellow tissue fluorescence and a dense accumulation of bright yellow-white glial nuclei. Zone II, which was much wider than zone I, also displayed yellow tissue fluorescence but contained much fewer fluorescent glial nuclei and some fluorescent neuronal nuclei. In contrast to the findings in the TB injection areas, this central part of the NY injection area showed relatively normal tissue texture in the cresyl violet sections. Zone III showed no tissue fluorescence and contained only dull fluorescent glial and neuronal nuclei. Zones I and II involved the injected half of the cord, while zone III extended into the contralateral half (Fig. 1). Rostro-caudally zones I and II involved the injected segments, while zone III extended into the segment rostrally and caudally bordering the injected ones.

The DY injection area in the C5-C8 spinal segments (cases 3, 4, 6 and 8) was smaller than the NY injection area and showed the following characteristics (Fig. 1). The ends of the needle tracks were enlarged by a mass of brown yellow fluorescent material and pieces of yellow fluorescent necrotic tissue containing many brightly labeled cellular nuclei (Fig. 1). The end of the needle tracks were surrounded by two concentric zones in the same way as in the TB injection area. Zone I displayed yellow blueish tissue fluorescence (360 nm excitation wavelength) and contained a dense accumulation of bright glial nuclei. In cresyl violet sections this zone had lost its normal histological texture. Zone II was wider than zone I and showed no tissue fluorescence but only yellow fluorescent glial and neuronal nuclei, which decreased peripherally in number and brightness. This zone II faded into the area of normal tissue with little or no fluorescence. Zone I of the DY injection area involved the injected half of the cord and zone II extended from the injected half of the cord over a short distance into the contralateral half (Fig. 1).

Fig. 4. *Upper row:* Photomicrographs of NY (or DY) labeled rubrospinal neurons, double labeled with TB from cerebellum. Note golden yellow granules in the blue cytoplasm of the TB-DY double labeled neurons. *Middle row:* Photomicrographs of a single NY and a single DY labeled rubrospinal neuron. Note: the DY labeled nucleus is more diffusely fluorescent than the NY labeled one and the fluorescent ring around the nucleolus in the DY labeled nucleus is less pronounced than in the NY labeled one. *Bottom row:* Photomicrographs of single TB labeled rubro-cerebellar neurons. Note: they display only a blue TB fluorescent cytoplasm and nucleolus and do not display any yellow fluorescence of the nucleus.

tract. The ependymal cells lining the fourth ventricle were not fluorescent except in case 3 in which fluorescent ependymal cells were present but only in the area where zone II extended up to the ventricle (Fig. 1).

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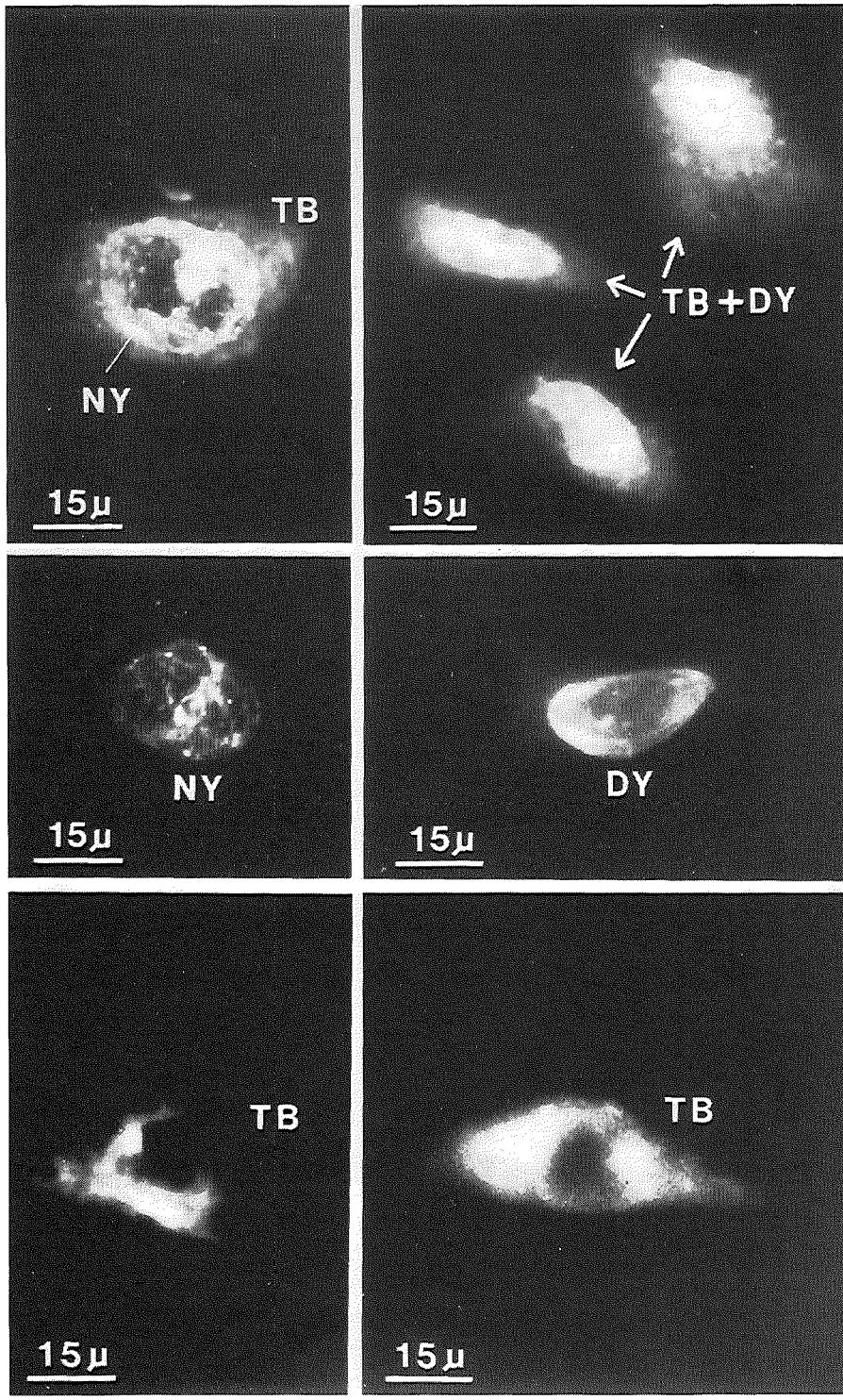
Fig. 4. *Upper row:* Photomicrographs of NY (or DY) labeled rubrospinal neurons, double labeled with TB from cerebellum. Note golden yellow granules in the blue cytoplasm of the TB-DY double labeled neurons. *Middle row:* Photomicrographs of a single NY and a single DY labeled rubrospinal neuron. Note: the DY labeled nucleus is more diffusely fluorescent than the NY labeled one and the fluorescent ring around the nucleolus in the DY labeled nucleus is less pronounced than in the NY labeled one. *Bottom row:* Photomicrographs of single TB labeled rubro-cerebellar neurons. Note: they display only a blue TB fluorescent cytoplasm and nucleolus and do not display any yellow fluorescence of the nucleus.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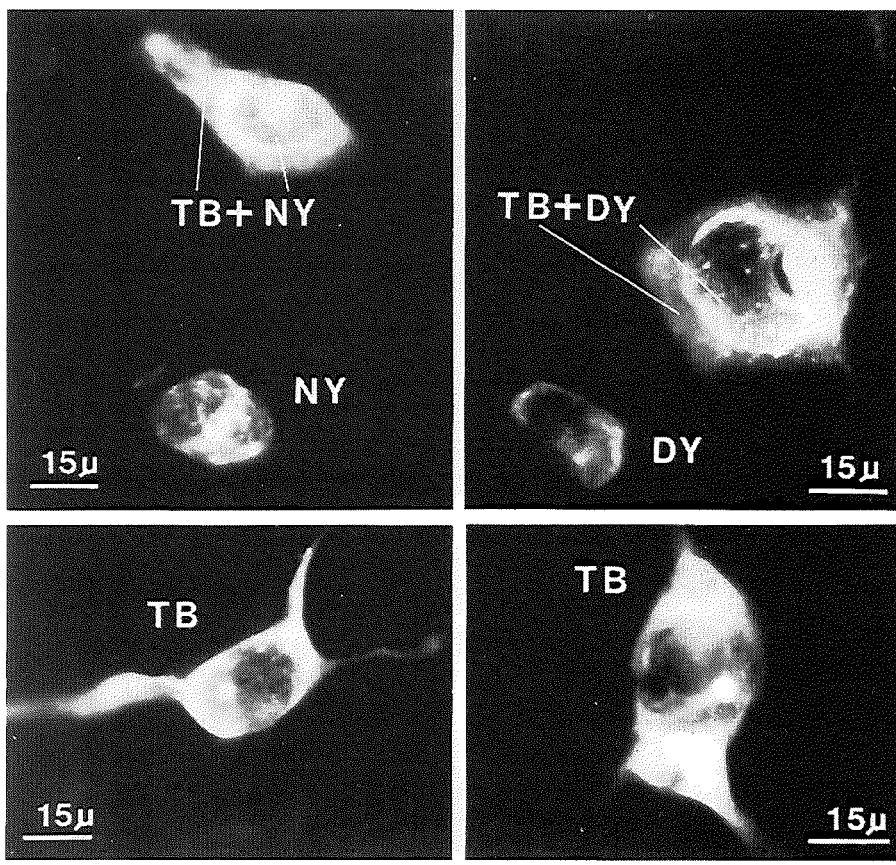


Fig. 5 *Upper row:* Photomicrographs of single NY (or DY) labeled rubrospinal neurons and of NY (or DY) labeled rubrospinal neurons double labeled with TB from cerebellum. Note: the fluorescence of the DY labeled nucleus is more diffuse and the TB-DY double labeled neuron contains golden yellow granules in the blue labeled cytoplasm. *Bottom row:* For comparison: photomicrographs of single TB-labeled rubrospinal neurons with TB fluorescent cytoplasm and nucleolus. Note: the absence of nuclear fluorescence.

Rostrocaudally the first and second zones were restricted to the injected segments.

The DY injection area was more restricted than the NY injection area in both the transverse and in longitudinal directions. Therefore it may be concluded that DY, which is less water soluble than NY, diffuses less through the tissue at the injection site.

