The time course of retrograde transsynaptic transport of tetanus toxin fragment C in the oculomotor system of the rabbit after injection into extraocular eye muscles

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Summary. The aim of this study was to determine the optimal survival time for labelling those neurons that monosynaptically terminate on extraocular motoneurons, i.e. the premotor neurons, after an injection of tetanus toxin fragment C, a retrograde transsynaptic tracer substance, into the eye muscle of the rabbit. Concentrated fragment C was injected into the inferior rectus or inferior oblique muscle and detected immunocytochemically in the brain after survival times of 8 h, 17 h, 2 d, 3 d, 4 d, 5 d, 6 d, 8 d and 12 d. Immunoreactivity was confined to granules within motoneuronal and premotor neuronal cell bodies, but became associated with punctate profiles outlining the somata with longer survival times. The strongest and most consistent labelling of premotor cell bodies was seen after 4 days survival time. The transsynaptic labelling pattern was shown to vary for individual premotor pathways.

Abbreviations: III oculomotor nucleus, IV trochlear nucleus, Vmes mesencephalic trigeminal nucleus, Vmt motor trigeminal nucleus, VI abducens nucleus, VIacc accessory abducens nucleus, VII facial nucleus, BC brachium conjunctivum, co cochlear nucleus, CR restiform body, d dentate nucleus, DAB diamino-benzidine-tetrahydrochloride, HRP horseradish peroxidase, iC interstitital nucleus of Cajal, iv inferior vestibular nucleus, lgn$_d$ lateral geniculate nucleus dorsalis, lgn$_v$ lateral geniculate nucleus ventralis, lv lateral vestibular nucleus, mgn medial geniculate nucleus, MLF medial longitudinal fasciculus, mv$_p$ medial vestibular nucleus pars parvocellularis, mv$_m$ medial vestibular nucleus pars magnocellularis (= ventral part of the lv), NIII oculomotor nerve, NV trigeminal nerve, NVII facial nerve, NVIII vestibular nerve, PC posterior commissure, pg periaquaeductal grey, ppH nucleus praepositus hypoglossi, riMLF rostral interstitial nucleus of the medial longitudinal fasciculus, rn red nucleus, sc superior colliculus, sn substantia nigra, so superior olive, sv superior vestibular nucleus, sv$_c$ superior vestibular nucleus contralateral, sv$_i$ superior vestibular nucleus ipsilateral, TR tractus retroflexus, Y Y-group, zi zona incerta

Introduction

After injection into a muscle, tetanus toxin has been shown to move retrograde transsynaptically into presynaptic terminals of the motoneurons (Schwab and Thoenen 1976; Price et al. 1977; for review: Mellanby and Green 1981; Wellhörner 1982). Some fragments of tetanus toxin, such as BII$_b$ (Bizzini et al. 1977), or C (Helting and Zwisler 1977), have the same transport properties as the intact toxin, but are themselves not toxic. These can be used to label those neurons that monosynaptically terminate onto motoneurons, called premotor neurons, without toxic side-effects in studies involving long survival times. The main advantage of using a transsynaptic tracer such as tetanus toxin fragments rather than using simple retrograde tracers such as horseradish peroxidase (HRP) to locate premotor neurons is that fragment C can be injected into a muscle, rather than centrally in the motor nucleus, which is less specific. One disadvantage is that the interpretation of the pattern of retrograde labelling in the motoneurons and premotor neurons is difficult, since the location of label changes rapidly with survival time (Evinger and Erichsen 1986; Fishman and Carrigan 1987).

Non-toxic tetanus toxin fragments have been shown to label neuronal cell bodies retrograde transsynaptically in the oculomotor (Büttner-Ennever et al. 1981; Evinger and Erichsen 1986) and sympathetic system (Manning et al. 1987; Cabot et al. 1987), but up to now there has been no study of the pattern of labelling in premotor neurons and its changes with time. Since monosynaptic connections in the oculomotor system from premotor areas to motoneurons have been fully described (Graybiel and

Hartwieg 1974; Steiger and Büttner-Ennever 1979; for review: Evinger 1988; Büttner-Ennever and Büttner 1988; Highstein and McCrea 1988), we have chosen it as a suitable system in which to investigate the time course of transsynaptic transport of tetanus toxin fragment C.

The aim of this study is to investigate the pattern of labelling in motoneurons and premotor neurons after an extraocular eye muscle injection with fragment C, and to determine the optimal survival time for the retrograde transsynaptic labelling of premotor neurons in the oculomotor system. We chose to inject the inferior rectus or inferior oblique eye muscle of the rabbit which move the eyes in the vertical plane. The results of this work also provide data on the localization of premotor neurons controlling vertical eye movements in the rabbit.

