Pergamon

Biochemical Pharmacology, Vol. 47, No. 1, pp. 53–62, 1994.

0006-2952/94 $6.00 + 0.00

RETROGRADE AXONAL TRANSPORT OF NEUROTENSIN IN RAT NIGROSTRIATAL DOPAMINERGIC NEURONS

MODULATION DURING AGEING AND POSSIBLE PHYSIOLOGICAL ROLE

MARIE-NOËLLE CASTEL,*† ALAIN BEAUDET‡ and PIERRE M. LADURON§

*Laboratoire de Génétique Moléculaire de la Neurotransmission et des Processus Neurodégénératifs (LGN), CNRS, 1 Avenue de la Terrasse, 91198 Gif-sur-Yvette Cédex, France,
‡Neuroanatomy Laboratory, Montreal Neurological Institute, McGill University, 3801 University Street, Montreal, Quebec, Canada H3A 2B4, and
§Laboratoire de Neurochimie et Service de Neurobiologie, Université Catholique de Louvain, 10 Avenue Hippocrate, B 1200 Brussels, Belgium

Abstract—Biochemical and anatomical data are reported which demonstrate for the first time the existence of a retrograde axonal transport process for a neuropeptide, neurotensin, in rat brain. Neurotensin receptors are mainly located in the striatum on nerve terminals of the nigrostriatal dopaminergic pathway. Thus, the association of specific neurotensin receptors on a well defined pathway provides an excellent model to investigate the existence of such a process. Two hours after the intrastriatal injection of iodinated neurotensin, radioactivity started to accumulate in the ipsilateral substantia nigra. The levels were maximal during the fourth hour. The appearance of this labelling was prevented by injection of a large excess of unlabelled neurotensin or of neurotensin 8–13, an active neurotensin fragment, but not by neurotensin 1–8 which had no affinity for neurotensin receptors. These results suggest that the appearance of radioactivity in the ipsilateral substantia nigra was dependent on the initial binding of this peptide to its receptors in the striatum. HPLC studies demonstrated that the radioactivity found in the substantia nigra corresponded to intact neurotensin and to degradation products of this peptide. Moreover, it has been shown that this retrograde transport was microtubule-dependent and occurred in dopaminergic nigrostriatal neurons. Light and electron microscopic data confirmed and extended the present results. Four and a half hours after intrastriatal injection of iodinated neurotensin, silver grains were mainly detected in dopaminergic perikarya of the substantia nigra pars compacta. The vast majority were associated with neuronal elements and their localization within cell bodies suggests that retrogradely transported neurotensin may be processed along a variety of intracellular pathways including those mediating recycling in the rough endoplasmic reticulum and degradation in lysosomes. However, the presence of silver grains over the nucleus, as well as the increase in tyrosine-hydroxylase mRNA expression in the ipsilateral substantia nigra 4 hr after intrastriatal injection of neurotensin support the concept that neurotensin alone, or associated with its receptor, might be involved in the regulation of gene expression. Finally, we have demonstrated that in old rats the quantity of retrogradely transported neurotensin was significantly decreased as compared to that observed in young adult rats. This retrograde axonal transport of a neuropeptide may represent, as already suggested for growth factors, an important dynamic process conveying information from nerve terminals to the cell body.

The machinery necessary for protein synthesis, i.e. ribosomes and endoplasmic reticulum is only present in cell bodies and dendrites of neurons. The distances between the perikaryon and nerve terminals (e.g. ~1 m in the human sciatic nerve) require the existence of a special transport system to bring newly formed membrane and secretory products from the Golgi apparatus to the end of the axon. Fast anterograde axonal transport allows molecules synthetized within cell bodies and dendrites to be transported to the nerve terminals at a rate of about 400 mm/day [1].

Ligature experiments in peripheral nerves have demonstrated that rapid transport also occurs in the retrograde direction from nerve endings towards the cell body (see Ref. 2). Receptors, trophic factors and toxins can be retrogradely transported [1, 2]. The rate of this transport is about one-half to two-thirds that of fast anterograde transport and is dependent on the integrity of microtubules [1]. This retrograde transport mainly seems to have a scavenger function [3]. However, the study of growth factors, particularly the NGF‖ suggests that this retrograde transport plays an important role during development in informing the perikaryon about events that occur at the distant ends of axons. Moreover, it has been shown in rat brain that NGF can be retrogradely transported in cholinergic neurons of the basal forebrain after specific binding to presynaptic NGF receptors [4]. In Alzheimer's disease, these neurons markedly degenerate. Since administration of NGF prevents the death of these

† Corresponding author. Tel. (33) 1 69 82 36 52; FAX (33) 1 69 82 35 80.

‖ Abbreviations: NT, neurotensin; DA, dopamine; TH, tyrosine hydroxylase; NGF, nerve growth factor; SN, substantia nigra; SNc, substantia nigra pars compacta.

Table 1. Radioactivity accumulated in different cerebral areas 4 hr after the intrastriatal injection of iodinated NT (0.16 pmol) alone or in association with a 1000-fold excess of unlabelled NT or NT fragments (fragments 1–8 and 8–13)

	^{125}I-NT	^{125}I-NT + NT	^{125}I-NT + NT 8–13	^{125}I-NT + NT 1–8
Ipsilateral SN	360 ± 25	0	0	357 ± 78
Contralateral SN	0	0	0	0
Cerebellum	0.6 ± 0.2	0.6 ± 0.05	0.3 ± 0.07	0.4 ± 0.07
Ipsilateral Hippoc.	13 ± 3	15 ± 5	16 ± 2	14 ± 5

Results are expressed in dpm/mg of tissue ± SEM (N = 5–10). Hippoc., hippocampus.

neurons in experimental models [5–7], one hypothesis is that reduced amounts of NGF or other trophic factors in the brain may be one important element in the progression of this degenerative disease.