Red Nucleus

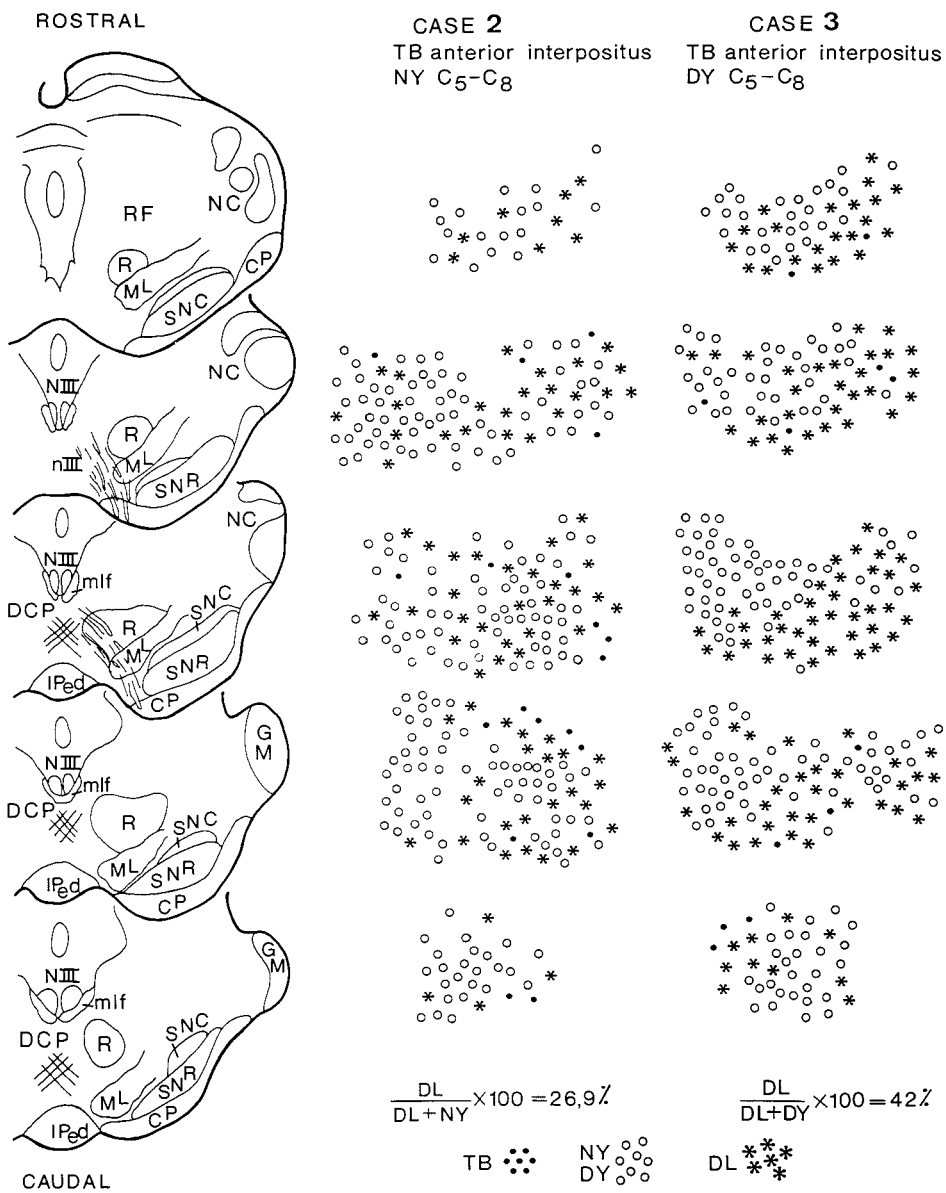
In the 8 rats (cases 1-8) in which NY or DY was injected in the spinal cord, many NY- or DY-labeled rubrospinal neurons were present in the dorsomedial, intermediate and ventrolateral parts of the contralateral red nucleus. These neurons, when single labeled[3,24] showed a yellow fluorescence of the nucleus with some yellowish fluorescence of the cytoplasm (Figs. 4 and 5). In the one control rat with only a TB injection in the cerebellum, many single TB labeled neurons were present in the red nucleus. They were located throughout the red nucleus both in the transverse and longitudinal directions. They showed only a blue cytoplasmic and nucleolar labeling without any indication of a yellow labeling of the nucleus (Fig. 4). In the cases 1-8 with injections in the cerebellum and in the spinal cord relatively few single TB labeled neurons ($\pm$ 70) were present in the contralateral red nucleus. However, a relatively large proportion of the NY- or DY-labeled rubrospinal neurons were double labeled with TB from the cerebellum, such that they showed a yellow fluorescent nucleus and a blue TB fluorescent cytoplasm (Figs. 4 and 5).

In general, the double labeled neurons were present throughout the rostrocaudal extent of the red nucleus (Fig. 6). However, in the most caudal end of the nucleus mainly single NY- or DY-labeled rubrospinal neurons were present and only relatively few double labeled neurons occurred (Fig. 6). The double labeled neurons were more or less evenly distributed throughout the cross-sectional profile of the red nucleus, except in cases 2 and 3 in which more double labeled neurons were present in the intermediate and lateral portions of the nucleus, including the lateral horn described by Reid et al. 1975[35] than in the medial parts (Fig. 6). In order to determine the percentage of the NY- or DY-labeled rubrospinal neurons which were TB double labeled from the cerebellum the following procedure was adopted. In each case the NY- or DY-labeled neurons as well as the TB-NY or TB-DY double labeled ones were counted in every fourth section through the red nucleus. These numbers were then added, multiplied by four and treated according to the following formula.

$$\frac{\text{TB-NY double-labeled neurons}}{\text{single NY + TB-NY double-labeled neurons}} \times 100\%$$

(In the animals in which DY was used read DY for NY)

Fig. 6. Semidiagrammatic representations of the distributions of retrogradely labeled neurons in red nucleus of cases 2 and 3 after TB injections contralaterally in the anterior interpositus nucleus (NIA) and a NY (case 2) or DY (case 3) hemiinfiltration contralaterally at C5-C8. Note: the double labeled neurons are more or less evenly distributed throughout the rostrocaudal extend of the red nucleus; in case 3 the double labeled neurons show a preponderance laterally in the nucleus.
Abbreviations: CP, cerebral peduncle; DCP, decussation of superior cerebellar peduncles; GM, medial geniculate body; I Ped, interpeduncular nucleus; ML, medial lemniscus; mlf, medial longitudinal fasciculus; NC, cochlear nuclei; N III, oculomotor nucleus; n III oculomotor nerve; R, red nucleus; RF, reticular formation; SNR, substantia nigra, pars reticulata; SNC, substania nigra, pars compacta.


ROSTRAL
CASE 2
TB anterior interpositus
NY C5-C8
CASE 3
TB anterior interpositus
DY C5-C8
RF
NC
R
ML
SNC
CP
NIII
nIII
SNR
mlf
DCP
IPed
GM
DL/(DL+NY) ×100 = 26,9%
DL/(DL+DY) ×100 = 42%
TB
NY
DY
DL
CAUDAL

	TB injection area (zones I and II) in the cerebellum	NY and DY labeled rubrospinal neurons, including double labeled ones	TB-NY and TB-DY double labeled neurons in the red nucleus	percentages of yellow (NY or DY) labeled neurons, which were double labeled with TB
CASE 1	zone I involved the anterior interpositus nucleus (NIA)	2380 (NY)	484	20,4%
CASE 2	zone I involved NIA	2168 (NY)	584	26,9%
CASE 3	zone I involved NIA	2468 (DY)	1036	42 %
CASE 4	zone I involved NIA	2824 (DY)	812	28,7%
CASE 5	zone I bordering on NIA zone II involving NIA	2184 (NY)	336	15,4%
CASE 6	zone I bordering on NIA zone II involving NIA	1996 (DY)	316	16 %
CASE 7	zones I and II dorsal to NIA	2246 (NY)	46	2 %
CASE 8	zones I and II dorsomedial to NIA	2344 (DY)	52	2 %

Table II shows: the localization of the TB injection area in the cerebellum (column 1), the numbers of NY and DY labeled rubrospinal neurons (column 2), the number of NY and DY rubrospinal neurons, double labeled with TB from the cerebellum (column 3) and the percentages of NY and DY labeled rubrospinal neurons, double labeled from the cerebellum (column 4).

In the four cases (cases 1,2,3,4) in which zone I of the TB injection was centered in the NIA the following findings were obtained. In the cases injected with NY, the total numbers of NY-labeled rubrospinal neurons, including the NY-TB double labeled ones were: 2380 (case 1) and 2168 (case 2). In the cases injected with DY, the total numbers of DY-labeled neurons including the DY-TB double labeled ones were 2468 (case 3) and 2824 (case 4)(Table II). In the two rats with the NY injections 20.4% (case 1) and 26.9% (case 2) of the NY labeled rubrospinal neurons were double labeled from the cerebellum. In the two rats with the DY injections, 42% (case 3) and 28.7% (case 4) of the DY labeled neurons were double labeled from the cerebellum (Fig. 6, Table II). The higher percentage in case 3 as compared to cases 1, 2 and 4 may be explained by the fact that in case 3 a large quantity of TB was injected in the cerebellum than in the other cases (cf. Table I) and correspondingly a larger injection area was found.

In the four other cases (cases 5,6,7 and 8), in which the TB zone I was located outside the NI the following findings were obtained. In the red nucleus approximately the same number of NY or DY labeled rubrospinal neurons was found as in cases 1,2,3 and 4 (Table II), and these neurons displayed the same distribution as in the cases 1 to 4. However, the percentages of the NY and DY labeled neurons which were NY-TB or DY-TB double labeled varied according the location of the cerebellar injection area. Thus, in cases 5 and 6, in which only zone II involved the NI (Table II), 15.4% and 16% of the rubrospinal neurons was double labeled from the cerebellum. However, in cases 7 and 8 in which neither zone I nor zone II involved the NI, virtually no single TB labeled neurons were found in the red nucleus and in both cases only 2% of the rubrospinal neurons were double labeled (Table II).

These retrograde double labeling findings lead to the conclusion that almost all ($\pm$90%) of the rubro-cerebellar fibers to the anterior interpositus nucleus represent collaterals of rubrospinal neurons. In the 8 cases a maximum number of 2824 rubrospinal neurons were labeled (case 4, Table II). In case 3 with the largest cerebellar TB injection area the maximum number of rubrospinal neurons was double labeled from the cerebellum (i.e. 1036, Table II). According to the formula used in the present study 37% of the rubrospinal neurons were double labeled from the cerebellum. Within the restraint of the present method these findings suggest that at least 37% of the rubrospinal neurons give rise to rubrocerebellar collaterals.