Methods

Five pigmented (Chinchilla) and seven albino (New Zealand) rabbits were used in this study. Prior to injection the fragment C (Calbiochem) was concentrated by ultrafiltration in a microconcentrator (Amicon; 30000 molecular weight cutoff) to at most 15% as limited by the 34° angle of the fixed-angle rotor of the centrifuge. The final concentration of the fragment C solution was calculated from the end-volume. For eye muscle exposure the rabbits were anaesthetized with Ketamin (35 mg/kg) and Rompun (5 mg/kg) intramuscularly. The eye muscles were exposed by retracting the eyelids, collapsing the eye ball, and making a conjunctival incision. All animals received an injection of 15 µl concentrated fragment C into the right inferior rectus or inferior oblique eye muscle. To prevent intraorbital spread of fragment C, the muscle to be injected was enclosed by a small piece of plastic film prior to injection: in some cases the injection hole made by the syringe in the muscle was locally cauterized as an additional precaution. After survival times of 8 h, 17 h, 2 d, 3 d, 4 d, 5 d, 6 d, 8 d and 12 d the rabbits were deeply anaesthetized and transcardially perfused with saline (37° C) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed from the skull, postfixed for another 5 h at 4° C and then stored either in 0.1 M phosphate buffer for Vibratome sections or in 30% sucrose at 4° C for frozen sections. The brainstem was transversely cut at 35–40 µm. The free-floating sections were immunocytochemically treated according to the avidin-biotin peroxidase method (Hsu et al. 1981) using a monoclonal antibody (now available from Boehringer Mannheim) that had been raised against fragment C (Evinger and Erichsen 1986) at a dilution of 1 : 3000. The avidin-biotin peroxidase complex was visualized by a 15 min incubation in 0.05% diamino-benzidine-tetrahydrochloride (DAB) and 0.01% H_2O_2. After mounting and drying the sections were osmified in a 0.05% osmium tetroxide solution for 20 seconds before dehydration. A modified DAB-reaction in the presence of 0.6% Ni^{2+} in acetate buffer (pH 6.0) (Hancock 1984) was proved to intensify the immunocytochemical staining significantly and it was used in some cases for the visualization of the weak transsynaptic label. A series of every fifth section was counterstained with 1% Cresyl violet. The tissue was examined and photographed under brightfield with a light microscope. Labelled cells were plotted onto camera lucida drawings by using a drawing tube that was connected to the microscope. The nuclear boundaries were drawn from Nissl-stained sections and the nomenclature for the vestibular nuclei was taken from Epema et al. (1988).

Results

Motoneurons

After an injection of fragment C into the inferior oblique or inferior rectus muscle the motoneuron pools of these muscles in the ipsilateral oculomotor nucleus (III) were strongly labelled. Additional retrograde label was found in the motoneurons of the medial rectus muscle in the ipsilateral III, in the ipsilateral abducens nucleus (VI) and accessory abducens nucleus (VIacc) containing motoneurons of the lateral rectus and the retractor bulbi muscles, and in the motoneuron subgroup of the facial nucleus (VII) that supplies the orbicularis oculi muscle of the injected side (Fig. 3). The spread of fragment C proved very hard to avoid as judged from the motoneuronal labelling.

The labelling pattern of all the motoneurons changed rapidly with survival time: after 8 h single motoneurons were distinctly labelled. The DAB-reaction product was located in the cytoplasm – associated with granules – while the nucleus was free of staining (Figs. 1a and 2a). The amount of filling within the cell bodies and proximal dendrites increased over the next 10 h, at which time a diffuse label was observed in the surrounding neuropil, that could not be correlated with neuronal elements using light microscopy (Figs. 1b and 2b). In preparations with survival times longer than 2 days the fragment C immunoreactivity was no longer restricted to neuronal somata, but in addition was seen associated with punctate profiles outlining the cell bodies and dendrites of the motoneurons (Figs. 1c–f and 2c, d). At this stage the staining of the oculomotor nucleus took on a reticulated appearance, which did not change even with the longest survival time in our study, 12 days, except that the intensity decreased slowly with time. Motoneurons containing no immunoreactive granules could be found within the oculomotor nucleus after survival times longer than 4 days (large arrows in Figs. 2c and d).

Premotor neurons

After at least 2 days survival time a weak immunoreactivity was found in several additional areas that are known to send monosynaptic afferents to the motoneurons of vertical eye muscles: the contralateral y-group, the superior vestibular nuclei (sv) of both sides, the lateral border region of the parvocellular medial vestibular nucleus (mv_p) and the magnocellular part of the medial vestibular nucleus (mv_m) mainly contralateral, the ipsilateral interstitial nucleus of Cajal (iC), and the rostral interstitial nucleus of the medial longitudinal fasciculus (riMLF) in the rostral mesencephalon. In addition transsynaptic label was found within the contralateral VI presumably associated with internuclear neurons. Figure 3 illustrates the location of all labelled premotor neurons after 4 days survival time following an inferior rectus muscle injection with fragment C.

