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Fragment C tetanus toxin: A putative activity-dependent neuroanatomical tracer

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Abstract. The non-toxic C fragment of tetanus toxin fused to the β-galactosidase enzyme was analysed as a neuroanatomical tracer. After intramuscular injection in rat tongue, its location in the hypoglossal network was compared with other classic tracers such as neurotropic viruses. The hybrid protein reached second and higher-order neurons after crossing several synapses. It appears to be a powerful tool to map neuronal circuits since the protein is easy to handle and detect and its transsynaptic transport is potential activity-dependent.

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INTRODUCTION

Tracing neuronal connections has been a challenge in neuroanatomy, with profound implications both in the study of neural function and in the developmental and adult plasticity of the nervous system (Vercelli et al. 2000). Several anterograde and retrograde tracers have been used to provide a more detailed resolution of neuronal circuitry but with mixed results (Kobbert et al. 2000, Vercelli et al. 2000). The "ideal" tracer should allow easy, fast and stable detection, just-retrograde or anterograde transport, allow to label long circuits, provide specificity in neuronal network labeling and be safe to handle and harmless for animals. Finally, it would be an advantage to combine tracing protocols and neurophysiological paradigms (Lanciego and Wouterfood 2000).

Tetanus toxin is a potent neurotoxin produced by *Clostridium tetani*. Tetanus toxin prevents the release of inhibitory neurotransmitters from spinal cord interneurons by a specific mechanism of cell intoxication (for review, see: Schiavo et al. 2000). The pathological mechanism has been shown to involve retro-axonal and transsynaptic transport of the tetanus toxin. The transsynaptic movement of tetanus toxin was first demonstrated by autoradiographic localization in spinal cord interneurons after injection in muscle (Schwab and Thoenen 1976). However, previous studies of transsynaptic passage of tetanus toxin from motoneurons were limited by the rapid development of clinical tetanus and the death of the experimental animal (Price et al. 1977, Schwab and Thoenen 1976, Schwab and Thoenen 1977). The nontoxic proteolytic C fragment of tetanus (TTC peptide) is responsible for binding and internalization of the tetanus toxin into neurons (Ginalski et al. 2000, Halpern and Neale 1995, Herreros et al. 2000, 2001). This fragment has the same ability as the native toxin to bind nerve cells and be retrogradely transported through a synapse, but without causing clinical symptoms (Bizzini et al. 1977, Fishman and Carrigan 1987). For this reason, TTC has been used in several studies as a neuronal tracer (Cabot et al. 1991, Evinger and Erichsen 1986, Horn and Buttner-Ennever 1990, Meckler et al. 1990). However, it has limitations since TTC is diluted at each synapse, which makes antibody detection difficult. In order to solve this problem, other groups have amplified the signal using the β-galactosidase enzyme as a marker that is genetically fused to the TTC (Coen et al. 1997).

Another interesting characteristic of TTC is that its uptake at the neuromuscular junction depends on neuronal activity (Miana-Mena et al. 2002). A similar process may occur at the synaptic cleft, making TTC a very interesting tracer of active circuits to the CNS.

In this report, β-galactosidase-TTC (β-gal-TTC) hybrid protein was used as a retrograde neuroanatomical tracer to try to map the complex interconnections of the central nervous system (CNS). The behavior of β-gal-TTC was analysed in the known hypoglossal network of the rat, where it was able to cross several synapses. The results are compared with previous patterns obtained by injection of different neurotrophic viruses. Technical aspects of the application, uptake mechanisms, intracellular transport of the molecule and the problems of subsequent signal detection are also discussed.

METHODS

Protein injection

All experiments were carried out in accordance with European Community guidelines for laboratory animal handling. Twenty Wistar six-week-old rats were obtained from Iffa Credo Laboratories. Animals were anaesthetised for 10 minutes by halothane inhalation and were given an intramuscular injection (in the tongue) of β-galactosidase-TTC recombinant hybrid protein (30 µl, 1 µg/µl), obtained and purified as in Coen et al. (1997).