In some of the cases, in which the TB injections were centered in the NIA, the efferent fibers from the cerebellum to the contralateral red nucleus

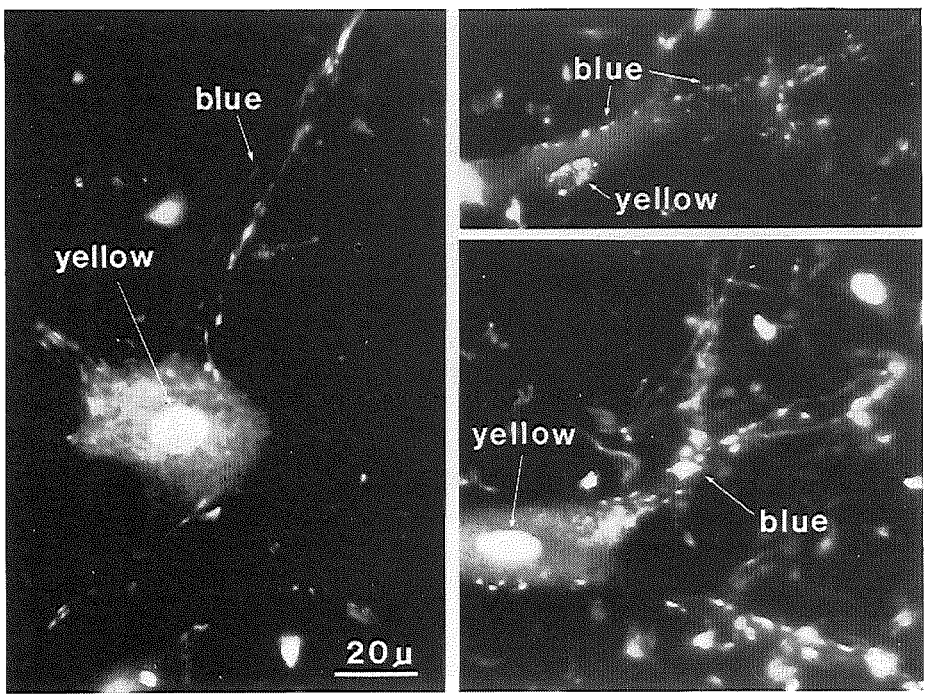


Fig. 7. Photomicrographs of FB fluorescent fibers in cat red nucleus, which are anterogradely labeled from the contralateral cerebellar interpositus nucleus. Note: the fiber varicosities, which seem to be in contact with the surface of cell body and dendrites of the single NY labeled rubrospinal neurons.

were anterogradely labeled and appeared as very thin blue threads in the red nucleus. However, in some additional experiments in cat with „Fast Blue" (FB)[3] injections in the NIA and NY injections in the spinal cord, such anterograde labeling of cerebello-rubral fibers was much more pronounced. Thus in these cases, which will be reported separately, blue FB fluorescent fibers could be traced from the cerebellum to the cell body and dendrites of retrogradely NY labeled rubrospinal neurones (Fig. 7).

Discussion

Earlier anatomical studies[10,12] showed that in cat fibers from the red nucleus descend through the rubrospinal decussation to the level of the pontine trigeminal nuclei[15] and then proceed along the fibers of the brachium conjunctivum into the cerebellum. These rubrocerebellar fibers approach the deep cerebellar nuclei from the ventral side.[12] Brodal and Gogstad (1954)[6] on the basis of retrograde changes concluded that in the cat the bulk of these fibers terminate in the lateral cerebellar nucleus. However, Courville and Brodal (1966)[12] on the basis of anterograde degeneration demonstrated that the termination area of the rubrocerebellar fibers is largely restricted to the interpositus nucleus (NI) chiefly its anterior part (NIA) and that only a few fibers terminate in the posterior interpositus nucleus (NIP). This discrepancy in regard to the termination of rubrocerebellar fibers may be due to differences in the definition of the border between the interpositus and lateral nucleus used in these two studies and to differences in technique. In view of the anterograde degeneration findings of Courville and Brodal[12] it has been assumed in the present study that the bulk of the rubrocerebellar fibers both in cat and rat terminate in the rostral portion of the NI (i.e. NIA). In this study an attempt was made to determine whether these crossed rubrocerebellar fibers represent collaterals of rubrospinal neurons. For this purpose in rats 'True Blue' (TB) was injected in the cerebellum and 'Nuclear Yellow' (NY) was injected ipsilaterally in the spinal cord. In some cases a new tracer 'Diamidino Yellow' (DY) was used instead of NY. This new tracer produces roughly the same retrograde labeling as NY but has the advantage that it migrates much more slowly out of the retrogradely labeled neurons than NY. Therefore DY does not require a short survival time[4] and can be injected with TB in the same session.

The present study essentially comprises 9 cases. In one of them, injections (TB) were made in only the cerebellum and in the other 8 cases the injections were made both in the cerebellum (TB) and the spinal cord (NY or DY). In the one case with only a TB injection in the cerebellum, many single TB labeled neurons were present throughout the contralateral red nucleus. These neurons displayed the typical TB labeling described earlier[2] and contained only a blue TB fluorescent cytoplasm and a bright blue fluorescent nucleolus. However, they clearly did not display any yellow fluorescence of the nucleus. In the other eight cases only few such single TB labeled neurons were present while the majority ($\pm$90%) of the neurons with a TB fluorescence of the cytoplasm were double labeled such that they also showed a pronounced yellow fluorescence of the nucleus. On the basis of these double labeling findings it has been concluded that the rubrocerebellar fibers mainly represent collaterals of rubro-

spinal neurons. This is in keeping with the earlier anatomical and electrophysiological findings in cat.[6,1]

In order to determine the percentage of rubrospinal neurons which give rise to collaterals to the cerebellar interpositus nucleus it was necessary to obtain retrograde labeling of a maximum number of rubrospinal neurons as well as of a maximum number of rubrocerebellar neurons.

In order to obtain retrograde labeling of a maximum number of rubrospinal neurons use was made of the fact that NY and DY are transported retrogradely both from terminal areas and from damaged axons.[9,24] Therefore, the NY and DY injections in C5-C8 were made by means of multiple pipette penetrations both into the grey matter and the white matter. The injections in the grey matter were expected to produce retrograde labeling of rubrocervical neurons, while those in the white matter were expected to produce retrograde labeling of rubrospinal neurons from damaged rubrospinal fibers passing through the C5-C8 dorsolateral funiculus to more caudal levels of the cord.[7,40,32,33] This strategy appeared to have been successful since in all cases a large number of rubral neurons was retrogradely labeled. Moreover, they were distributed over the dorsomedial, intermediate and ventrolateral parts of the cross-sectional profile of the red nucleus, which parts project to the cervical, the thoracic and the lumbosacral cord respectively.[18,30,19,34] In view of these findings, it may be concluded that, within the restraints of the present method, a maximum number of rubrospinal neurons was retrogradely labeled.

In order to judge whether the cerebellar TB injections produced a maximum number of TB labeled rubrocerebellar neurons the following points should be taken into account. The TB injection areas consist of two concentric zones, i.e. zone I and II, the former of which seems to give rise to the highest degree of retrograde labeling. The NI injections were always made from above, while according to the earlier anatomical findings[12] the rubrocerebellar fibers penetrated the nucleus from below, i.e. through its hilar region. As a consequence it was expected that the TB injections which were restricted to the NI produce retrograde labeling of rubrocerebellar neurons mainly from the termination area of the rubrocerebellar fibers, while injections which also involved the hilar region of the NI would in part produce retrograde labeling of rubral neurons from damaged rubrocerebellar fibers. In keeping with these expectations it was found that in the cases 7 and 8 in which the TB injection area (zones I and II) was located dorsal or medial to the NI and largely spared the nucleus, only a very small percentage of rubrospinal neurons was double labeled. However, in the cases 5 and 6 in which zone I was located outside the NI, but zone II involved the nucleus, a larger percentage of rubrospinal neurons was double

labeled, and in the cases 1 to 4 in which zone I was centered in the NIA a much larger percentage of rubrospinal neurons was double labeled. Moreover, in case 3, in which zone I was also centered in the NIA, but in which the TB injection area most extensively involved the hilar region of the nucleus, the maximum number of rubrospinal neurons was double labeled, i.e. 1036 (Table II).

In the 8 cases a maximum number of 2824 rubrospinal neurons were labeled (case 4, Table II). In case 3 with the largest cerebellar TB injection area involving both the nucleus and the hilar region a maximum number of 1036 rubrospinal neurons was double labeled from the cerebellum (Table II). According to the formula used in the present study 37% of the rubrospinal neurons were double labeled from the cerebellum.
With the restraint of the present method these findings suggest that at least 37% of the rubrospinal neurons give rise to rubrocerebellar collaterals.

The double labeled neurons in the red nucleus, distributing divergent axon collaterals to both the cerebellar interpositus nucleus and the spinal cord, were more or less evenly distributed throughout the red nucleus both in the transverse and longitudinal directions except in cases 2 and 3 in which more double labeled neurons were present in the intermediate and lateral parts of the nucleus than in the medial parts (Fig. 6). This is in general accordance with the findings of Brodal and Gogstad (1954)[6] that after cerebellar lesions retrograde changes occur in large and medium sized neurons, which are located in the same parts of the red nucleus as the retrogradely affected neurons after spinal lesions. However, after cerebellar lesions Brodal and Gogstad found a slight preponderance of retrogradely affected neurons medially in the nucleus, while in the present study the double labeled neurons showed a slight preponderance laterally in the nucleus which in cases 2 and 3 was more pronounced than in the other cases (Fig. 6). This difference may be due to the following. In the study of Brodal and Gogstad[6] mainly large to intermediate neurons were taken into account, because in such neurons the retrograde changes can be recognized most reliably. However, this may produce a bias, because in the rostral part of the red nucleus such neurons tend to be concentrated in the medial and intermediate parts. On the other hand, in the present study also many relatively small rubrospinal neurons were double labeled, not only in the medial and intermediate parts of the nucleus, but also in the ventrolateral parts, including the lateral horn described by Reid et al..[35]

In respect to the present findings it is of interest to note that the interpositus nucleus represents the main source of afferents to the rubrospinal neurons which projection in addition is somatotopically organized.[11,17,14]
The rubrospinal collaterals to the interpositus nucleus therefore appear to

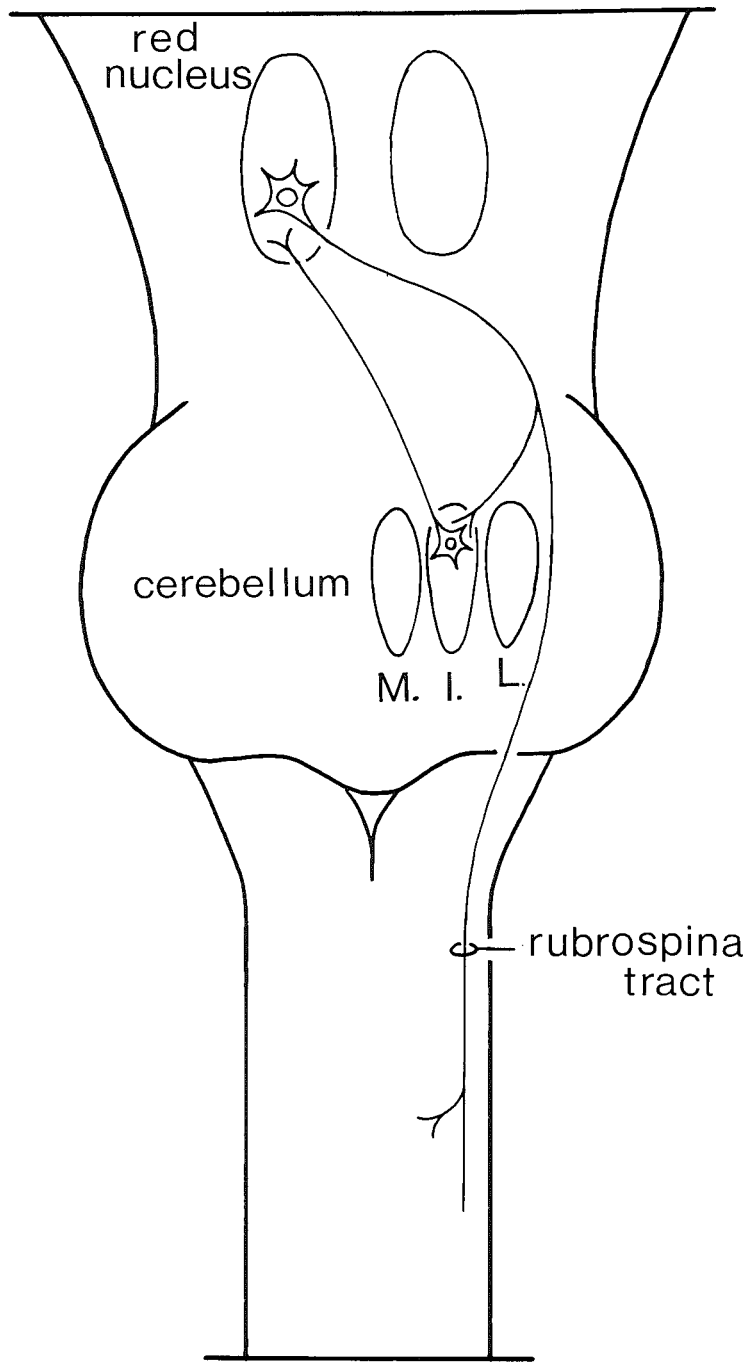


Fig. 8. Diagram of the interconnections between red nucleus and contralateral cerebellar anterior interpositus nucleus. *Note* that the rubro-interpositus connections are established by collaterals of rubrospinal neurons.