It was striking that the staining pattern differed widely between premotor regions. For instance the most impressive transsynaptic labelling was always seen in the y-group, while the iC contained only very few weakly stained cells. For the detailed description of the transsynaptic labelling pattern 3 premotor structures have been chosen as examples: 1. the ipsilateral sv, 2. the contralateral sv, 3. the ipsilateral riMLF.
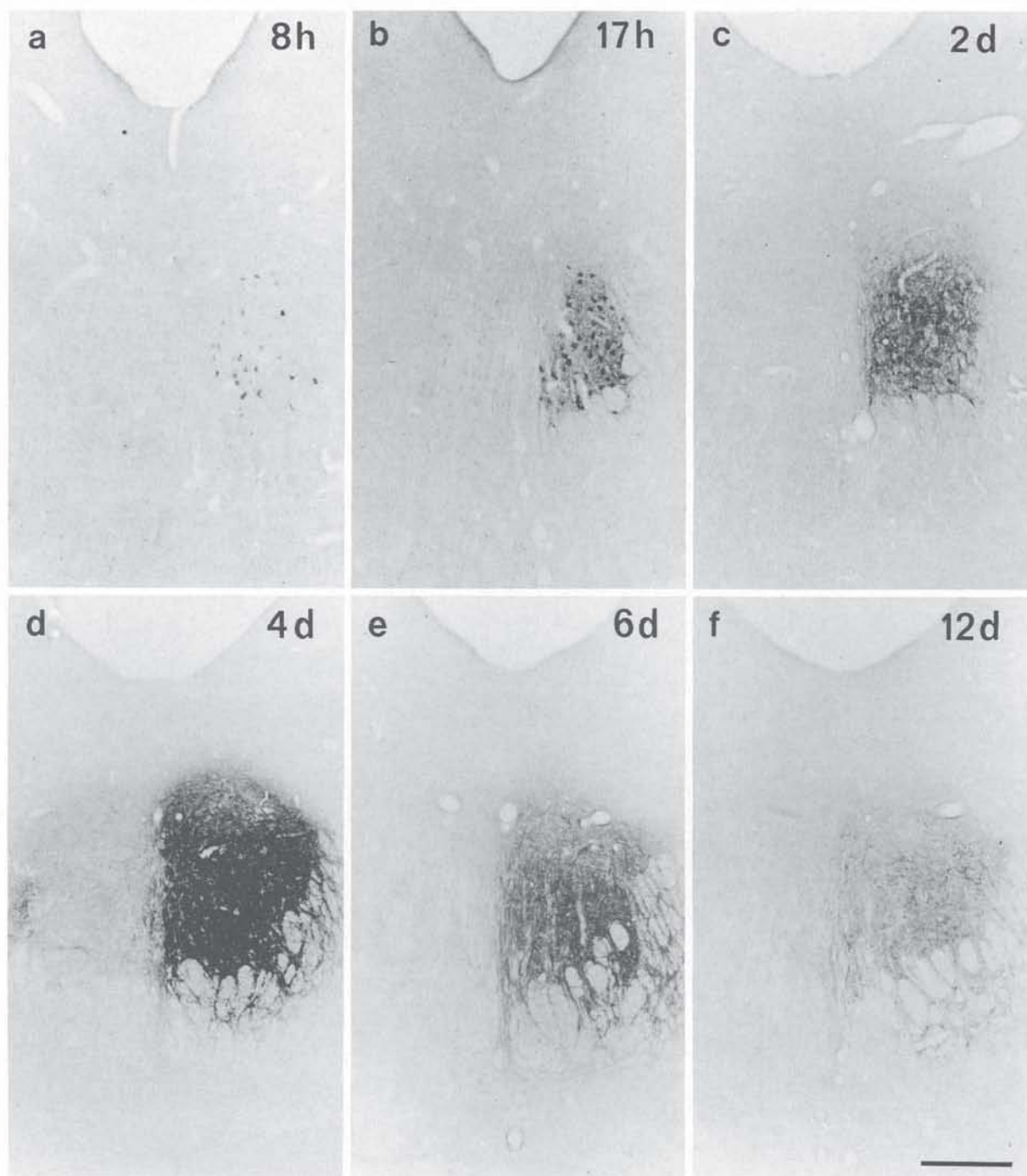
Fig. 1a–f. Photographs of transverse sections through the oculomotor nucleus to show the pattern of labelling of the motoneurons and neuropil after survival times of **a** 8 h, **b** 17 h, **c** 2 days, **d** 4 days, **e** 6 days, **f** 12 days. **a** Distinctly labelled motoneurons are visible after 8 hours survival time. **b–d** There is a gradually increase in labelling of the neuropil with survival times up to 4 days. **e, f** The staining intensity of motoneurons and neuropil decreases. Calibration = 500 μm