X-Gal and Immunohistology

After survival times of 2, 6, 12, 24 or 48 hours, the animals were deeply anaesthetised (pentobarbital 75 mg/kg). Then they were perfused *via* intracardiac with 75 ml of PBS and afterwards with 75 ml of 4% paraformaldehyde in PBS for fixation. Fixed brains were removed and rinsed in PBS before being frozen and cryosectioned into 20 µm-thick slices for X-Gal staining or immunohistological methods.

In X-Gal staining, slices were incubated in X-Gal solution (4 mM $K_3(FeCN)_6$, 4 mM $(CN)_6FeK_4 \cdot 3H_2O$, 8 mg/ml X-Gal and 2 mM $MgCl_2$) at 37°C overnight and counter-stained for 5 minutes in Fast Nuclear Red.

In the immunohistological methods, slices were incubated in a 1:5,000 dilution of anti-TTC antibody as described by Coen et al. (1997). Antibodies were detected

using the Vectastain ABC alkaline phosphatase kit with diaminobenzidine color development. Slices were counter-stained for 5 minutes in Emerald Green.

After light microscopy analysis, labeled structures were identified in relation to the nuclei as defined in the atlas of the rat brain (Paxinos and Watson 1986).

Electron Microscopy

The removed fixed brain was cut into 150 μm vibratome sections and incubated in X-Gal solution. The X-Gal precipitate is electron dense and can be detected by direct examination (Bonnerot et al. 1987). Selected sections were post-fixed in 2.5% glutaraldehyde in PBS for 6 hours, rinsed with PBS for 10 minutes, and reacted with 1% osmium tetroxide in water for 30 minutes and subsequently with 1% uranyl acetate in water for 1 hour. Sections were dehydrated in increasing series of acetone and then embedded in Epon at 60°C for 48 hours, cut in 50 nm ultra-thin sections and examined with a Zeiss transmission electron microscope. The ultra-structural studies were performed on ten CD1 mice.

RESULTS

After intramuscular injection in the rat tongue, the distribution of β-gal-TTC protein in the CNS (Fig. 1) was analysed by X-Gal staining at several post-injection times (2, 6, 12, 24 and 48 hours). The retrograde transneuronal transport of β-gal-TTC was time dependent (Table I; Fig. 3) and observed in all animals, with a minimal variability.

At 2 hours post-injection, hypoglossal motoneurons (XIIMNs) and hypoglossal nerve (XIIn) labeling were easily found but relatively weak and only involved a few motoneurons. No other stained structure was detected in the CNS at this time, implying a lack of transsynaptic passage.

At 6 hours post-injection, both cell bodies of the XIIMNs and the closest part of their dendrites were very densely stained. The distribution of the second and third order transneuronally labeled cells in various regions of the brain was clearly defined and reproducible in all animals. Labeling appeared granular and confined to the soma of neuronal cell bodies in XIIMNs and transneuronally labeled neurons, while the cell nuclei were never labeled. The granular appearance is a result of the distribution of the β-gal-TTC protein in the endoplasmic reticulum membrane around the nucleus,