represent a direct, tightly coupled return projection to cells of origin of the afferents of these rubral neurons (Fig. 8). The precise functional role of these return projections is still unclear. However, they must be of critical importance since such return projections occur in several places in the cerebellar circuitry. Thus, for example the efferent fibers from the deep cerebellar nuclei to the red nucleus and the thalamus display a similar arrangement and give off direct return collaterals to the cerebellar cortex,[37,38] from where they receive the bulk of their afferents. Moreover, the inferior olive, which gives rise to projections to the cerebellar cortex and the deep cerebellar nuclei[39] also receives return projections from these deep cerebellar nuclei. In this context it is of interest to note that in parallel with the return collaterals from the rubrospinal neurons to the interpositus nucleus also another return projection from the red nucleus to the cerebellar cortex exists. This return projection is established by way of rubral fibers to the lateral reticular nucleus,[41,10] which nucleus projects to the cerebellum.[5] In light of the present findings the question arises as to whether these rubrolateral reticular connections are also established by collaterals of rubrospinal neurons.

In a preceding fluorescent retrograde labeling study[22,23] it was demonstrated that in rat, cat and monkey the rubrospinal and raphe spinal fibers give rise to collaterals along their trajectory throughout the spinal cord. However, the rubrospinal system was found to produce much fewer of such collaterals than the raphe spinal system. Thus, in rat 20% and in cat and monkey 3.8% and 6.6% of the rubrocervical neurons were found to provide collaterals to segments caudal to L1, while in all these species 31-48% of the raphe cervical neurons provide collaterals to the segments caudal to L1 and only a slightly smaller percentage (27%-35%) to segments caudal to S1 (cf. Histograms1 and 2, Fig. 9). It was therefore concluded that the rubrospinal tract represents a relatively focussed system, which distributes its individual fibers to restricted groups of spinal segments, while the raphe spinal tract represents a diffuse system, which distributes almost half of its fibers throughout the length of the spinal cord. Moreover, the degree of collateralization of the rubrospinal system in rat appeared to be much higher than in cat and monkey, since in rat as compared to cat and monkey a much higher percentage of rubrocervical neurons give rise to collaterals to the spinal cord caudal to L1 (Fig. 9, histogram I). This, together with observations in pigeon[42] and opossum suggests that the rubrospinal tract as a focussed system emerges only gradually during phylogeny.

In view of the above interspecies differences in the degree of collaterali-

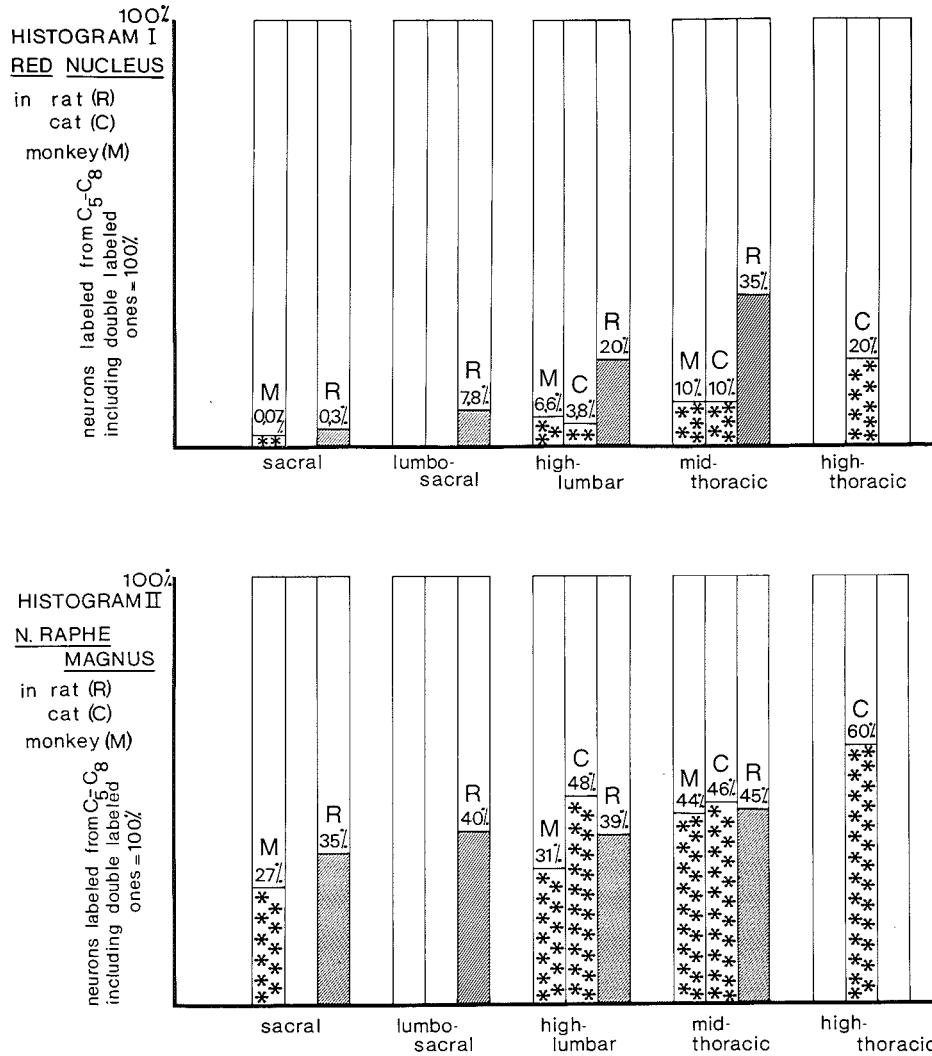


Fig. 9. *Histogram I*, showing the percentages of rubrocervical neurons, which distribute descending collaterals passing through more caudal levels of the cord in rat (R), cat (C) and monkey (M). Note that in cat and monkey a smaller percentage of the rubrocervical neurons distribute collaterals through more caudal levels of the cord than in rat (c.f. Huisman et al., [22, 23]).
Histogram II, showing the percentages of raphe-cervical neurons from Tabers et al. [35] Nucleus Raphe Magnus and adjoining reticular formation, which distribute collaterals passing through more caudal levels of the cord in rat (R), cat (C) and monkey (M). Note that a large portion (approx. 1/3) of the rubrocervical neurons give rise to collaterals which descend throughout the length of the spinal cord.

zation of the rubrospinal system in the cord the question arose as to whether such interspecies differences also exist in respect to the rubrospinal collaterals to the cerebellum. Therefore a further interspecies comparison of these rubrospinal collaterals would be of interest. However, it would seem unlikely that the degree of rubrospinal collateralization to the cerebellum would run parallel with that of the rubrospinal collateralization in the cord, since these two types of rubrospinal collaterals probably subserve an entirely different function.

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CHAPTER VI: GENERAL DISCUSSION

VI. GENERAL DISCUSSION

VI.1. Technique

Classic anatomical studies using the Golgi technique (Cajal, 1952) demonstrated that many axons in the central nervous system give off axon collaterals along their trajectory through the brain. However, with the available tracing techniques, which are based on degeneration or intra-axonal transport (Cowan, 1972; Fink and Heimer, 1967; Kristensson, 1970; Kristensson et al., 1971; LaVail and LaVail, 1972; Nauta and Gygax, 1954) it is very difficult to distinguish divergent axon collaterals from unbranched fibers. Therefore in the late seventies several attempts were made to demonstrate the existence of axon collaterals by developing different retrograde tracers, which are transported retrogradely through the stem axon and through a collateral and which can be demonstrated independently in the parent cell body.

At the moment the following 3 methods for such retrograde double labeling of neurons by way of divergent axon collaterals are available.

A) The HRP-tritiated apo-HRP technique (Hayes and Rustioni, 1979 and 1981). This technique is based on the fact that both the enzyme HRP and the ^{3}H apo-HRP are transported retrogradely to the cell body (Geisert, 1976; Kristensson and Olsson, 1971; LaVail and LaVail, 1972). The presence of HRP is demonstrated histochemically, while the presence of the tritiated-apo HRP is then demonstrated by means of autoradiography. Thus, this technique requires a combined histochemical-autoradiographic procedure. However, because of the fact that by means of the autoradiographic technique the presence of label can only be detected in the upper 3 μm of the section, the presence of the double labeled neurons can only be demonstrated in these upper 3 μm. (Sidman, 1970).

B) The HRP-Iron dextran technique (Cesaro et al., 1979; Olsson and Kristensson, 1978). The presence of these two tracers in the parent cell body may be demonstrated histochemically by first processing the sections for HRP and then demonstrating the presence of ferric ions in the cell body by means of the Perl's reaction. However, double labeling may be underestimated when few granules of iron dextran or HRP are present in the cell body, which are overshadowed by a massive storage of the other reaction product. This

can be prevented by adapting the survival times to the fiber systems under study.

C) The fluorescent double labeling technique. This technique makes use of the fact that several fluorescent substances may be transported retrogradely through divergent axon collaterals to the same parent cell body where they can be visualized independently by means of fluorescence microscopy (Kuypers et al., 1977, 1980). Since this "fluorescent" technique requires only a relatively simple procedure (see Chapter II) and is one of the few anatomical techniquessuitable to study quantitatively the existence of collaterals in brain pathways, this technique has been used to try to clarify anatomically possible quantitative differences in collateralization in the different brainstem pathways, which descend through the dorsolateral funiculus i.e. the rubrospinal pathway, the descending pathways from the ventrolateral pontine tegmentum and from the raphe magnus including the adjoining ventral reticular formation.