In the sv of both sides retrograde transsynaptically labelled neurons were first found after 2 days. While immunoreactive neurons contralateral to the injected side were located within the dorsal part of the sv, the labelled cells ipsilateral were distributed throughout the central parts of the nucleus. After 2 and 3 days the labelled neurons in the sv of both sides showed a similar but weak staining pattern, which resembled the pattern of direct labelling of motoneurons after an 8 h time period (Fig. 4a and b compare with Fig. 2a). The cells contained immunoreactive granules within their somata and proximal dendrites. This type of staining pattern did not change in the *contralateral sv* with longer survival times. After 4 and 5 days their somata were still distinctly labelled, and only few immunoreactive puncta were found in the surrounding neuropil (Fig. 4d and g). A dif-
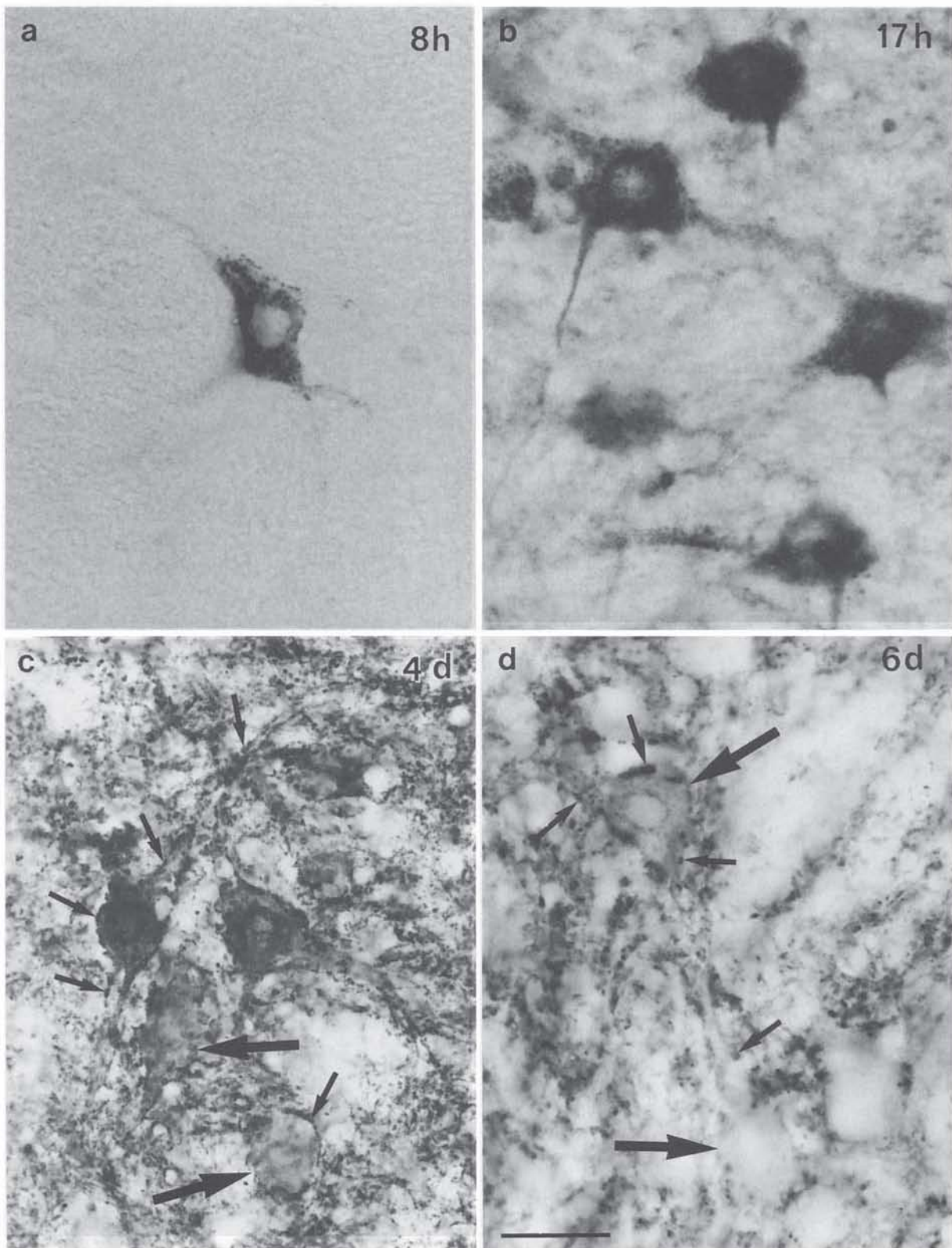
Fig. 2a–d. High power magnification of the motoneuronal labelling in the oculomotor nucleus with fragment C after different survival times. **a** Survival time of 8 h, the DAB-reaction product is associated with granules within the cytoplasm, while the nucleus is free. **b** Survival time of 17 **h**, a diffuse label is present in the neuropil. **c** Survival time of 4 days, the fragment C immunoreactivity is no longer restricted to somata (large arrows), but associated with punctate profiles (small arrows) outlining the cell bodies and dendrites. **d** Survival time of 6 days, "empty" motoneurons (large arrows) are associated with labelled puncta (small arrows). Calibration = 30 μm