Numerous studies have recently demonstrated that neuropeptides could play an important role in trophic processes (see Ref. 8). Since the retrograde transport of NGF is thought to be essential to its trophic action, we investigated if neuropeptides could also be retrogradely transported in specific neurons and subsequently induce alterations in gene expression after arrival in the cell body. A serious candidate to provide the evidence of such a transport was NT. NT is a tridecapeptide first isolated and sequenced from bovine hypothalami [9, 10]. More recently, the gene encoding NT was isolated in rat [11] and immunohistochemical studies have demonstrated that NT is widely and heterogeneously distributed in mammalian (including human) central nervous system [12–17]. NT fulfills many neurotransmitter criteria [18]. More particularly, specific and high-affinity NT receptors are present in several brain regions, especially in areas containing DA cell bodies and terminals [19]. In rat striatum, it has been demonstrated that these NT receptors are mainly located presynaptically on nerve terminals of the DA nigrostriatal pathway [19–22] which originates from SNc and which is markedly degenerated in Parkinson's disease. Thus, the existence of specific NT receptors on nerve terminals of a well defined pathway provides an excellent model to study the existence of a retrograde transport process for a neuropeptide. We also investigated the alterations of this process during ageing and tried to gain a better understanding of the physiological role of this retrograde transport of NT.

Biochemical evidence for the retrograde axonal transport of NT in the DA nigrostriatal pathway

Young adult male Sprague–Dawley rats (2–3 months old) were injected into the right striatum with monoiodo-Tyr3 NT (0.16 pmol, Amersham International, Amersham, U.K.) after pretreatment with a peptidase inhibitor, kelatorphan (30 μg) (for more details, see Ref. 23). Four hours later they were killed and the radioactivity accumulated in different brain areas was measured using a LKB gamma counter.

A substantial accumulation of radioactivity was detected in the ipsilateral SN whereas no labelling was observed in the contralateral one (Table 1). Other cerebral structures such as the cerebellum or hippocampus were also labelled (Table 1). This accumulation of radioactivity in the ipsilateral SN was totally prevented by a 1000-fold excess of the non-radioactive NT peptide co-injected with iodinated NT (Table 1), suggesting that the appearance of radioactivity in this structure, 4 hr after the intrastriatal injection of iodinated NT, is dependent on the initial binding of NT to its receptors located on striatal DA terminals. This was confirmed by the co-injection of iodinated NT with a 1000-fold excess of the fragment 8–13 of NT (Table 1). This NT fragment could bind to NT receptors with the same or even a higher affinity than NT [24–29]. Conversely, when iodinated NT was co-administered with the fragment 1–8 of NT which had no affinity for NT receptors, levels of radioactivity detected in the ipsilateral SN were similar to those reported after injection of iodinated NT alone (Table 1). The cerebellum and the hippocampus remained labelled whatever the treatment (Table 1), indicating that

Table 2. Effect of neurotoxic treatments on the labelling observed in different brain areas 4 hr after the intrastriatal injection of iodinated NT (0.16 pmol)

	^{125}I-NT	^{125}I-NT + 6-OH-DA	^{125}I-NT + colchicine
Ipsilateral SN	360 ± 25	0	0
Contralateral SN	0	0	0
Cerebellum	0.6 ± 0.2	0.2 ± 0.02	0.8 ± 0.2
Ipsilateral Hippoc.	13 ± 3	5 ± 2	15 ± 5

Results are expressed in dpm/mg of tissue ± SEM (N = 5–10). 6-OH-DA, 6-hydroxydopamine.

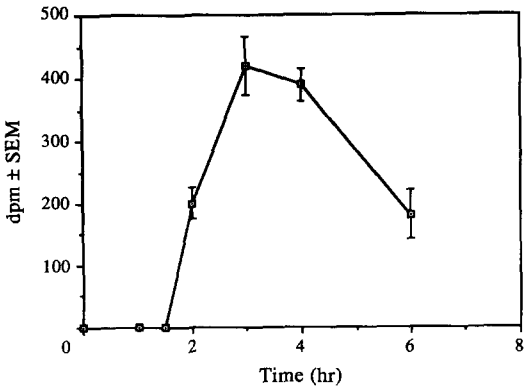

Fig. 1. Time-course of appearance of radioactivity in the ipsilateral SN after the intrastriatal injection of iodinated NT. Animals were killed immediately, 1, 1.5, 2, 3, 4 or 6 hr after the intrastriatal injection of iodinated NT. Results are expressed as mean dpm ± SEM (N = 4–10).

the accumulation of radioactivity in these structures was unrelated to NT receptors.

It has been shown that this retrograde transport of iodinated NT occurred within the DA nigrostriatal pathway [23], since no radioactivity was observed within the ipsilateral SN 4 hr after the intrastriatal injection of iodinated NT in rats pretreated with 6-hydroxydopamine into the medial forebrain bundle 3 weeks before (Table 2).