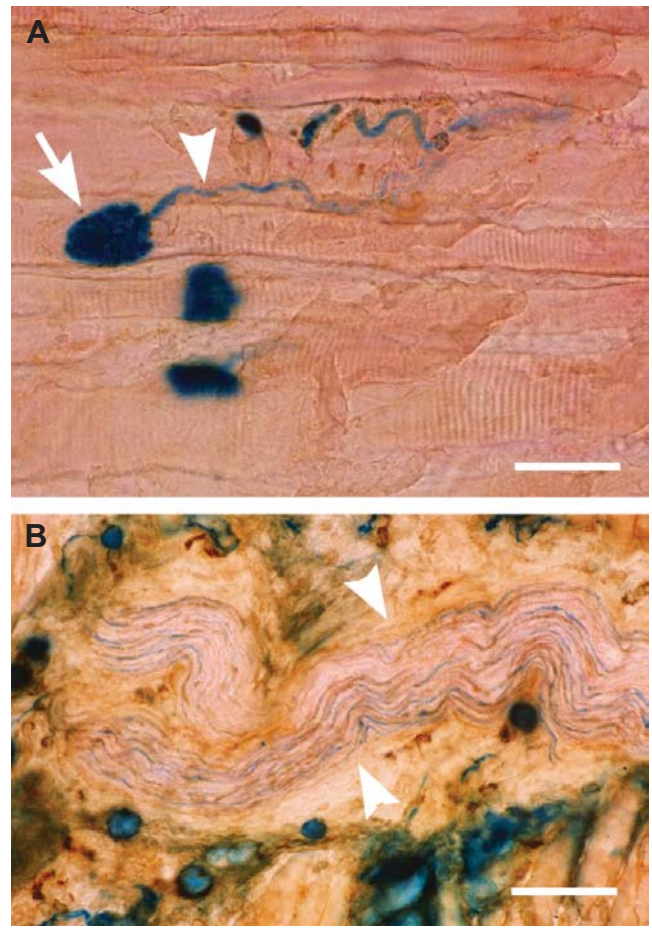

Fig. 1. β-galactosidase-TTC labeling in the tongue. A, tracer entering into the neuromuscular junction (arrow). Also observe an X-Gal labeled axon (arrowhead) (bar-50μm). B, X-Gal labeling in axons of a nerve in the tongue (arrowheads) (bar-5 μm).

as shown by electron microscopy (Fig. 2). Sometimes the axon and parts of the dendrites closest to neuronal bodies could be visualised after β-galactosidase labeling (Fig. 2).

Second-order labeled neurons (which connected with the XIIMNs) were found in several regions of the brain (Table I, Fig. 2). First, we examined structures surrounding XIIMNs as oral motor nuclei. The labeling was dense in the motor trigeminal nucleus (MoV); while other oral structures, the facial nucleus (VII) (Fig. 2) and its descending facial nerve (VIIn), and the adjoining accessory facial nucleus (AcsVII) were more lightly labeled.

As expected, strongly labeled neurons were also present in the parvocellular reticular nucleus (PCRt). The PCRt is located among the XIIMNs, MoV and VII. Most of the afferent projections to these motor oral nu-

Table I

Labeling of different cell types in the CNS after β-gal-TTC injection in the tongue.

	2 h	6 h	12 h	24 h	48 h
First-order neurons					
XIIMNs, Hypoglossal motoneurons	+/-	+++	+++	+/-	-
XIIn, Hypoglossal Nerve	+/-	++	++	-	-
Second-order neurons					
MoV, Motor Trigeminal n.	-	++	+	+/-	-
VII, Facial n.	-	+	-	-	-
VIIn, Facial Nerve	-	+	+/-	-	-
AcsVII, Accessory Facial n.	-	+/-	-	-	-
PCRt, Parvocellular Reticular n.	-	++	+	+/-	-
R, Caudal Raphe n.	-	+	+/-	+/-	-
CG, Mesencephalic Central Gray*	-	+	+/-	+/-	-
Sol, Solitary Tract n.	-	-	-	+/-	-
Putative third-order cell groups					
SC, Superior Colliculus	-	+	+/-	+/-	-
Cx, Cerebral Cortex	-	+	+	+/-	-
Gen, Geniculated n.	-	+	+	-	-
SN, Substantia Nigra	-	+/-	+/-	-	-
MeV, Mesencephalic Trigeminal n.	-	-	+	+/-	-
Mve, Medial Vestibular n.	-	-	-	+/-	-
Rn, Red Nucleus	-	-	-	+/-	-

(*)Second-order cell groups also containing third-order neurons. (-) No labeling. (+ to +++) Increasing density of labeling. (+/-) Only labeled in some cases or at very low density.

clei come from the brainstem reticular formation (Panneton and Martin 1979, Travers and Norgren 1983, Ugolini 1995a). Additionally, some neuronal cells were also labeled in the caudal raphe nuclei (R) and in the central grey nucleus (CG) placed in mesencephalon.