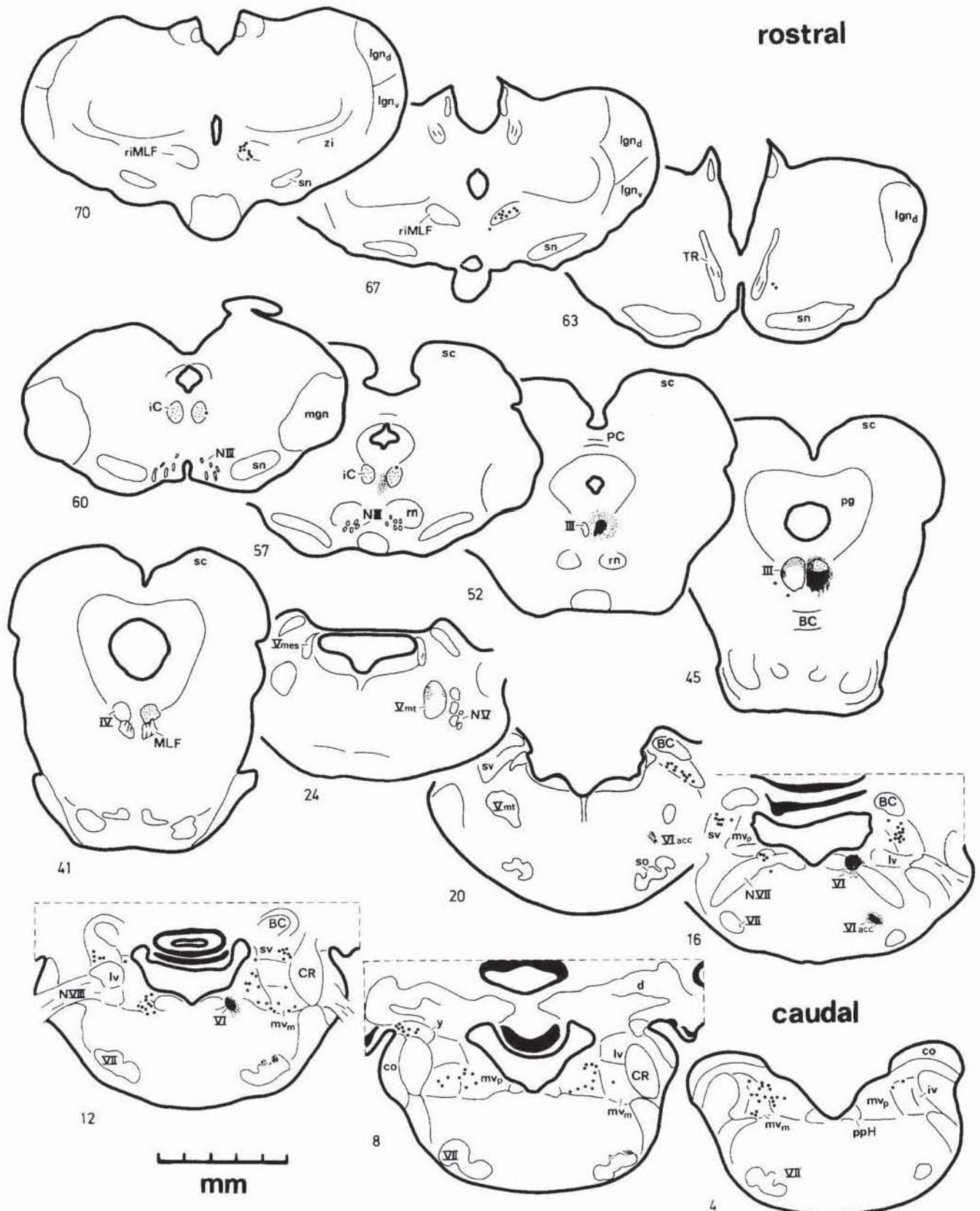
Fig. 3. Drawings taken from transverse sections of a rabbit brainstem to show the distribution of labelled motor and premotor neurons after an injection of fragment C into the right inferior rectus muscle; 4 days survival time. The distribution of small dots indicates the punctate labelling of presynaptic terminals. For clarity, the puncta around labelled cell bodies (e.g. Fig. 4e) are omitted. Each retrograde transsynaptically labelled premotor neuron is represented by a large dot. Consecutive section numbers are 200 μm apart
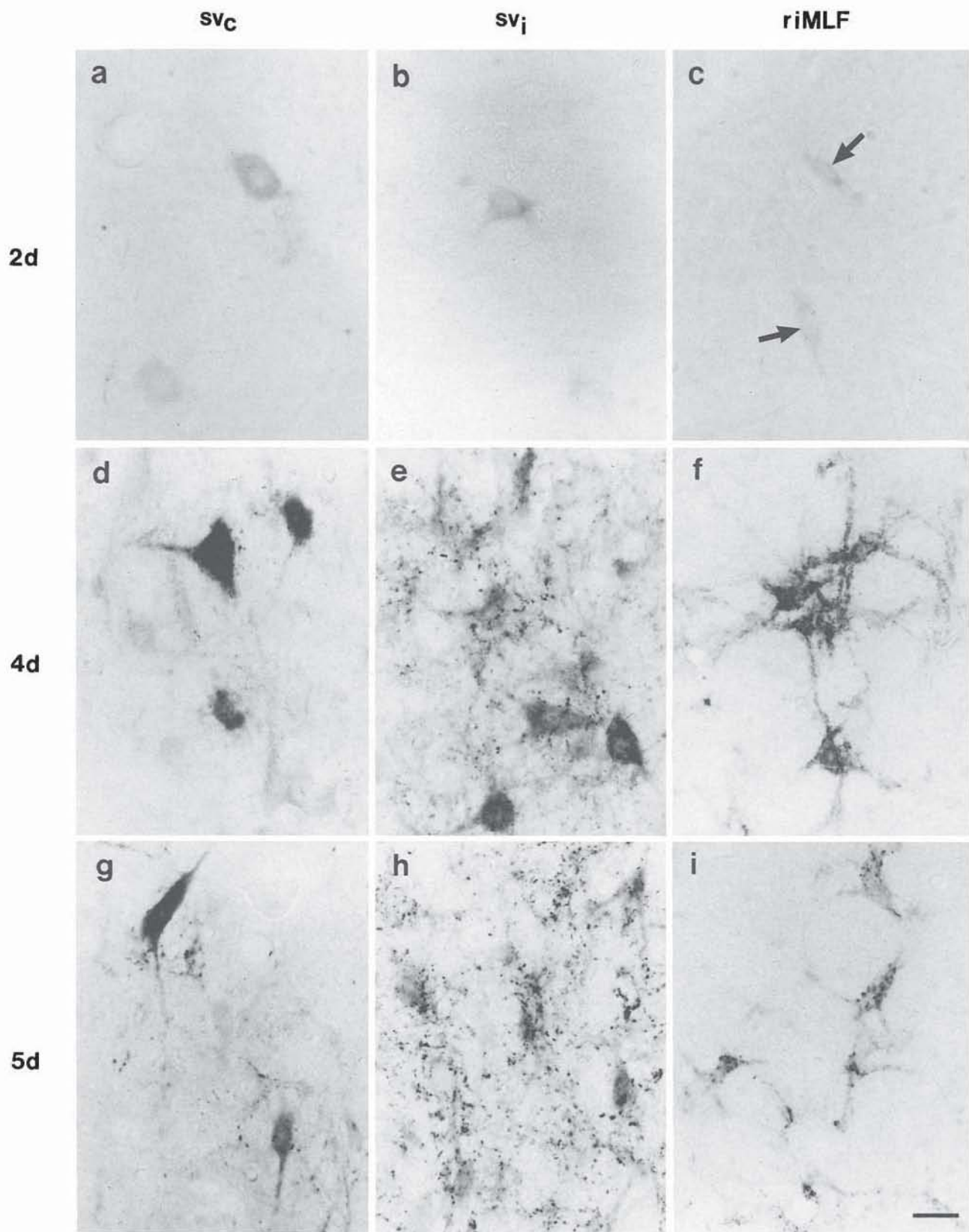
Fig. 4a–i. High power magnification of labelled premotor neurons in the superior vestibular nucleus contralateral (sv$_c$) **a, d, g;** in the sv ipsilateral (sv$_i$) **b, e, h;** rostral interstitial nucleus of the medial longitudinal fasciculus (riMLF) **c, f, i,** to show the changes of the pattern of labelling after survival times of 2 days in **a–c;** 4 days in **d–f;** 5 days in **g–i.** The strongest overall transsynaptic cell labelling is seen after 4 days. Note the difference in the pattern of labelling in sv$_i$ and sv$_c$ after 4 and 5 days survival time. Calibration = 30 μm