Subsequently, it has been demonstrated that this transport fulfilled the criteria of a classical fast axonal transport [1, 23]. First, animals were killed at different times after the intrastriatal injection of iodinated NT (immediately, or 1, 1.5, 2, 3, 4, 6 or 24 hr after) and it has been observed that the radioactivity started to accumulate in the ipsilateral SN after about 2 hr, with the highest levels detected during the fourth hour (Fig. 1). No labelling was observed after 24 hr (data not shown). This delayed appearance of labelling in the SN is in agreement with the velocity usually described for a fast retrograde axonal transport [1]. Moreover, the pretreatment of animals with colchicine, a molecule which blocks axonal transport by depolymerizing microtubules [30], 30 hr before the intrastriatal injection of iodinated NT totally prevented the appearance of radioactivity in the ipsilateral SN (Table 2). This suggests that the retrograde axonal transport of NT was dependent on the integrity of microtubules. Conversely, the labelling observed in the cerebellum and the hippocampus was detectable very quickly after the intrastriatal injection of NT and was not altered by colchicine (Table 2), indicating the existence of a diffusion phenomenon in these areas.

The last step of this biochemical characterization of the retrograde axonal transport of NT was to identify the nature of the radioactive material found in the ipsilateral SN after transport [31]. HPLC studies were performed from SNs of rats killed 2.5 or 4 hr after the intrastriatal injection of iodinated NT in two different systems of elution (for more

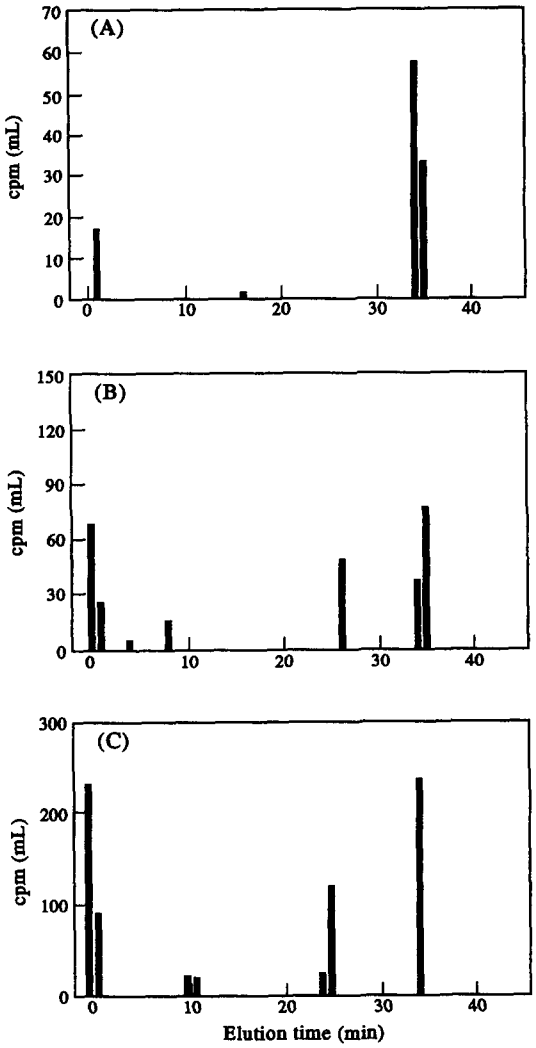

Fig. 2. Radioactivity profile of HPLC reverse-phase chromatograms performed in system 1 (see the text). (A) Radioactivity profile of monoiodo-Tyr 3 NT sample from Amersham used as reference. (B) HPLC profile of SN extract obtained 2.5 hr after the intrastriatal injection of iodinated NT. (C) HPLC profile of SN extract obtained 4 hr after the intrastriatal injection of iodinated NT. Results are expressed in cpm/mL.

details, see Ref. 31). Monoiodo-Tyr3 NT from Amersham was used as a reference. When the reference sample was analysed by HPLC, two peaks were observed in the first system (Eluent A: ammonium acetate 20 mM, pH 6.8; Eluent B: acetonitrile with ammonium acetate 100 mM, pH 6.8; 80:20). The first peak appeared in the unretained fraction (Fig. 2A) and may likely be free iodine according to Amersham specifications. Thus, one may conclude that the second peak which was the major one and had in this system a retention time of 34–35 min corresponded to intact NT (Fig. 2A). The chromatographic profile of the radioactivity performed from SN of animals killed 2.5 (Fig. 2B)

or 4 hr (Fig. 2C) after the intrastriatal injection of iodinated NT exhibited a peak with the same retention time as the NT reference sample. This observation was confirmed in a second system of elution (Eluent A: trifluoroacetic acid 0.07%; Eluent B: trifluoroacetic acid 0.07% in acetonitrile; data not shown) demonstrating that intact iodinated NT was transported retrogradely from the striatum towards the ipsilateral SN. The presence of other peaks with shorter retention times in both systems of elution as well as the smaller percentage of radioactivity eluted at 34–35 min from samples of rats killed after 4 hr (Fig. 2C; 36% of the total recovered radioactivity) as compared to those of animals killed after 2.5 hr (Fig. 2B, 44%) suggest that retrogradely transported intact NT may be partly degraded within the SN.

All these results indicate that NT injected into the striatum can selectively bind to its receptors located on DA terminals. Then, the ligand–receptor complex can be internalized and this has since been confirmed by *in vitro* studies performed on cell cultures [32, 33]. In a third step, the ligand associated or not with its receptor can be retrogradely transported to the cell body. However, the studies described above did not give us any information concerning the intracellular pathways following the arrival of NT in the SN, even if our HPLC studies suggest that at least a part of this intact iodinated NT was degraded in the cell body. In order to try to answer this question, the topographical and cellular localization of iodinated NT in the SN after its transport was investigated using both light and electron microscopic autoradiography [34].