The retrograde fluorescent tracers, used in this study (Chapter III and IV), were „True Blue" (TB) and „Nuclear Yellow" (NY) in rats (Chapter III) and „Fast Blue" (FB) and „Nuclear Yellow" (NY) in cats and monkeys (Chapter IV)(Bentivoglio et al., 1979; Bentivoglio et al., 1980a+b; Kuypers et al., 1980). These tracers are transported relatively effectively over long distances, although TB and FB more slowly than NY, and produce a fluorescent double labeling which can be observed at one excitation wavelength (i.e. at 360 nm a blue TB or FB labeling of the cytoplasm and a yellow NY labeling of the nucleus).

After relatively long survival times NY may migrate out of retrogradely labeled neurons into surrounding neurons, as signalled by the presence of NY labeled fluorescent glial nuclei (c.f. Chapter II). In order to avoid false retrograde labeling relatively short survival times should be used (Bentivoglio et al., 1980b). Therefore first TB or FB was injected in the C5-C8 dorsal spinal grey and subsequently in a later operation NY was injected at more caudal levels of the cord, a short time before the animal was sacrificed. Following this procedure, in all cases only dull NY glial labeling was present around the single NY- and double TB-NY or FB-NY labeled neurons. This indicates that only minimal migration of NY out of retrogradely labeled neurons occurred i.e. without any risk of false retrograde labeling. In exceptional cases single FB-labeled neurons in the central nervous system show some blue to white FB labeling of the nucleus which could make such neurons difficult to distinguish from FB-NY double labeled ones. In this respect it should be emphasized however, that in our experiments in the brain in which a relatively low percentage of FB was dissolved in distilled water only very seldom

a strong FB labeling of the neuronal nucleus was obtained (Bharos et al., 1981; Huisman et al., unpublished observations). Moreover, in this material the exceptional blue to white FB labeling of the nucleus could always be clearly distinguished from the yellow to green NY labeling of the nucleus. Therefore the combination of FB and NY has been consistently used in double labeling experiments in cats and monkeys. Applying this technique to the descending pathways in spinal cord (Chapter III and IV) is difficult, especially in rat, due to the close proximity between the area of termination of the fibers in the grey matter and their trajectory in the adjoining funiculus In order to obtain reliable results it is therefore imperative that the tracer injection is entirely restricted to the grey matter, because injection of some of the tracer in the lateral funiculus may result in its retrograde transport through damaged fibers. This in turn will lead to retrograde labeling of neurons in the red nucleus and the other brain stem cell groups which may not distribute collaterals to the injected part of the spinal grey This difficulty only applies to the injections in C5-C8 segments, because the injections at more caudal spinal levels were intentionally made in white and grey matter in order to damage many fibers in the lateral funiculus which would result in retrograde transport of the tracer through a maximum number of fibers descending in this funiculus at the injected levels. In light of the above considerations, in all cases each of the injection areas in C5-C8 grey were studied histologically in detail and only those cases were selected for study in which the fluorescent zones, from which retrograde transport occurs to the parent cell bodies, did not involve the lateral funiculus. The sparing of the fibers in the lateral funiculus was confirmed by the fact that the population of retrogradely labeled neurons from the cervical grey matter in the caudal part of the contralateral red nucleus was always restricted to its dorsomedial part, while the population of neurons which were retrogradely labeled from lower levels of the cord was always located separately in the ventrolateral part of the nucleus. This is in keeping with retrograde degeneration and retrograde HRP findings (Flumerfelt and Gwyn, 1974; Gwyn, 1971; Kneisley et al., 1978; Murray and Gurule, 1979; Pompeiano and Brodal, 1957). Sparing of the rubrospinal tract also implies sparing of the two other descending pathways, since their fibers are grouped together with the rubrospinal tract in the dorsolateral funiculus, where the raphe-spinal tract is located in the most peripheral zone (Basbaum and Fields, 1978 and 1979; Brown, 1967; Edwards, 1972; Holstege et al., 1979; Kuypers and Maisky, 1977; Martin et al., 1978 and 1979; Miller and Strominger, 1973; Nyberg-Hansen and Brodal, 1964; Petras, 1967; Tohyama et al;, 1979a+b; Waldron and Gwyn, 1971).

A comparison of the results obtained by the multiple retrograde fluorescent tracer technique on one hand and the HRP-^{3}H-apo-HRP technique (Hayes and Rustioni, 1979, 1981) on the other hand suggested that the retrograde fluorescent tracer technique produces a larger proportion of double labeled neurons (Hayes and Rustioni, 1981; c.f. Huisman et al., 1981; Huisman et al., 1982). This difference may be explained by the fact that in the autoradiographic material silver grains will only be elicited in the emulsion by sources of radioactivity which are located in the upper 3 μm layer of the 40 μm sections (Sidman, 1970). As a consequence, even under optimal circumstances, only a small percentage of the total population of HRP labeled neurons observed in each 40 μm section can be double labeled.

The fluorescent tracers are suggested to be more sensitive than HRP, as pointed out by the findings of Sawchenko and Swanson (1981) that the fluorescent tracer TB in rat labels approximately twice as many neurons than the HRP-TMB technique using HRP-polyacrylamide gel implants (Mesulam and Rosene, 1979). This is supported by a comparison of the present findings with those obtained by Condé and Condé (1982) (personal communications) which show that in our experiments after Fast Blue (FB) injections in inferior olive of the cat the number of FB labeled rubro-olivary neurons at a given level in the red nucleus was significantly larger than obtained with the HRP-BDHC technique (Condé and Condé, 1982; Mesulam, 1976,1978,1979) at approximately the same level (i.e. in a 30 μm section about 80 FB labeled neurons and in a 60 μm section about 40 HRP labeled neurons). On the other hand comparing the efficacies of FB and HRP in retrograde labeling of cortical neurons in cat after injections in the cervical cord including the white matter showed that twice as many cortical neurons were labeled with HRP than with FB (Keizer et al., in preparation b). However, this is true only for FB transport from damaged fibers, since after FB injections in termination areas i.e. in the spinal grey instead of in the dorsolateral funiculus, in which the bulk of the corticospinal fibers descend (Armand and Kuypers, 1980; Armand, 1982; Chambers and Liu, 1957) approximately the same number of FB labeled neurons are present in the corresponding cortical area as obtained with HRP. After FB injections in the dorsolateral funiculus much less FB labeled neurons were obtained than HRP (Keizer et al., in preparation b) even if FB is dissolved in dimethylsulfoxide (Huisman et al., 1982; Keefer, 1978). The efficacy of the fluorescent dye „Nuclear Yellow" (NY), which is related to „Bisbenzimide" (Bb) turned out to be comparable to that of HRP (TMB procedure) (Keizer et al., in preparation b; Sawchenko and Swanson, 1981). The fact that a larger number of cortical neurons is retrogradely labeled from damaged fibers with HRP than

with FB, may be explained by the fact that FB gives much more necrosis at the injection site than HRP, which necrosis may interfere with uptake and retrograde transport of the tracer. However, NY gives much less necrosis at the injection site than FB and TB. Yet in cat and monkey optimal retrograde NY transport from damaged fibers is only obtained when NY is dissolved in dimethylsulfoxide (Huisman et al., 1982). Remarkably enough this phenomenon was not observed in rat in which a very effective retrograde NY labeling of neurons was obtained both from damaged fibers as well as from termination areas (Huisman et al., 1981).

The fact that in all cases a difference in degree of collateralization was found between the rubrospinal tract on one hand and the raphe- and ventrolateral pontine tegmentospinal tracts on the other hand supports the reliability of this double labeling technique. Furthermore, the quantitative anatomical data of the present studies were all in good agreement with earlier electrophysiological studies, which will be discussed below.

In chapter V the use of a new retrograde tracer is reported: „Diamidino Yellow"(DY), which migrates only very slowly out of the cell and can be used in combination with TB or FB in double labeling experiments (Keizer et al., in preparation a). DY when combined with TB or FB is as effective as NY in double labeling of neurons by way of divergent axon collaterals. However, DY migrates much more slowly out of the retrogradely labeled neurons than NY and therefore does not require a short survival time. As a consequence DY may be injected at the same time as TB or FB, without the risk that DY leaks out of retrogradely labeled neurons into surrounding glial and neuronal nuclei, which could produce false double labeling.

After injecting DY in the cervical cord in rat and cat many single DY labeled neurons were present in both the dorsomedial and ventrolateral parts of the red nucleus, which indicates that DY as NY is transported both from axon terminals and from passing fibers (Keizer et al., in preparation; Huisman et al., in press). Moreover, in double labeling experiments in rat in which the collateralization of rubrospinal neurons to the cerebellum was studied quantitatively, the combination TB-NY was compared with the combination TB-DY (Chapter V). The findings in these cases led to the conclusion that in quantitative double labeling studies DY may be used instead of NY, since the combination TB-DY results approximately in the same

percentage of double labeled neurons as the combination TB-NY. This was confirmed by a more elaborate quantitative study in rat and cat, in which DY was compared with NY in the combination with TB in rat and with FB in cat (Keizer et al., in preparation a).

VI.2. The rubrospinal tract in rat, cat and monkey

VI.2.a. Somatotopic organization of red nucleus

In *rat* the retrograde labeling findings in the red nucleus (Chapter III Figs. 4 and 5) confirm its somatotopic organization as demonstrated by means of the retrograde degeneration technique (Flumerfelt and Gwyn, 1974) and the retrograde HRP transport technique (Murray and Gurule, 1979). However, contrary to these studies the present findings also demonstrate some degree of somatotopic organization in the rostral shell-shaped portion of the red nucleus. This rostral portion consistently contained the highest number of double-labeled neurons (Chapter III Fig. 4) which indicates that it harbors the bulk of the neurons distributing collaterals to different levels throughout the spinal cord. The presence of this considerable number of branching neurons in the rostral part of the red nucleus probably explains the fact that its somatotopic organization has escaped detection by means of other techniques (Flumerfelt and Gwyn, 1974; Gwyn, 1971; Murray and Gurule, 1979).

In *cat* the retrograde labeling findings in red nucleus confirm previous findings (Hayes and Rustioni, 1981; Pompeiano and Brodal, 1957) and showed that the rubrospinal neurons are mainly present in the caudal two-thirds of the nucleus. The findings in the experiments with FB injections in the inferior olive and NY injections in the cervical cord further showed that in cat the red nucleus neurons, which distribute fibers to the ipsilateral inferior olive (Courville and Otabe, 1974; Martin et al., 1975, 1980; Strominger et al., 1979; Walberg, 1956) represent a population, which is seperate from rubrospinal neurons and which is largely restricted to the rostral part of the nucleus including the area laterally adjoining the fasciculus retroflexus (Chapter IV Figs. 8, 10 and 11). This is in keeping with other HRP findings (Condé and Condé, 1982; Saint-Cyr and Courville, 1980 and 1981; Walberg and Nordby, 1981). The distribution of the rubrospinal neurons, which are retrogradely labeled from the cervical, thoracic and lumbar cord confirmed the somatotopic organi-

zation of this nucleus as demonstrated earlier by means of other techniques (Hayes and Rustioni, 1981; Pompeiano and Brodal, 1957; Tsukahara et al., 1967). Thus, in the caudal half of the magnocellular part of the rubrocervical, the rubrothoracic and the rubrolumbar neurons are located in the dorsomedial, intermediate and ventrolateral parts of the nucleus, respectively. On the other hand in the rostral half of the magnocellular part these neurons become intermixed (Chapter IV Fig. 8).