Several cell groups belonging to the third order were also labeled, such as the superior colliculus (SC) and geniculated nucleus (Gen) (with a relatively strong signal) and the substantia nigra (SN), which is related to the oral motor nuclei. These cells, although not directly connected to the XIIMNs, provide projections to XIIMNs connected cell groups. We emphasise that β-gal-TTC arrived to the cerebral cortex (Cx) 6 hours after injection (Fig. 2).

At 12 hours post-injection the spatial distribution of labeled neurons was similar to 6 hours (Table I). The labeling intensity in XIIMNs and XIIn was also similar to 6 hours but decreased in second-order structures (e.g., MoV, VIIn, PCRt, R and CG) and disappeared in VII and AcsVII. In structures containing third-order neurons, the intensity of the signal was maintained in Cx, Gen and SN and declined in SC. The only new structure detected was the mesencephalic trigeminal nucleus (MeV), which contains third-order neurons related to the oral motor nuclei.

After 24 hours post-injection, labeling was very weak. The β-gal staining decreased substantially in the XIIMNs, was not detectable in other structures such as XIIn, VIIn, Gen and SN. The solitary tract nucleus (Sol) appeared as a new positive second-order structure, which projects a few axons to XIIMNs, MoV and VII. As shown in Table I, the number of positive third-order structures increased with time, although the intensity of the signal weakened. Thus, labeling was weak in SC, MeV and CX, as at 12 hours post-injection, but also in the medial vestibular nucleus (Mve) and the red nucleus (Rn).

After 48 hours post-injection no structures were labeled, not even the XIIMNs.