Anatomical localization of retrogradely transported NT in the DA cell bodies

In animals killed 4.5 hr after the intrastriatal injection of iodinated NT, distinct accumulations of silver grains were apparent over neuronal cell bodies and intervening processes within the ipsilateral SN, the adjacent ventral tegmental area and further caudally in the retrorubral field [34]. Labelled neurons in each of these regions displayed a pattern of distribution similar to that demonstrated previously for DA mesostriatal neurons [35, 36], confirming that the retrograde transport of NT occurred in DA neurons. No labelling was detected on the contralateral side [34].

Light microscopic examination of semi-thin sections of the SNc 4.5 hr after the intrastriatal injection of iodinated NT revealed distinct accumulations of silver grains over a sub-population of neuronal cell bodies (Fig. 3a). These perikarya were fusiform, round, oval or multipolar in shape and some of them displayed a large nucleus with a prominent, centrally located nucleolus. Silver grains were mainly associated with the perinuclear cytoplasm, but a small proportion were also detected over the nucleus (Fig. 3a).

Electron microscopic analysis of autoradiographic labelling of the SNc confirmed and extended the light microscopic results. Quantitative evaluation of the electron microscopic data was performed using a modification of the 50% resolution circle method of Williams (for more details, see Ref. 34), which allows us to assign the autoradiographic labelling to specific cellular sources. Thus, 85% of silver grains sampled from the SNc (about 2100 grains counted) were associated with neuronal profiles (Fig. 4). A high percentage of this total number of grains was detected over myelinated and un-myelinated axons (Fig. 4) supporting the concept of an intra-axonal transport of NT between the striatum and the SN. Approximately, 13% of silver grains were observed over neuronal cell bodies where they were mainly overlying the cytoplasm (Fig. 4). A large proportion of these intracytoplasmic grains were detected over clearly identifiable organelles, particularly the rough endoplasmic reticulum and mitochondria and to a lesser extent in lysosomes, Golgi apparatus or multivesicular bodies (Fig. 3b). Sixteen per cent of the number of silver grains counted in cell bodies (or 2% of the total number of silver grains) were located over the nucleus (Figs 3b and 4), confirming the observations done on semi-thin sections and they were identified directly over or next to accumulations of heterochromatin (Fig. 3b). This association of NT with the nucleus may explain the relatively low degree of degradation of NT. Indeed, it has previously been demonstrated *in vitro* that different growth factors could after internalization be translocated to the nucleus where they bound to the chromatin and remained as a non-degraded form [37, 38]. Finally, almost 35% of silver grains were associated with dendritic profiles (Fig. 4). A similar localization has already been reported for NGF in the peripheral sympathetic nervous system [39]. In the case of NT, only 37% of the dendritic labelling (about 13% of the total number of grains counted) were intracytoplasmic and the majority of dendritic silver grains were detected over the plasma membrane (Fig. 4). However, the absence of any preferential localization apposite to any of the abutting structures makes the presence of silver grains within dendrites difficult to interpret.

These anatomical results together with our HPLC data lead us to propose a model for the sequence of intracellular events following the arrival of iodinated NT in DA cell bodies which is very close to that usually described after the endocytosis of receptor–ligand complexes [40, 41]. Subsequent to the internalization of iodinated NT in the striatum, the peptide alone or in association with its receptor can be transported within vesicles to DA cell bodies. Then, the vesicles may ultimately enter an acidic compartment equivalent to the endosome and the ligand–receptor complex can undergo dissociation into separate ligand and receptor components. Based on our anatomical observations, iodinated NT may be processed along a variety of intracellular pathways including those mediating degradation in lysosomes, and recycling in rough endoplasmic reticulum. However, the association of silver grains with the nucleus suggests that the retrograde axonal transport of NT may play an important role in conveying information from the nerve terminals to the cell body and subsequently generating alterations in gene expression as a response to distal synaptic events. To test this hypothesis, we investigated in a third part how NT can modulate in DA cell bodies of the SNc the expression of tyrosine hydroxylase (TH)