ferent time course of the staining pattern was seen in the *ipsilateral sv*. The cell somata became more weakly stained, and after 4 and 5 days they were only faintly visible, whereas numerous immunoreactive puncta in the surrounding neuropil gave the nucleus a reticulated appearance (Fig. 4e and h). Even after 12 days a few immunoreactive puncta were observed in the ipsilateral sv, while in the contralateral sv no immunoreactivity was detected after more than 6 days.

Retrograde transsynaptically labelled cells were found in the riMLF exclusively ipsilateral to the injected side. The immunoreactivity was weak and confined to numerous granules within the somata and proximal dendrites after 2 days (arrows in Fig. 4c), but after 3 and 4 days immunoreactivity was stronger and already associated with labelled punctate profiles outlining the cell bodies (Fig. 4f). One day later (5 days) the labelled riMLF neurons could only be identified by the immunoreactive puncta outlining the somata of the "empty" cells (Fig. 4i). This was clearly seen by focussing through the depth of the section, but is not obvious from the photograph (compare to motoneuronal labelling in 2c, d). After 12 days very few labelled puncta could be detected.

We never found any evidence for double transsynaptic transport; that is no labelled cells were found in those regions that are known to send monosynaptic projections onto premotor neurons, e.g. omnipause cells in the nucleus raphe interpositus in the paramedian pontine reticular formation, or cells in the deep layers of the superior colliculus, both of which are known to project to the riMLF (for review: Büttner-Ennever and Büttner 1988).