In *monkey* retrograde degeneration studies (Kuypers and Lawrence, 1967; Poirier and Bouvier, 1966) showed that the rubrospinal neurons are largely restricted to the magnocellular part. However, the present findings showed that the population of rubrospinal neurons extends further rostrally and continues along the lateral aspect of the parvicellular part (Chapter IV Fig. 13) up to the level, where the cross-section through the fasciculus retroflexus is located ventral to the nucleus. This seems in keeping with the retrograde HRP finding of Kneisley and collaborators (1978) as inferred from their illustrations. The location of these rubrospinal neurons appears to correspond with that of neurons with coarse Nissl bodies (Miller and Strominger, 1973). This supports the idea that rubrospinal neurons are characterized by their internal configuration more or less independent of the size of the neurons (King et al., 1971). The present findings support the earlier reported somatotopic organization in the caudal magnocellular part of the monkey red nucleus (Kneisley et al., 1978). They also indicate that in the rostral extension of the population of rubrospinal neurons along the lateral aspect of the parvicellular part, the rubrocervical neurons are located dorsal to the rubrolumbar ones and that the latter neurons continue more rostrally than the former (Chapter IV Fig. 13).

VI.2.b. Collateralization of the rubrospinal tract in rat, cat and monkey

Earlier electrophysiological studies in cat have dealt with the collaterals of the rubrospinal pathway. Shinoda et al. (1977), reported the number of red nucleus neurons, which could be antidromically invaded from C3-C8 grey matter and the percentage of these neurons, which could be antidromically invaded by stimulation of the fibers passing through different more caudal segments of the cord. In order to facilitate a comparison between our anatomical data and

these physiological data the anatomical experiments were set up in a comparable manner. Thus the number of TB or FB labeled neurons which project to the C5-C8 dorsal grey matter were counted and the percentages of these neurons which were also labeled with NY from lower levels of the cord were computed. These percentages as computed from the anatomical findings in cat, were found to be of the same order of magnitude as those inferred from the earlier electrophysiological findings in this animal. Thus according to the electrophysiology in cat (Shinoda et al., 1977) 5% of the rubrocervical neurons projecting to C3-C8 distribute collaterals to segments caudal to L1 and 50% distribute collaterals to segments caudal to T2. According to the anatomical findings about 3% of the rubrocervical neurons projecting to C5-C8 distribute collaterals to the segments caudal to L2 and about 20% distribute collaterals to the segments caudal to T3 (Chapter IV Table IV). In monkey a similar arrangement was found anatomically: 9,3% of the rubrocervical neurons projecting to C5-C8 were found to distribute collaterals to the segments caudal to T8 and 6,6% to the segments caudal to L1 (Chapter IV Table V). The slight differences between the percentages obtained by means of the electrophysiological and the anatomical techniques may be due to the fact that in the two types of experiments slightly different cervical segments were studied and slightly different thoracic and lumbar segments were stimulated and injected, respectively. The differences may also be due to differences in the rostro-caudal extent of the nucleus explored in the two types of experiments.

A comparison of the anatomical findings in cat and monkey with those obtained in rat (Chapter IV and III) respectively, indicates that the rubrospinal system in rat displays a higher degree of collateralization than in cat and monkey. Thus in rat 20% of the rubrocervical neurons projecting to C5-C8 distribute collaterals to segments caudal to L1 versus only about 3% in cat and about 6% in monkey. Further, in rat about 35% of the rubrocervical neurons distribute collaterals to segments caudal to T7 versus about 10% in cat and monkey. The rubrospinal tract in cat and monkey therefore appears to be more focussed than in rat, such that in the former species a larger proportion of the rubrospinal neurons distribute fibers to a restricted part of the spinal cord than in the latter. This conclusion is further strengthened by the fact that the high degrees of collateralization of the raphe-spinal and the crossed pontospinal tract are of the same order of magnitude in the three species (see VI.2.c.).

The comparison of the anatomical findings in opossum and rat (Huisman et al., 1981; Martin et al., 1981[a]) strongly suggests that the rubrospinal tract in opossum shows an even higher degree of collateralization than in rat. This would imply that the rubrospinal tract in rat, in turn, is more focussed than

in opossum. The fact that the red nucleus in opossum displays a much less rigid somatotopic organization than in rat (Huisman et al., 1981; Martin et al., 1974, 1981) implies that with an increase in collateralization the somatotopic organization becomes less sharply defined. If this is correct the absence of any somatotopic organization in the red nucleus in pigeon (Wild et al., 1979) and in reptiles (ten Donkelaar and De Boer-van Huizen, 1978) would imply that in these species the rubrospinal tract displays an even more pronounced collateralization than in opossum. In the tadpole this tract develops just at the stage when limbs are developing (ten Donkelaar, 1982), while in snakes this tract seems to be lacking (ten Donkelaar, 1976a+ b). It therefore appears that the rubrospinal tract, as a highly focussed fiber brain stem system, is only present in higher mammals. It would be of interest to determine whether in respect to the rubrospinal collateralization the „ontogeny mimics the phylogeny", such that e.g. in new-born kittens a higher degree of collateralization occurs than in adult cat. This question is prompted by the fact that in other structures such an ontogenetic decrease in collateralization has been established (Innocenti, 1981; O'Leary et al., 1981).

In respect to the decrease in rubrospinal collateralization from higher to lower mammals it is of interest to recall that in cat and monkey (Gorska and Sybirska, 1978; Kohlerman et al., 1980; Kuypers, 1964; Lawrence and Kuypers, 1968; Smith, 1970) this tract together with the corticospinal tract contributes to the capacity to execute relatively independent movements of the extremities in particular their distal parts. This is probably a reflection of the fact that in these animals a relatively limited degree of rubrospinal collateralization exists such that many of the rubrospinal neurons project to very restricted parts of the spinal cord. The suggested decrease in collateralization of this tract from reptiles to pigeon, opossum, rat, cat and monkey makes it likely, that the capacity of the rubrospinal tract to assist in the execution of relatively independent movements of the individual extremities is present only in the higher mammals. This is exemplified by the fact that this capacity which is clearly present in monkey and cat, seems to be lacking in reptiles.

VI.2.c. Collateralization of the rubrospinal tract compared with the collateralization of the raphe spinal and ventrolateral pontine tegmentum spinal tracts in rat, cat and monkey.

The descending pathway from the nucleus raphe magnus (NRM)(i.e. the nucleus raphe magnus as defined by Taber (1960,1961) and the lateral adjoining ventral reticular formation dorsal to the pyramidal tract) and the crossed descending pathway from the ventrolateral pontine tegmentum (VLPT) descend together with the rubrospinal tract through the dorsolateral funiculus. Moreover as the rubrospinal tract they distribute fibers to the dorsal spinal grey (Basbaum et al., 1978; Basbaum and Fields, 1979; Goode et al., 1980; Holstege et al., 1979; Kuypers and Maisky, 1975 and 1977; Leichnetz et al., 1978; Martin et al., 1979; Martin et al., 1981; Tohyama et al., 1979a+b). This arrangement provided the opportunity to compare the collateralization of these three tracts in the same experiment. As compared to the findings in the red nucleus a relatively large proportion of NRM-cervical neurons was found to be double labeled from more caudal levels of the cord in all three species (c.f. Chapter III Table I; Chapter IV, Table IV and V; Chapter V Fig. 9). Thus in rat about 40%, in cat about 55-60% and in monkey about 40% of raphe-cervical neurons were double labeled from several more caudal levels of the cord. These high percentages occurred more or less independent of the level of the caudal injections varying between 30-50% in rat, 40-60% in cat and 30-40% in monkey. Moreover, no clear-cut somatotopic organization in the distribution of the single and double labeled neurons was observed. (Chapter III Fig. 6; Chapter IV Figs. 12 and 16). These findings in rat, cat and monkey (Huisman et al., 1981; Huisman et al., 1982; Chapter III and IV) are in keeping with those in opossum (Martin et al., 1981a+b). The raphe spinal system therefore resembles the reticulospinal system since according to an electrophysiological study (Peterson et al., 1975). 66% of the reticulocervical neurons distribute collaterals to the segments caudal to L1.

In respect to the raphe spinal connections, the anatomical findings suggest that a large percentage of the raphe spinal neurons in NRM distribute collaterals throughout the length of the spinal cord, but that the remainder project to restricted parts of the cord. This would imply that the raphe spinal system comprises both a focussed component and a diffuse component. One of these two components may possibly subserve the pain modulating function of the

raphe spinal pathway (Basbaum et al., 1976a+b, 1977; Basbaum and Fields, 1978 and 1979; Beall et al., 1976; Fields et al., 1977; Fields and Basbaum, 1978; Giesler et al., 1981; Guilbaud et al., 1977; Hayes et al;, 1978; Oliveras et al., 1975 and 1977) which function is presumably provided by serotonergic raphe spinal neurons (Dahlström and Fuxe, 1965; Proudfit and Anderson, 1973 and 1974; Proudfit et al., 1980). However, the focussed or the diffuse component may also represent the serotonergic raphe spinal fibers to the autonomic cell groups throughout the spinal cord (Amendt et al., 1979; Basbaum et al., 1978; Bowker et al., in press; Coote and Macleod, 1974; Dahlström and Fuxe, 1965; Loewy and McKellar, 1981; Steinbusch, 1981).

The crossed pontospinal tract (Busch, 1964; Papez, 1926) from VLPT neurons in rat, cat and monkey (Basbaum and Fields, 1979; Kuypers and Maisky, 1975; Leichnetz et al., 1978) which tract is located in the dorsolateral funiculus ventromedial to the rubrospinal tract (Busch, 1961), seems to occupy a position between the rubrospinal and the raphe spinal system in respect to the degree of collateralization. Thus, it shows little somatotopic organization and a relatively high degree of collateralization (Chapter III Fig. 6 and Table I; Chapter IV Figs. 12 and 16 and Table IV). In contrast to the findings in the nucleus raphe magnus the percentages of VLPT-cervical neurons, which are double labeled from more caudal levels of the cord tend to show some decline when the injections are placed progressively more caudally in the cord. This suggests that the crossed pontospinal system which has been regarded as the pontine component of the rubrospinal system (Busch, 1964) displays a certain degree of specialization in that the bulk of the collaterals of the neurons projecting to C5-C8 tend to distribute to the thoracic and lumbosacral levels but not to the sacral cord. Further studies, however, are necessary to confirm this impression and to give clues to the function distribution of this system.