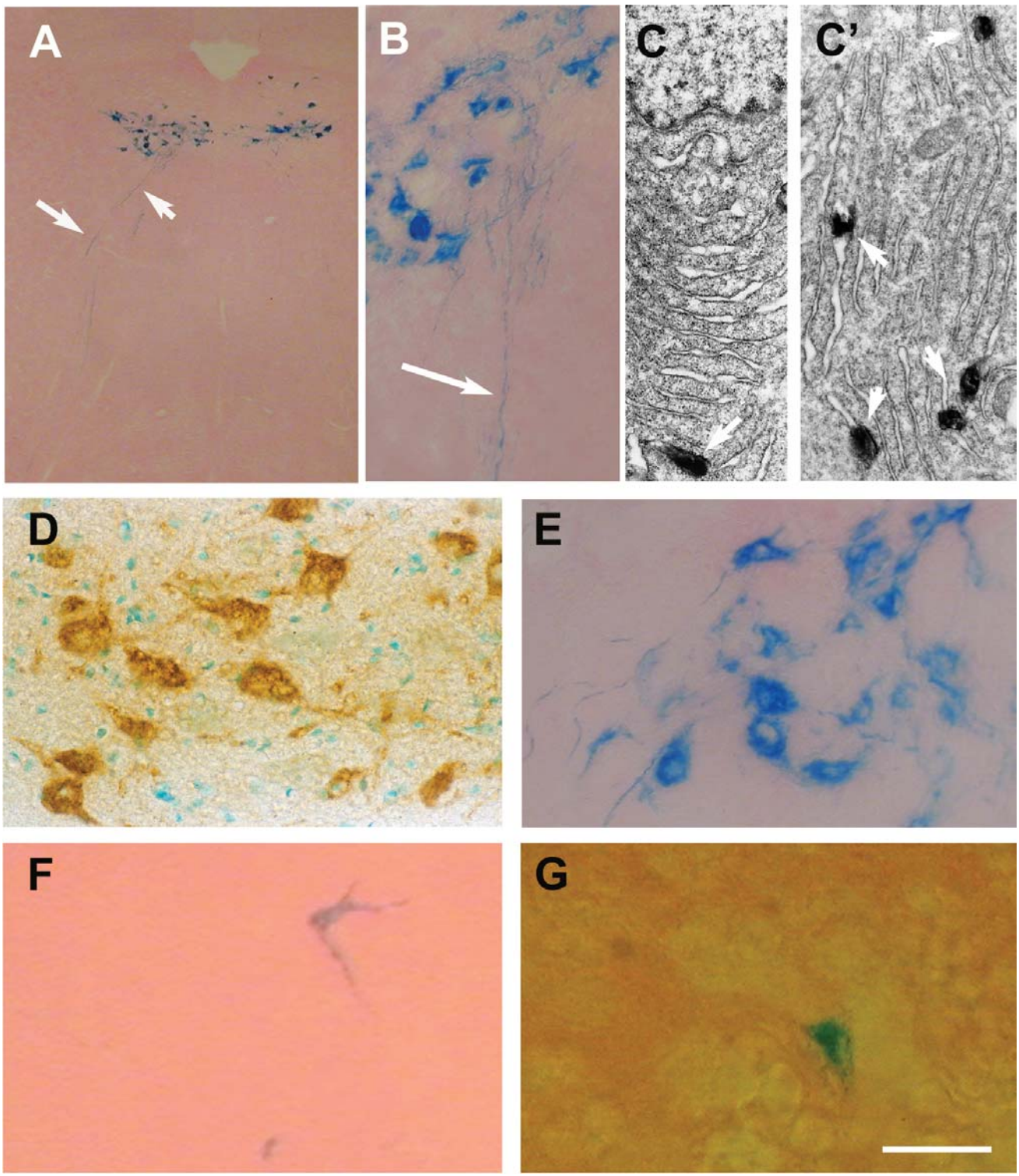

Fig. 2. A, β-galactosidase-TTC labeling in the hypoglossal nucleus. The arrows point to the hypoglossal nerve (bar-1mm); B, magnification of the hypoglossal nucleus and the axons of the hypoglossal nerve arriving to the motoneurons (arrow) (bar-0.5 mm); C and C', ultrastructural detail of the motoneuron body. Labeling was observed in the RER (bar-1 μm); D, TTC was detected in the hypoglossal nucleus using anti-TTC antibodies (bar-20 μm); E, β-galactosidase-TTC labeling of the trigeminal nucleus, related with the hypoglossal nucleus (bar-20 μm); F, labeled motoneuron of the facial nucleus (bar-20 μm); G, labeled motoneuron of motor cortex (bar-10 μm).