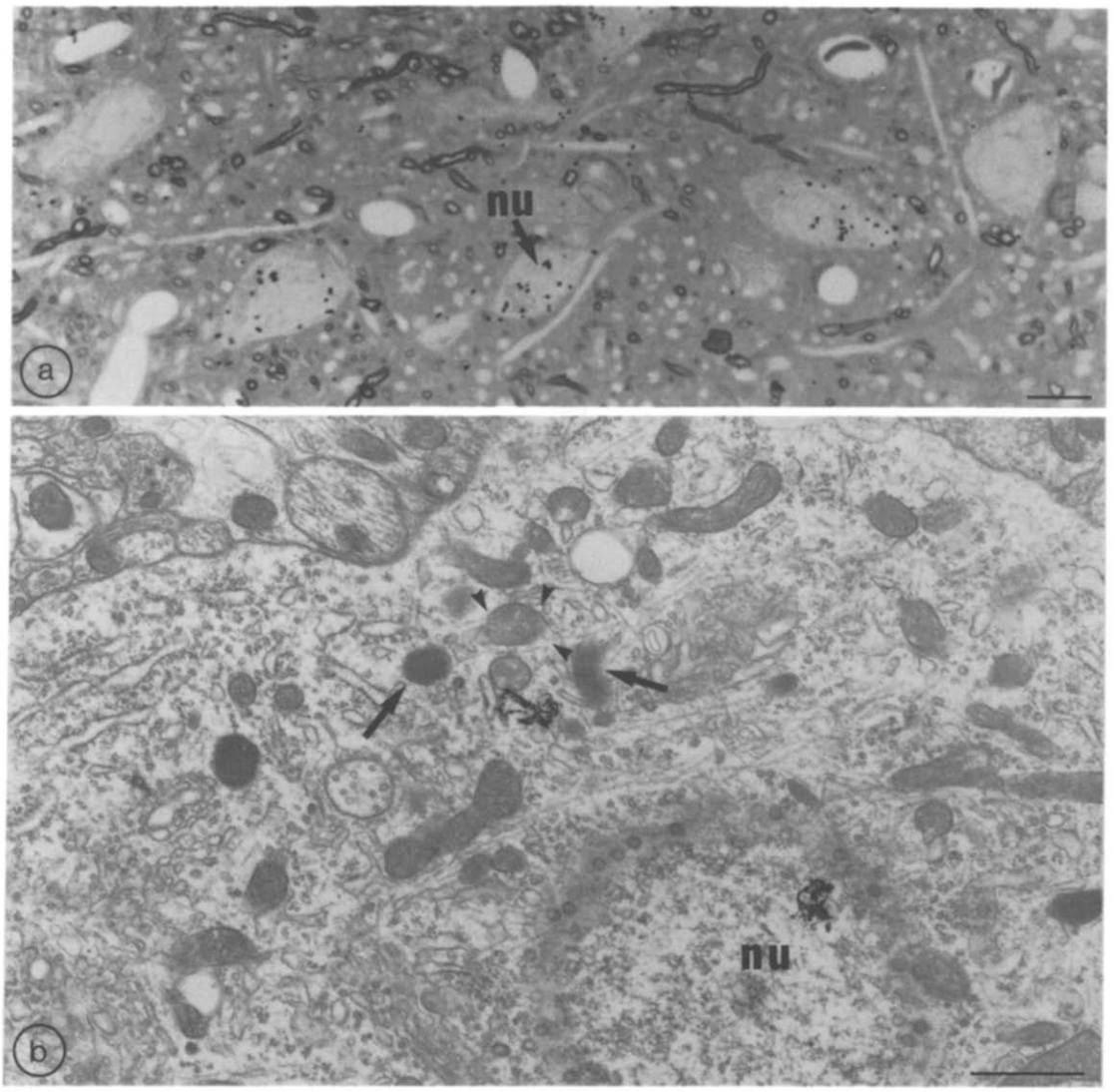

Fig. 3. Distribution of retrogradely transported radioactivity in semi-thin (a, 1 μm) and ultra-thin (b, 80 nm) sections through the ipsilateral SNc processed 4.5 hr after the intrastriatal injection of iodinated NT. (a) Silver grains are mainly concentrated over the perinuclear cytoplasm of neuronal cell bodies. Some are also apparent over nuclei (nu) and stray grains are detected over the neuropil including myelinated fibre bundles. Scale bar = 10 μm. (b) Cross-section of a neuronal cell body displaying autoradiographic labelling over the nucleus (nu) and in the vicinity of a mitochondrion (arrowhead) and vesicular elements (arrows). Scale bars: 1 μm.

mRNA, the step-limiting enzyme of catecholaminergic neurons [42].

Effect of intrastriatal injection of NT on the expression of TH mRNA in the SNc

In a first step of the experiments, adult male Sprague–Dawley rats were simultaneously injected with unlabelled NT (16 nmol), into one striatum, and with saline into the other one, after a pretreatment with kelatorphan. In this way, each animal could act as its own control excluding the possibility that the alterations in TH mRNA levels resulted from the influence of external factors. The animals were killed 4 hr later and their brains rapidly removed and frozen in isopentane at −25°. Ten micrometre thick mesencephalic sections were hybridized with the pEM-TH19 oligonucleotide probe [43].

TH mRNA-expressing cells were virtually detected over the SNc and the adjacent ventral tegmental area. A more intense labelling was observed on the side injected with NT than on the control side [42]. The same observation was done when NT was replaced by the active fragment 8–13, whereas the other striatum was injected with the inactive fragment 1–8 instead of saline. In order to quantify more precisely the differences between the right and the left SN, grain counting was carried out using a