VI.2.d. Collateralization of the rubrospinal tract to the cerebellum

Since the red nucleus also distributes fibers to the contralateral cerebellar anterior interpositus nucleus (NIA)(Courville and Brodal, 1966) and since these fibers originate from the same rostrocaudal parts of the red nucleus as the rubrospinal fibers (Brodal

and Gogstad, 1954) it was of interest to study the collateralization of rubrospinal neurons to the cerebellum by means of the retrograde fluorescent double labeling technique and to compare this with the collateralization of the rubrospinal tract in the spinal cord (Huisman et al., 1981, 1982 and Huisman et al., in press., Chapter III, IV and V). It was found that in rat, almost all rubrocerebellar fibers represent collaterals of rubrospinal neurons (Chapter V Fig. 6), which is in keeping with earlier anatomical and electrophysiological findings in cat (Anderson, 1971; Brodal and Gogstad, 1954) and that at least 37% of the rubrospinal neurons give rise to such cerebellar collaterals. The double labeled neurons in the red nucleus, distributing divergent axon collaterals to both the cerebellar NIA and the spinal cord, were more or less evenly distributed throughout the red nucleus both in the transverse are longitudinal directions except in cases 2 and 3 in which more double labeled neurons were present in intermediate and lateral parts of the nucleus (Chapter V Fig. 6). This is in accordance with the findings of Brodal and Gogstad (1954) that after cerebellar lesions retrograde changes occur in neurons, which are located in the same parts of the red nucleus as the retrogradely affected neurons after spinal lesions.

In respect to the findings that almost all rubrocerebellar fibers are collaterals of rubrospinal ones it is of interest to note that the interpositus nucleus represents the main source of afferents to the rubrospinal neurons which projection in addition is somatotopically organized (Courville, 1966; Dekker, 1981; Flumerfelt et al., 1973; King et al., 1973). The rubrospinal collaterals to the interpositus nucleus therefore appear to represent a direct, tightly coupled return projection to cells of origin of the afferents of these rubral neurons (Chapter V Fig. 8). The precise functional role of these return projections is still unclear. However, they must be of critical importance since such return projections occur in several places in the cerebellar circuitry. In this context it is of interest to note that in parallel with the return collaterals from the rubrospinal neurons to the interpositus nucleus also another return projection from the red nucleus to the cerebellar cortex exists. This return projection is established by way of rubral fibers to the supraspinal portion of the lateral reticular nucleus (i.e. the part of the nucleus not in receipt of fibers from the spinal cord (Brodal, 1943; Walberg, 1958). This lateral reticular nucleus is also designated as the nucleus of the lateral funiculus (Brodal, 1943), which name makes it distinct from the reticular formation. In light of the present findings the question arises as to whether these rubro-lateral reticular connections are also established by collaterals of rubrospinal neurons.

As was shown in Chapter III and IV (Huisman et al., 1981 and 1982), the rubrospinal tract represents a relatively focussed system, which distributes its individual fibers to restricted groups of spinal segments. Moreover, the degree of collateralization of the rubrospinal system in rat appeared to be much higher than in cat and monkey, since in rat as compared to cat and monkey a much higher percentage of rubrocervical neurons give rise to collaterals to the spinal cord caudal to L1 (Chapter V Fig. 9, histogram I). This, together with observations in pigeon (Wild, 1979) and opossum (Martin et al., 1981c) suggests that the rubrospinal tract as a focussed system emerges only gradually during phylogeny. In view of the above interspecies differences in the degree of collateralization of the rubrospinal system in the cord the question arose as to whether such interspecies differences also occur in respect to the rubrospinal collaterals to the cerebellum. Therefore a further interspecies comparison of these rubrospinal collaterals would be of interest. However, to some extent, it would seem unlikely that the degree of rubrospinal collateralization to the cerebellum would run parallel with that of the rubrospinal collateralization in the cord, since these two types of rubrospinal collaterals probably subserve an entirely different function.

VI.3. References

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Summary

Classical anatomical studies using the Golgi technique demonstrated that many axons in the central nervous system give off axon collaterals along their trajectory through the brain. For a long time little attention has been paid anatomically to these collaterals mainly because modern tracing techniques did not make it possible to distinguish divergent axon collaterals from unbranched fibers. However, during recent years electrophysiological studies have re-opened the issue of the existence of axon collaterals especially in respect to the descending pathways. Recently the existence of divergent axon collaterals has also been studied anatomically by means of the retrograde neuronal double labeling techniques using for example two fluorescent retograde tracers which, after being transported retrogradely through an axon and its collateral, label in different colors different features of the parent cell (Kuypers et al., 1980).

In the present quantitative anatomical study, which deals with the collateralization of descending brainstem pathways from red nucleus (RN), raphe magnus (NRM) and ventrolateral pontine tegmentum (VLPT) in rat, cat and monkey this retrograde fluorescent double labeling technique was employed (c.f. chapter III and IV). The descending brainstem pathways from RN, NRM and VLPT all descend throughout the spinal cord in the dorsolateral funiculus and terminate in the dorsal half of the spinal grey matter. In order to study the collateralization of these pathways, „True Blue" (TB) in rat or „Fast Blue" (FB) in cat and monkey was injected in the dorsal half of the C5-C8 spinal grey avoiding the adjoining dorsolateral funiculus. Subsequently in the various animals NY was injected at more caudal levels in the cord. TB and FB produce a blue fluorescent labeling of the cytoplasm while NY after short survival times relative to the transport distance, produces only a golden yellow fluorescence of the nucleus. This fluorescent retrograde labeling obtained with all three tracers can be observed at the same 360 nm excitation wavelength. In all cases in which the TB or FB deposit was restricted to the grey matter, the number of TB or FB labeled neurons and the number of TB-NY or FB-NY double labeled ones in the above brain stem cell groups was counted. These data made it possible to approximate both the numbers of neurons in the three different brain stem cell groups in rat which distribute fibers to the dorsal half of C5-C8 spinal grey and to compute the percentages of these cells, which distribute fibers to the various, progressively more caudal portions of the neuraxis.

The retrograde fluorescent labeling findings in the red nucleus confirm its somatotopic organization as demonstrated by means of other anatomical and

physiological techniques (Kneisley et al., 1978; Murray and Gurule, 1979; Pompeiano and Brodal, 1957; Tsukahara et al., 1967). The double labeling findings show a relatively limited degree of collateralization of rubrocervical neurons to more caudal levels of the cord indicating that the red nucleus represents a rather focussed system which distributes its individual fibers to specific groups of spinal segments. On the other hand, the NRM-spinal and the VLPT-spinal pathways show little somatotopic organization and a relatively high degree of collateralization. These double labeling findings are in keeping with electrophysiological findings in cat (Peterson et al., 1975; Shinoda et al., 1977). A comparison of the anatomical findings in cat and monkey, with those obtained in rat indicates that the rubrospinal system in rat displays a higher degree of collateralization than in cat and monkey, while the high degree of collateralization of the NRM-spinal and the VLPT-spinal pathways are of the same order of magnitude in all three species (c.f. chapter III and IV). This indicates that the rubrospinal tract as a highly focussed brain stem fiber system, emerges only gradually during phylogeny.

In another double labeling study in rat the collateralization of rubrospinal neurons to the cerebellar anterior interpositus nucleus (NIA) was studied and compared with the collateralization of the rubrospinal tract in the spinal cord (c.f. chapter V). For this purpose TB was injected in the NIA and NY or „Diamidino Yellow" (DY) in the cervical spinal cord. It was found that in rat almost all rubrocerebellar fibers represent collaterals of rubrospinal neurons, which is in keeping with earlier anatomical and electrophysiological findings in cat (Anderson, 1971; Brodal and Gogstad, 1954) and that at least 37% of the rubrospinal neurons give rise to such cerebellar collaterals. The double labeled neurons in the red nucleus, distributing divergent axon collaterals to both the cerebellar NIA and the spinal cord, were more or less evenly distributed throughout the red nucleus, which is in general accordance with the retrograde degeneration findings of Brodal and Gogstad (1954). In this same study DY was compared with NY in double labeling with TB. From the findings it was concluded that in quantitative double labeling studies DY may be used instead of NY, since the combination TB-DY results in approximately the same percentage of double labeled neurons as the combination TB-NY. This was confirmed by a more elaborate quantitative study in rat and cat, in which DY was compared with NY in the combination with TB in rat and with FB in cat (Keizer et al., in preparation)

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Samenvatting voor leken

Om mijn studie te plaatsen in het perspectief van de kennis en concepten over het centraal zenuwstelsel grijp ik ver in de tijd terug.
In de Griekse oudheid was het Plato (427-347 voor Christus) die drie zielsvormen lokaliseerde in het lichaam: het denken in de hersenen, de levensadem in het hart en het instinkt in de lever. Het denken werd door de Grieken geplaatst in de hersenholtes (=ventrikels), die omgeven werden door hersensubstantie, die niet van betekenis geacht werd. Op grond van deze „ventrikelleer" heeft Galenus, die vanaf 162 in Rome praktizeerde, een medisch oevre geschreven, dat eeuwenlang zijn invloed heeft doen gelden. In die tijd werden de hersenen dan ook afgebeeld als schematisch afgebakende kompartimenten en later als bolvormige holtes. De sensus communis, een begrip dat van Aristoteles afkomstig is, en door hem in het hart gelokaliseerd, werd in die tijd onder andere in één van die holtes geplaatst. Dit begrip is later te herkennen in het Engelse common sense. In de Renaissance wordt de schematische hersenholte afbeelding vervangen door meer realistische weergaves zoals o.a. door Leonardo da Vinci (1452-1519) die behalve kunstenaar ook een wetenschapsman was. In de 16e eeuw gaat de aandacht in de hersenanatomie verschuiven van de hersenholtes naar de hersensubstantie zelf, die om de ventrikels heen ligt. Deze verschuiving is met name te bespeuren in de afbeeldingen van de hersenen. Zo laat Vesalius (1514-1564) in een houtsnede bijzondere strukturen zien, die wij nu als capsula interna zouden herkennen en laat Varolio (1543-1575) de pons (brug) zien. Ook Descartes (1596-1650) en Willis (1621-1675) laten natuurgetrouwe afbeeldingen zien. Zij houden echter allen nog vast aan de Griekse theorie dat de belangrijkste processen plaatsvinden in de hersenholtes en dat de hersensubstantie zelf een onbelangrijke rol speelt. De Deen Niels Stensen (1638-1686) onderstreept echter, dat kennis van hersenen alleen te verkrijgen is via onbevooroordeelde studie van anatomie van het zenuwstelsel, met name van de vezelstrukturen in de witte stof. Met de komst van de lichtmicroscoop (van Leeuwenhoek 1632-1723) werd het mogelijk om de struktuur van hersenweefsel te bestuderen. In de huidige tijd beantwoorden modellen als een „telefooncentrale" of een „computer" het meest aan de gestelde vragen over struktuur en funktie van de hersenen. Deiters (1834-1863) beschreef voor het eerst de zenuwcellen, waaruit het centraal zenuwstelsel is opgebouwd. Een zenuwcel (=neuron) bestaat uit een zenuwcellichaam met een groot aantal korte uitlopers (=dendrieten) en één lange uitloper, (zenuwvezel of axon), die zich kan opsplitsen in zenuwvezelvertakkingen (Fig. 1). De zenuwvezeleindigingen staan in contact met een volgende zenuwcel of spiercel. De informatie overdracht loopt