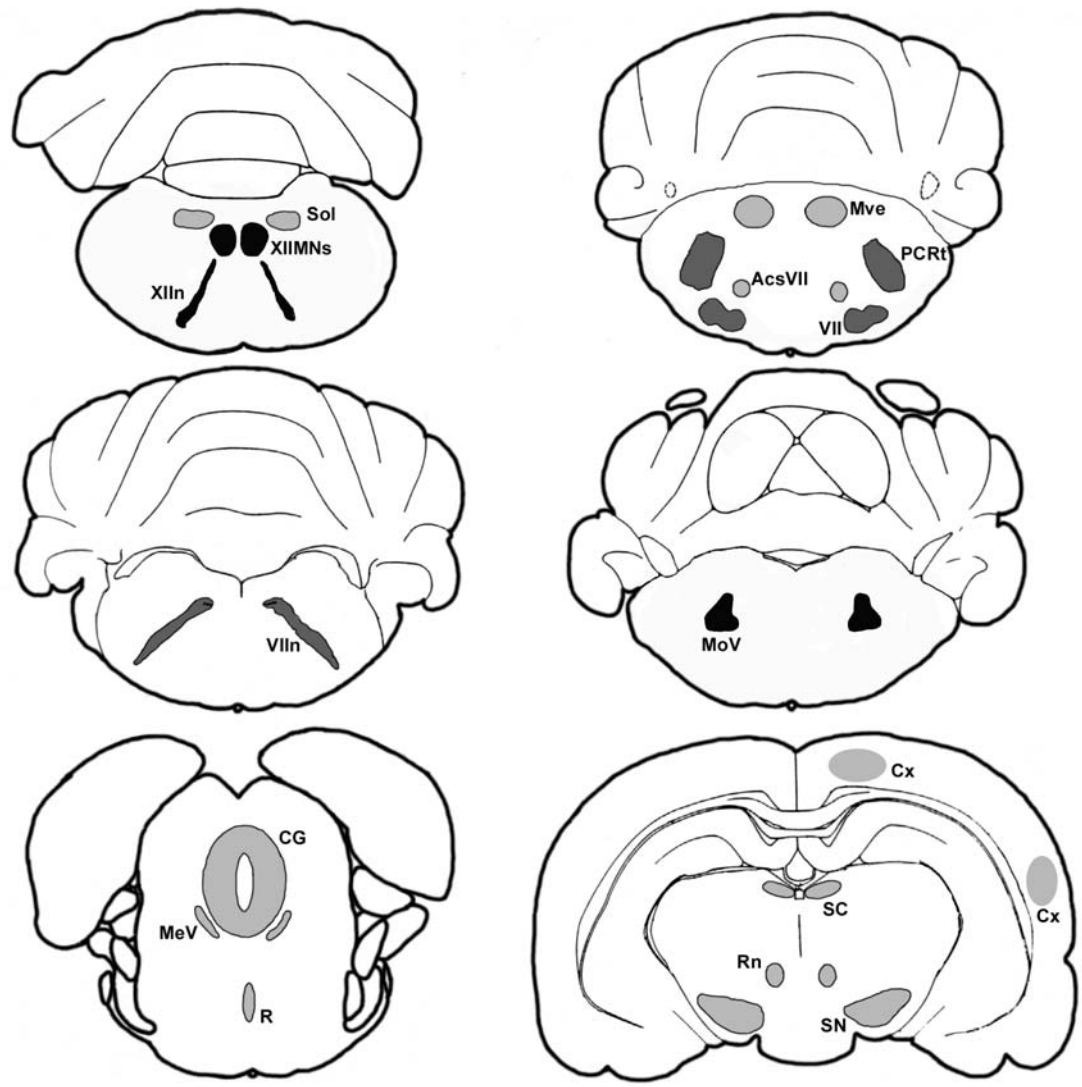

Fig. 3. Labeled structures in the brain. Oral motor nuclei (XIIMNs, MoV and VII) were labeled intensely. Third-order cell groups (MeV, SC, SN, Rn, R or Cx) were weakly labeled.

DISCUSSION

In this study we analysed the use of β-gal-TTC recombinant hybrid protein to map the neuronal circuits in the rat CNS. In spite of easy manipulation, it was difficult to detect due to the limitation of fragment C use. When a limited amount of protein is injected (such as TTC), only a fraction will be transported through a synapse and the signal will be diluted along the arborisation of the neuronal circuitry. This pitfall is partially solved by fusing the TTC to β-galactosidase (Coen et al. 1997). The signal is amplified by enzymatic activity, which produces a blue precipitate after joining the X-gal substrate. Moreover, the precipitate is electron-dense (Bonnerot et al. 1987), which allows direct ultra-structural study by electron microscopy.

The spatial distribution of labeling in the CNS after β-gal-TTC protein injection was consistent with other studies using classic retrograde tracers such as germ agglutinin-horseradish peroxidase complex (Krammer et al. 1979, Panneton and Martin 1979, Travers and Norgren 1983) and transsynaptic neurotropic viruses, such as α-herpes (Ugolini 1995b) or the rhabdoviruses (Ugolini 1995a).