Only after 3–5 days survival time were *all* premotor populations labelled following an eye muscle injection with fragment C. We found the strongest and most consistent labelling of cell bodies in all premotor areas with 4 days survival time. These findings were critically dependent upon the injection of highly concentrated fragment C, i.e. 15%. Lower concentrations (approx. 10%) were used in several cases and were found to be less suitable for this study. These cases required longer survival times (5–6 days) to see any transsynaptic label at all. There was also no survival time at which all premotor neuronal cell bodies were labelled at the same time: while some regions contained clear cell body labelling, only punctate labelling was seen in others. It is important to emphasize that for this reason only those cases that receive an eye muscle injection of the same batch of fragment C concentrate can be qualitatively compared to each other as in Fig. 4.

Discussion

The precautions that were usually adequate to prevent leakage into the orbit after HRP or wheatgerm agglutinin (WGA) injections into an eye muscle (Büttner-Ennever and Akert, 1981), were not effective in these experiments with fragment C. Uptake by neighbouring eye muscles always occurred, and resulted in additional labelling of corresponding motoneurons in III, VI and VII (Courville 1966; Gray et al. 1981; Satoda et al. 1987; Evinger 1988). This probably reflects a more efficient uptake system for the tetanus toxin fragment compared

to other macromolecules used as neuroanatomical tracers (Wan et al. 1982; Trojanowski et al. 1982). It did not interfere with the results of this study because: 1) the premotor connections of VII (Takada et al. 1984) and VIacc (for review: Evinger 1988) were not labelled, 2) the premotor neurons of the medial rectus motoneurons were labelled (i.e. internuclear neurons in the contralateral VI, Highstein and Baker 1978; Labandeira-Garicia et al. 1989b), but did not involve the 3 premotor regions (sv ipsilateral, sv contralateral, riMLF) that we chose to study in detail (Evinger 1988).

The present study confirms the observation of other investigators (Stöckel et al. 1975; Fishman and Carrigan 1987) that tetanus toxin fragments undergo very fast intraaxonal retrograde transport, since ocular motoneurons were directly labelled within 8 h: demonstrating a minimum transport rate of 5 mm/h for fragment C. This is in agreement with the rate of transport of tetanus toxin calculated at 5–10 mm/h (Habermann 1978; Carrol et al. 1978) and 7–7.5 mm/h (Stöckel et al. 1975) in the motoneurons of rodents.

The immunoreactive granules we observed in the light microscope inside the cytoplasm of the motoneurons at 8 h to about 4 days and of premotor neurons probably represent the membrane compartments containing the tracer that were described in electron microscopic studies (Schwab and Thoenen 1978; Schwab et al. 1979; Cabot et al. 1987). The rapid change of the labelling pattern over the first 2 days indicates that after reaching the motoneuronal somata there is only a short time period of tracer accumulation within the cell bodies and dendrites, before additional transsynaptic transfer into presynaptic terminals starts. This transfer is indicated by the appearance of punctate profiles, and is already seen 2 days after the eye muscle injection. Previous studies have shown that tetanus toxin injected into the spinal cord accumulated within synaptic terminals (Price et al. 1977; Price and Griffin 1981), where the specific receptor is located (van Heyningen 1974), rather than within cell bodies. Schwab and Thoenen (1976) showed for the first time that after a muscle injection tetanus toxin accumulates not only within corresponding cell bodies, but also in presynaptic terminals. In their electron microscopic studies the authors did not observe any significant label over the cell membrane or in the extracellular space (Schwab and Thoenen 1977; 1978; Schwab et al. 1979).

The olivary pretectal nucleus sends monosynaptic projections onto the Edinger-Westphal nucleus in the oculomotor complex but not onto motoneurons, and it remained unlabelled in all our experiments, although it is labelled in HRP-studies of the oculomotor nucleus (Graybiel and Hartwieg 1974; Steiger and Büttner-Ennever 1979). Such results, as well as previous studies (Büttner-Ennever et al. 1981; Evinger and Erichsen 1986), provide further evidence for a real transsynaptic transfer of tetanus toxin fragments into presynaptic terminals rather than a non-specific leakage out of the cells by the fact that transsynaptic labelled cell bodies were exclusively found in those areas that are known to specificly target onto motoneurons.

The patterns of transsynaptic labelling were described on the basis of three pathways with well known properties: the sv contains two sets of premotor neurons that synapse on oculomotor neurons as part of the vestibulo-ocular three neuronal arc. The central part of the sv gives rise to an inhibitory projection that ascends via the ipsilateral MLF and terminates ipsilateral in the oculomotor and trochlear nucleus on vertical motoneurons. Whereas dorsally located sv neurons send an excitatory projection via the brachium conjunctivum (BC) onto vertical motoneurons of the contralateral side (Yamamoto et al. 1978; for review: Büttner-Ennever 1981; Highstein and McCrea 1988). The positive labelling of both sv neuronal populations in our experiments emphasizes for the first time the capability of tetanus toxin fragments to travel across inhibitory as well as excitatory synapses (see also Büttner-Ennever et al. 1981; Evinger and Erichsen 1986).