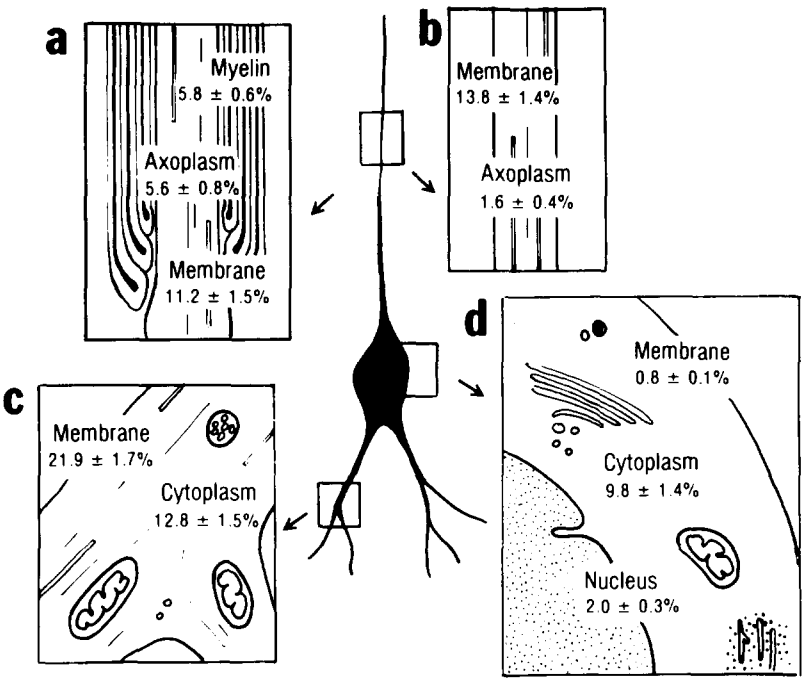

Fig. 4. Schematic drawing showing the repartition of retrogradely-transported radioactivity 4.5 hr after the intrastriatal injection of iodinated NT. Ultra-thin sections of four different animals were systematically scanned using the electron microscope and every silver grain or group of silver grains photographed yielding a sampling of about 2100 silver grains whose cellular distribution was documented following visual analysis of the electron micrographs. Results are expressed as a percentage of the total number of grains counted. (a, b) Axonal labelling. Silver grains were detected over both myelinated (a) and unmyelinated (b) axonal profiles. They were mainly associated with plasma membrane. (c) Dendritic labelling. Most of the dentritic grains were associated with the plasma membrane. However, these membrane-associated grains displayed no preferential localization apposite to any one of the abutting structures. (d) Perikarya labelling. Silver grains predominantly overlaid the cytoplasm where they were often detected over intracellular organelles including rough endoplasmic reticulum, Golgi apparatus, mitochondria and lysosomes. Some labelling was also observed over the nucleus.

computer image analysing system (HISTO 2000 by Biocom, France). Quantification was performed in a rectangle of 1 mm^2 positioned on identical areas of each SN on the same slice (for more details, see Ref. 42). Four hours after the intrastriatal injections, a highly significant increase in the number of cells expressing a detectable level of TH mRNA (+39%) was detected in the SNc ipsilateral to NT-injected striatum as compared to the saline-injected side (Fig. 5). Similarly, significant differences were observed in the SNc when rats received the active NT fragment (NT 8–13; +39%) in one side and saline in the other one (Fig. 5), or when animals were injected with NT versus the inactive NT fragment (NT 1–8; +43%) (Fig. 5). Conversely, no difference in TH mRNA levels was observed when animals received NT 1–8 versus saline (Fig. 5).

These results indicate that NT injected into the striatum is able to increase the number of cells expressing a detectable level of TH mRNA in the ipsilateral SNc. Since a majority of cells contained in the SNc are dopaminergic [44], a possible explanation for our results is that NT is able to increase the TH mRNA expression in cells where no labelling was seen in control experiments probably due to their too low levels of TH mRNA. However, no significant alterations in the mean grain density over these positive cells were observed in the SNc ipsilateral to NT injection as compared to the control side (data not shown). This suggests that NT may only act on a sub-population of DA cells. Moreover, the experiments performed with the different NT fragments clearly show that NT receptors are involved in the increase of TH mRNA levels induced by NT.

In a second set of experiments, we investigated if this effect of NT on TH mRNA levels coincides with the presence of the peptide in the SNc after its retrograde transport. Thus, animals were killed 1, 4 or 16 hr after the injection of NT into one striatum and of saline into the other one, and levels of TH mRNA were measured in both SNs for these three time-points.

No difference was observed between the two SNs at 1 and 16 hr post-injection (Fig. 6). Conversely, the up-regulation of TH mRNA levels was confirmed at 4 hr (Fig. 6). These results suggest that the presence of NT in the SNc may explain the modifications observed on the nigral TH mRNA expression. However, further experiments will be

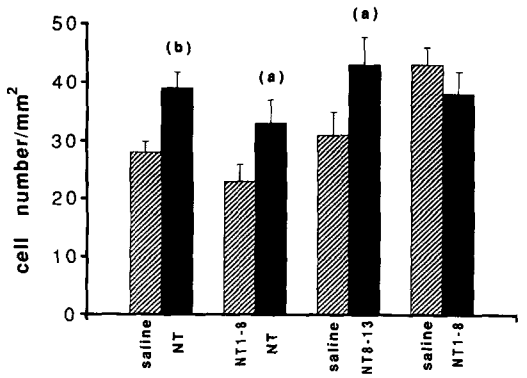

Fig. 5. Effect of simultaneous intrastriatal injections of NT and NT fragments or saline on TH mRNA expression in the SN. The results are expressed in terms of positive cell number/mm² ± SEM. Data were analysed using the Student's *t*-test for paired values (a: $P < 0.05$; b: $P < 0.001$). A significant increase in the number of cells expressing a detectable level of TH mRNA was observed in the SNc ipsilateral to the injection of NT or the active NT fragment 8–13 as compared to the saline or the inactive NT fragment 1–8 injected side. Conversely, no difference was seen when rats were injected with saline into one striatum and NT 1–8 into the other.