The granular shape of the labeling could have emerged because the migration of TTC inside the CNS is associated with the membraneous compartments (Miana-Mena et al. 2002). Moreover, previous studies have shown that

the markers remaining in vesicles produce a granular appearance in the cell (Kobbert et al. 2000).

The extent of retrograde transport of hybrid protein in the hypoglossal circuit indicated that only a subset of interconnected neurons was detected, compared with neurotropic viruses. However, we could identify most areas containing second or higher-order of neurons using β-gal-TTC. This phenomenon could be explained by: a) unspecific labeling of the virus, b) β-gal-TTC protein dilution through the circuit, or c) β-gal-TTC activity dependent transport.

In the first case, the transneuronal pathway of neurotropic viruses has not yet been elucidated. Even some viruses (such as α-herpes) lyse the neuronal cell, allowing random release and non-specific uptake by other non-connected neurons close by, which could explain the wide distribution of these viruses in the CNS (Loewy 1995, Norgren and Lehman 1998). On the other hand, despite the presence of β-galactosidase enzyme, the signal was diluted through time along the arborisation of the neuronal circuitry, as the amount of injected protein was limited. Finally, the neuronal activity could influence protein transport. It has been shown that β-gal-TTC requires neuronal activity to be internalised and transported to the neuromuscular junction (Miana-Mena et al. 2002). This suggests that synaptic activity is required for transneural transport of β-gal-TTC in the CNS. Therefore, the hybrid protein would only detect active neuronal circuits (i.e., no labeling is found in the inferior olive, which is crossed by XIIn but is not connected to the XIIMNs). The specificity of the activity could also explain why there are fewer structures labeled by β-gal-TTC than by neurotrophic viruses.

Currently, the most efficient transsynaptic method (in terms of the extent of transport and sensitivity of detection) involves neurotropic viruses (Kelly and Strick 2000, Kobbert et al. 2000, Norgren and Lehman 1998, Ugolini 1995a, Ugolini 1995b). These methods are very sensitive because each time a virus infects a new cell it replicates and amplifies the signal to help visualise higher order neurons in a chain. The disadvantage of viral methods is their potential toxicity. The development and institutionalisation of appropriate biosafety and animal care protocols are primary concerns when designing experiments with these neurotropic viruses (Kelly and Strick 2000). Even the relatively innocuous rabies virus (Ugolini 1995a), falls into the "continuous risk" category because it is often present in high concentrations can be contracted by bites or *via* aerosol (Kelly and Strick 2000). The animals also often become increasingly ill after viral injection and die within one week (Norgren and Lehman 1998).

TTC is a non-toxic fragment of the tetanus toxin responsible for retrograde transsynaptic transport of the native molecule, without causing symptoms (Bizzini et al. 1977). However, some authors (Gill 1982) have pointed out that the presence of small quantities of intact tetanus toxin (that appear when TTC is obtained by papain digestion of the protein) could still produce tetanus, which would be a serious inconvenience. We have eluded this potential problem by using a recombinant β-gal-TTC molecule. It was obtained in the laboratory without the intact tetanus toxin and, as a result, none of the animals developed tetanus.

Finally, since most tracers are not electron dense, they are not very useful in ultrastructural analysis and require labour- and time-consuming post-staining processing (Lanciego and Wouterfood 2000). Fortunately, β-gal-TTC produces an electrondense precipitate which can be detection directly by electron microscopy (Bonnerot et al. 1987).

CONCLUSION

In summary, we propose that the genetic fusion of the fragment C tetanus toxin and β-galactosidase enzyme could be a powerful retrograde tracer to analyse neuronal pathways after intramuscular injection. This method is not toxic and the transport and transsynaptic passage is quite fast. Moreover, since it can be detected directly by means of enzymatic activity, it can be used in electron microscopy studies. Finally, this method could also be used to map active neuronal networks since the transport may depend on neuronal activity.

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