The riMLF is involved in the generation of vertical saccadic eye movements (Büttner et al. 1977) and has been shown to send direct projections to vertical motoneuron subgroups (Büttner-Ennever and Büttner 1978; Graybiel 1977; Nakao and Shiraishi 1983, 1985) in cat and monkey. The present study confirms the recent description of the homologue in the rabbit (Labandeira-Garcia et al. 1989a), and it implies a purely ipsilateral connection to the oculomotor nucleus in this species.

The premotor neuronal label was weaker and occurred later than motoneuronal labelling. The changes in the pattern of the transsynaptic staining in premotor neurons resembled the changes in motoneuronal labelling described above: a short period of label accumulation within the soma is followed by a longer lasting punctate labelling around the premotor neurons. It is therefore difficult to distinguish labelled motoneurons from premotor neurons from the pattern of labelling alone. Other criteria such as the labelling intensity, the survival time, and the concentration of the injected fragment C (see below) must be taken into account as well.

Four days were chosen as the optimal survival time for labelling premotor neurons in the oculomotor system with fragment C, since this was the peak of tracer accumulation within cell bodies (Fig. 4). However this optimal time for premotor labelling was found to depend on the high concentration of the fragment C injected. We had the impression that the usage of lower concentrations could to some extent be compensated for by longer survival times, enabling sufficient tracer accumulation in premotor cells, or rather in their afferent terminals, for visualization (see Evinger and Erichsen 1986). A quantitative comparison of the binding and neuronal transport capabilities of tetanus toxin and its fragments showed that under physiological pH-conditions 50–100 times more of the fragments C or BII_b must be injected into a muscle in order to obtain neuronal transport similar to that seen with the whole tetanus toxin (Weller et al. 1986).

The punctate labelling in the vicinity of immunoreactive premotor neurons seen in the light microscope (see Fig. 4g and h), taken together with the knowledge from electron microscopic studies of the motoneuronal labelling, implies that fragment C has passed a third synapse, although confirmation by ultrastructal analysis remains to be done. The failure to find any labelling in regions that are known to send afferents to premotor neurons can be attributed to the dilution effect caused by the increasing divergence of afferent systems.

A comparison of the transsynaptic staining in 3 selected premotor regions described in detail here, shows that the time course of the changes in the pattern of labelling is different for individual pathways. For example we found that in the ipsilateral sv the pattern of transsynaptic label changed quickly from somatic to punctate, whereas in the contralateral sv the pattern remained almost exclusively confined to the cell bodies (compare Fig. 4g and h). Such variations must be related to certain individual properties of the premotor pathways: one possibility is the strength of the synaptic input from a given premotor source onto the motoneurons in the oculomotor nuclei that is reflected by the axonal termination pattern. For the horizontal oculomotor system of cats it has been shown that the excitatory contralateral projecting vestibulo-ocular fibers had a more widespread axonal arborization within the motonucleus with a large number of synaptic boutons, whereas the inhibitory ipsilateral projecting vestibulo-ocular fibers tend to terminate within restricted areas with less boutons (Ishizuka et al. 1980; Ohgaki et al. 1988). On the other hand higher numbers of inhibitory terminals were found to contact lower numbers of motoneurons indicating a stronger convergence for the inhibitory vestibular input onto motoneurons than for the excitatory input (Ohgaki et al. 1988). The distance from premotor neurons to motoneurons appeared not to be an important parameter, since both vestibular projections showing a different staining pattern are about the same length, but they do differ in the location of their synaptic inputs onto the oculomotor neurons. Electron microscopic degeneration studies demonstrate that the excitatory contralateral vestibular input terminates on the distal dendrites of extraocular motoneurons, whereas the inhibition of the ipsilateral vestibular pathway is mainly mediated by axosomatic synapses (Bak et al. 1976; Destombes and Rouvière 1981). Another possibility for variations in the transsynaptic staining patterns is the firing rate of certain premotor neurons. The rate of retrograde transsynaptic transport of WGA has been shown to be enhanced by electrical stimulation of afferent axons (Jankowska 1985). A similar correlation between neuronal activity and the rate of transport has been described for tetanus toxin: the "muscle pump" hypothesis (Ponomarev 1928) was tested by Wellhörner et al. (1973), who showed that the rate of ascent of ^{125}I-tetanus toxin into the spinal cord was increased by electrical stimulation of the nerve of the curarized muscle.

In conclusion we found that the optimal transsynaptic labelling of premotor neurons in the oculomotor system with fragment C was seen after 4 days survival time. The factors that were found to most influence the time course and pattern of labelling were: the concentration of the injected fragment C and the type of pathway to be studied. The parameters that control its transport are not

yet completely clear and this makes fragment C a less attractive tracer for the strict definition of neural pathways. However its properties do make it a highly useful neuroanatomical tool for locating synaptically related structures, whereby the nature of the connection must be investigated by other methods.

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