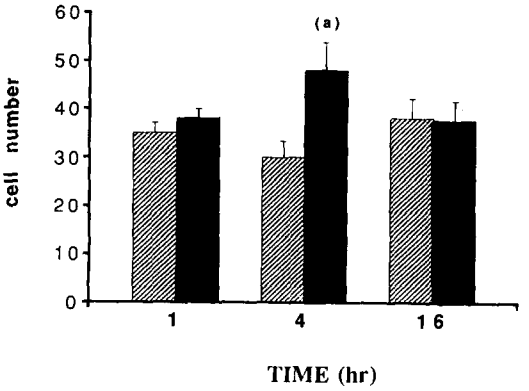

Fig. 6. Time-course of NT effect on TH mRNA expressing neurons. Results are expressed in terms of number of cells expressing a detectable level of TH mRNA in SNc of rats killed 1, 4 and 16 hr after the injection of NT into one striatum (black bars) and saline into the other one (hatched bars). A significant increase in the number of TH mRNA cells was observed only for the 4 hr time-point (a: $P < 0.001$, Student's *t*-test for paired values).

necessary to determine exactly the mechanism of action of NT on TH mRNA expression. Many hypotheses can be proposed. First, NT can reach the cell body and, after dissociation from its receptor, it may bind to specific receptors located on the nuclear membrane. The evidence of high-affinity NT binding sites detected over the nuclei of midbrain neurons [45], and more notably, those of cholinergic neurons comprising the rat nucleus basalis of Meynert [46] supports this hypothesis. A nuclear translocation of the ligand–receptor complex cannot be ruled out, since NT receptors possess a nuclear localization signal (NLS) which has been shown to be necessary for the passage of large proteins through nuclear pores (see Ref. 47). Then, the complex ligand–receptor may enter the nucleus and may bind to specific nuclear binding proteins to alter the expression of different genes, including TH. This mechanism of nuclear translocation of occupied cell surface receptors has been proposed to explain some of the long-term effects of insulin [48]. Of course, one cannot exclude the possibility that the effect of NT on TH mRNA expression is not direct, but is mediated through another messenger molecule generated by the activation of NT receptor. Thus, a likely explanation may be that the activation of NT receptors by NT can promote the induction of some immediate-early genes in the SNc, as it has been observed for NGF on PC12 cell cultures, which may subsequently modify the expression of TH mRNA (see Ref. 49). Further experiments will also be necessary to see if this transient effect of NT on TH mRNA expression is followed by an increase in TH protein levels and in the enzymatic activity. The use of mutant NT receptors, as has been previously done for interleukin receptors [49] may perhaps be an interesting approach to determine how NT receptor can modulate gene expression.

Finally, we will report results concerning the alterations of the retrograde axonal transport of NT during ageing [49].

Retrograde axonal transport of NT in old rats

Young (2–3 months) and old (20–23 months) rats were injected into the striatum with iodinated NT (0.16 pmol) and were killed 1, 2, 3, 4 or 6 hr after. Radioactivity was measured in SNc in a LKB gamma counter.

The quantity of radioactivity detected in the ipsilateral SN of young adult rats as well as the time-course of accumulation were similar to those described above (Fig. 7). The radioactivity started to accumulate after about 2 hr and was maximal at 4 hr, decreasing at 6 hr (Fig. 7). In old rats the time-course was similar to that observed in young animals, but a decrease in the levels of radioactivity was seen for each time-point. This reduction was significant for the third and the fourth hour (about 40% decrease; $P < 0.05$; Wilcoxon's test, Fig. 6).

These results indicate that the retrograde axonal transport of NT is altered during ageing. These modifications concerned the quantity of the retrogradely transported neurotensin, but the rate of this transport remains unchanged. Numerous experiments have demonstrated that fast anterograde axonal transport can be altered by ageing, or by a process associated with senescence, in both peripheral and central nervous systems [51–55]. These studies revealed a reduction in the amount and/or rate of molecules transported. However, the mechanism which is responsible for these modifications of the transport during ageing has not yet been elucidated. For instance, it has been demonstrated that acetylcholinesterase exists in four major molecular forms in mammalian and bird peripheral nerves; the

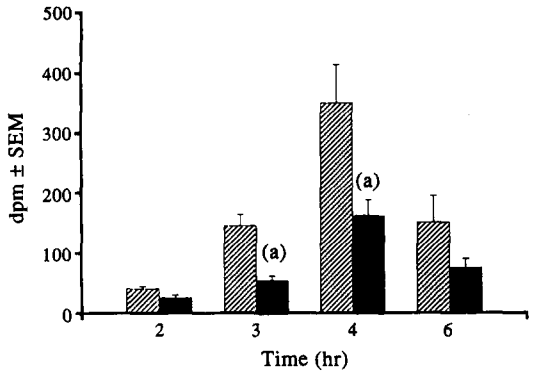

Fig. 7. Radioactivity measured in the SN 2, 3, 4 and 6 hr after the intrastriatal injection of iodinated NT in young adult (hatched bars) and old rats (black bars). Results are expressed as dpm ± SEM. A significant decrease in the radioactivity detected in the SN of old rats was observed compared to young adult animals during the third and fourth hours. (a: $P < 0.05$, Wilcoxon's test).

A12 and G4 form being components of the fast phase of axonal transport and the G1 and G2 being conveyed more slowly [52]. In old rats, the rapid axonal transport of the G4 form was decreased, whereas no modification was found for the A12 form, suggesting that the alteration of fast axonal transport during ageing is not a general phenomenon [52]. In the case of the retrograde axonal transport for NT, different explanations may account for the modifications observed in old rats. Since this retrograde transport of NT first involves the binding of the peptide to its receptors, the reduction in the retrogradely transported radioactivity in old rats could reflect a decrease in the number of NT receptors during ageing. In fact, Govoni *et al.* [56] have shown a 20% reduction in the number of NT receptors during senescence. On the other hand, these NT receptors mediating the retrograde axonal transport of NT are located on DA terminals and the modifications observed in old rats may be due to a reduction of DA neurons during ageing. However, there is as yet no clear evidence for the disappearance of DA neurons in old rats, even if there are some arguments supporting this hypothesis. For instance, Scherman *et al.* [57] demonstrated a significant decrease in the binding of tetrabenazine, a specific ligand of the vesicular monoamine transporter, in the caudate-putamen of senescent human brain, suggesting an age-dependent reduction in striatal DA innervation. It has recently been demonstrated that the retrograde transport of reserpine, another marker for monoamine vesicles was reduced during senescence [58]. Morphological changes in DA neurons, such as in sciatic nerves of old rats [52] may also be involved in this reduction of transport during ageing. However, this explanation seems unlikely, since in this case, one may expect a decrease in the rate of the transport possibly together with a reduced quantity of retrogradely transported material.