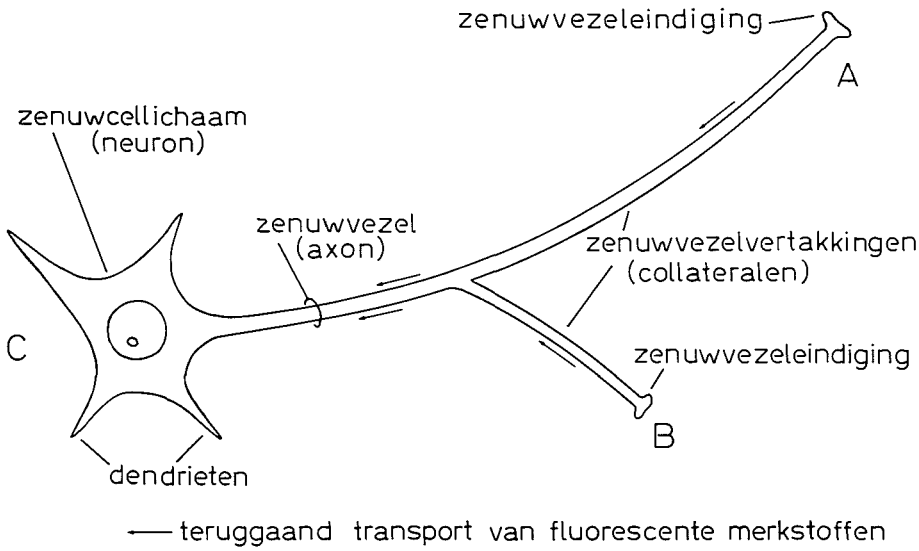

Fig. 1.

van het zenuwcellichaam via de zenuwvezel naar de zenuwvezeleindiging(en) en vindt plaats door elektrische veranderingen (potentiaal veranderingen) in de wand (membraan) van de zenuwvezel. Allerlei technieken zijn sindsdien ontwikkeld om een zenuwcellichaam met zijn zenuwvezel zichtbaar te maken om zodoende te bepalen waar die zenuwcel via zijn vezel, zijn informatie naartoe stuurt. Aanvankelijk werd gebruik gemaakt van de zilver impregnatie techniek van Golgi (1844-1926), die selectief enkele zenuwcellen in een gebied kleurt terwijl de rest van de cellen ongekleurd blijft. Met behulp van deze techniek heeft Cajal (1852-1934) het zenuwstelsel bestudeerd (Cajal, 1952) waarbij hij beschreef dat zeer veel zenuwvezels zijtakken afgeven (Fig. 1). Vervolgens werd gebruikt gemaakt van degeneratieve technieken. Deze technieken zijn gebaseerd op het feit dat na beschadiging van een zenuwcellichaam, de bijbehorende zenuwvezel degenereert (afsterft) en dat na beschadiging van een zenuwvezel, het bijbehorende zenuwcellichaam degenereert. Deze degeneratieve veranderingen in zenuwvezel of zenuwcellichaam zijn na een bepaalde behandeling van het weefsel zichtbaar onder de microscoop (Marchi en Algeri, 1885;

Rasdolsky, 1925; Nauta en Gygax, 1954; Fink en Heimer, 1967). Gedurende de veertiger jaren werd door Weiss en Hiscoe (1948) aangetoond, dat er in een zenuwcel een vloeistofstroom bestaat die vanuit het zenuwcellichaam de zenuwvezel ingaat. Eind zestiger jaren werd ontdekt, dat m.b.v. deze stroom radioactief gemerkte stoffen door de zenuwvezel getransporteerd kunnen worden en aldus vezelverbindingen in de hersenen opgespoord kunnen worden (Lasek en medewerkers, 1968; Cowan en medewerkers, 1972). Kristensson en Olsson (1970, 1971) en de LaVails (1972) toonden aan, dat er in de zenuwvezel ook een teruggaande vloeistofstroom bestaat, d.w.z. van het vezeluiteinde teruggaand in de richting van het zenuwcellichaam. Deze stroom is dus tegen de richting in van de electrische informatie-overdracht. Met behulp van het enzym mierikswortel peroxidase (horseradish peroxidase: HRP) wat teruggaand getransporteerd wordt kunnen de zenuwcellichamen van de verschillende zenuwvezels geidentificeerd worden, waardoor vezelverbindingen bepaald kunnen worden. Behalve HRP zijn er momenteel vele andere stoffen bekend, die teruggaand in zenuwcellen getransporteerd worden.

Onze onderzoeksgroep onder leiding van Professor Kuypers heeft recent een aantal merkstoffen beschreven, die met een fluorescentie microscoop zichtbaar te maken zijn. Een aantal van deze stoffen is zeer geschikt om te onderzoeken of één individuele zenuwcel vertakkingen (collateralen) naar verschillende gebieden in de hersenen stuurt. Hiertoe worden twee stoffen, die in de fluorescentie microscoop van elkaar te onderscheiden zijn op twee verschillende plaatsen in de hersenen ingespoten. Als na zo'n dubbele injectie op b.v. punt A en B, in gebied C door teruggaande stroom dubbelgemerkte zenuwcellen gevonden worden, dan betekent dit dat die cellen in gebied C een zenuwvezel vertakking naar punt A en een andere naar punt B sturen (Fig. 1). Ieder van deze cellen geeft dus tegelijkertijd informatie door naar twee verschillende gebieden in de hersenen, wat in functioneel opzicht van groot belang is om te weten. Immers als meer bekend wordt over hoe „de telefooncentrale" of „computer" geconstrueerd is, wordt daardoor ook duidelijker hoe deze eventueel werkt. Met behulp van deze techniek heb ik onderzocht in welke mate de „rubrospinale vezels" (dit zijn vezels, die vanuit de celgroep „nucleus ruber", gelegen in de hersenstam, door het hele ruggemerg heen afdalen) op zijn traject door het ruggemerg heen zijtakken afgeeft. De anatomische bevindingen van mijn vergelijkende studie in diverse diersoorten geven meer inzicht in de functie die deze „rubrospinale vezels" hebben in de besturing van de motoriek. Vereenvoudigd zou geconcludeerd kunnen worden dat de „rubrospinale vezels" een functie hebben in de besturing van de middelgrove motoriek van armen en benen, d.w.z. die motoriek die tussen de grove motoriek van de romp en de fijne motoriek van de individuele vingers instaat.

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List of abbreviations

A	nucleus ambiguus
AC	cerebral aquaduct
AChE	acetylcholinesterase
ACU	area cuneiformis
BA	bovine albumine
Bb	Bisbenzimide
BC	brachium conjunctivum
BDHC	benzidine dihydrochloride
C	nucleus coeruleus
CG	central grey
CI	inferior colliculus
CMP	posterior commissure
CP (or Cped)	cerebral peduncle
DAO	dorsal accessory olive
DAPI/Pr	DAPI/Primuline
DCP	decussation of superior cerebellar peduncles
DL	double labeled
dl	dorsal lamella of principal olive
DMSO	dimethylsulfoxide
dr	nucleus dorsalis raphe
DY	Diamidino Yellow
EB	Evans Blue
EPSP	excitatory postsynaptic potential
FB	Fast Blue
FLM	medial longitudinal fasciculus
FPT	transverse pontine fibers
FR	fasciculus retroflexus
G	central grey
GB	Granular Blue
G+C	nucleus gracilis and cuneatus
GM	medial geniculate body
HRP	Horseradish peroxidase
HRP-TMB	Horseradish peroxidase-tetramethylbenzidine dihydrochloride
IP	interpeduncular nucleus
IPSP	inhibitory postsynaptic potential
K	cap of Kooy
L	lateral nucleus of cerebellum

LL	lateral lemniscus
LM	medial lemniscus
LVN	lateral vestibular nucleus
MAO	medial accessory olive
M	medial nucleus of the cerebellum
MB	mammillary bodies
ML	medial lemniscus
MLF (or mlf)	medial longitudinal fasciculus
MVN	medial vestibular nucleus
NIII	oculomotor nucleus
nIII	oculomotor nerve
NVII	facial nucleus
nVII	facial nerve
NXII	hypoglossal nucleus
nXII	hypoglossal nerve
$NXII_p$	nucleus prepositus hypoglossi
NC	cochlear nuclei
NCE	external cuneate nucleus
NCI	nucleus of inferior colliculus
NCu	cuneate nucleus
NIA	anterior interpositus nucleus
NLL	nucleus of lateral lemniscus
NP	pontine nuclei
NRL	lateral reticular nucleus
NRM	nucleus raphe magnus
NRP	reticular nucleus of pontine tegmentum
NTS V	nucleus of spinal V tract
NVL	lateral vestibular nucleus
NVM	medial vestibular nucleus
NY	Nuclear Yellow
PCI	inferior cerebellar peduncle
PCM	medial cerebellar peduncle
ped	cerebral peduncle
PI	Propidium Iodide
Pr	Primuline
ps	propriospinal
R (or RN)	red nucleus
RF	reticular formation
RFl	lateral reticular formation

rfl fasciculus retroflexus
RM nucleus raphe magnus
Rm red nucleus, magnocellular part
Rp red nucleus, parvicellular part
RS rubrospinal tract
rs rubrospinal
S nucleus and tractus solitarius
SC nucleus subcoeruleus
SNC (or snc) substantia nigra, pars compacta
SNR substantia nigra, pars reticulata
ST spinothalamic tract
TB True Blue
TCS corticospinal tract
TR (or TRS) rubrospinal tract
trV tract of mesencephalic V nucleus
TSV spinal V tract
VIII third ventricle
VIV fourth ventricle
vest. compl. vestibular complex
vl ventral lamella of principal olive
vt ventral tegmental nucleus
VLPT ventrolateral pontine tegmentum
X dorsal motor nucleus of vagus

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Curriculum Vitae

Anne-Margriet Huisman werd op 27 augustus 1952 te Breda geboren. Zij bezocht van 1965-1972 het Stedelijk Gymnasium te Breda, waar zij in 1972 het diploma gymnasium-β behaalde. Van 1972-1979 studeerde zij geneeskunde aan de Erasmus Universiteit te Rotterdam; het doctoraalexamen legde zij af in 1978 en het artsexamen, cum laude, in 1979. Naast haar studie heeft zij studentassistentschappen op de afdelingen pathologische anatomie en maatschappelijke gezondheidszorg vervuld, waar zij assisteerde bij het pre- en postkandidaats onderwijs. Vanaf november 1979 is zij als wetenschappelijk medewerkster werkzaam op de afdeling neuroanatomie van de Erasmus Universiteit, waar zij onder leiding van Prof.Dr. H.G.J.M. Kuypers onderzoek verrichtte naar de collateralisatie van descenderende hersenstambanen. De resultaten van dit onderzoek staan beschreven in dit proefschrift. In november 1983 begint zij met de opleiding inwendige geneeskunde in het Academisch Ziekenhuis Dijkzigt te Rotterdam.