Conclusion

Our results clearly demonstrate that a neuropeptide, NT can be retrogradely transported in DA nigrostriatal neurons. This transport first involves the binding of NT to its receptors presynaptically located on DA nerve terminals of the nigrostriatal pathway. Then, the ligand–receptor complex can be rapidly internalized, as demonstrated on cell cultures [32, 33]. Our experiments did not allow us to draw conclusions about the association of NT with its receptor during the transport. However, the demonstration of a bidirectional transport of NT receptors within the vagus nerve is supporting this hypothesis [59]. Electron microscopic data together with the effect of colchicine demonstrate that this transport occurs inside axons and is microtubule dependent. The fact that the ligand–receptor complex is transported inside vesicles still remains to be elucidated, since only a few silver grains were detected in the vicinity of vesicles. Nevertheless, one cannot exclude that some grains because of their size rendered difficult the observation of vesicles. Moreover, favouring the hypothesis of the involvement of vesicles in the retrograde axonal transport of NT are the recent results obtained by Beaudet *et al.* [60]. Indeed, the use of confocal microscopy allowed us to show the internalization mechanism of a fluorescent derivative of NT in different types of cell cultures, and subsequently, the presence of fluorescence within granular, vesicular profiles (Beaudet *et al.* [60]). Finally, the complex enters the perikaryon and based on our electron microscopic and HPLC studies, it seems to be processed along a variety of intracellular pathways, including degradation in lysosomes, recycling in rough endoplasmic reticulum and Golgi apparatus, but also translocation to the nucleus. The modifications of TH mRNA expression in the ipsilateral SNc following intrastriatal injection of NT, as well as the parallel time-course between this effect and the presence of NT in the SN after retrograde transport, and the observation of silver grains over the nucleus suggest that retrograde axonal transport may serve to carry information coming from nerve terminals and leading to adaptative modifications in gene expression within the cell body. A dysfunctioning in such long-distance retrograde signalling system, such as that observed in old rats [48] may have important physiological consequences during ageing or in pathologies such as Alzheimer's and Parkinson's disease.

Acknowledgements—I wish to thank Drs Woulfe, Faucher, Burgevin and Malgouris for their contribution to the different experiments reported in the present article and Drs Barthe and Kresse for their help in preparing the manuscript.

REFERENCES

1. Grafstein B and Forman DS, Intracellular transport in neurons. *Physiol Rev* **60**: 1167–1283, 1980.
2. Laduron PM, Axonal transport of neuroreceptors: possible involvement in long-term memory. *Neuroscience* **22**: 767–779, 1987.
3. Bisby MA, Functions of retrograde axonal transport. *Fed Proc* **41**: 2307–2311, 1982.

4. Seiler M and Schwab ME, Specific retrograde transport of nerve growth factor (NGF) from neocortex to nucleus basalis in the rat. *Brain Res* **300**: 33–39, 1984.
5. Williams LR, Varon S, Peterson GM, Wictorin K, Fisher W, Bjorklund A and Gage FH, Continuous infusion of nerve growth factor prevents basal forebrain neuronal death after fimbria fornix transection. *Proc Natl Acad Sci USA* **83**: 9231–9235, 1986.
6. Hefti F, Nerve growth factor promotes survival of septal cholinergic neurons after fimbrial transections. *J Neurosci* **6**: 2155–2162, 1986.
7. Kromer LF, Nerve growth factor treatment after brain injury prevents neuronal death. *Science* **235**: 214–216, 1987.
8. Hökfelt T, Neuropeptides in perspective: the last ten years. *Neuron* **7**: 867–879, 1991.
9. Carraway R and Leeman SE, The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalamus. *J Biol Chem* **248**: 6854–6861, 1973.
10. Carraway R and Leeman SE, The amino acid sequence of a hypothalamic peptide, neurotensin. *J Biol Chem* **250**: 1904–1918, 1975.
11. Kislauskis E, Bullock B, McNeil S and Dobner PR, The rat gene encoding neurotensin and neuromedin N. *J Biol Chem* **263**: 4963–4968, 1988.
12. Uhl GR and Snyder SH, Regional and subcellular distribution of brain neurotensin. *Life Sci* **19**: 1827–1832, 1977.
13. Uhl GR, Bennet JP and Snyder SH, Neurotensin, a central nervous system peptide: apparent receptor binding in brain membranes. *Brain Res* **130**: 299–313, 1977.
14. Jennes L, Stumpf W and Kalivas PW, Neurotensin: topographical distribution in rat brain by immunohistochemistry. *J Comp Neurol* **210**: 213–224, 1982.
15. Manberg PJ, Youngblood WW, Nemeroff CB, Rossor MN, Iversen LL, Prange AJJ and Kizer JS, Regional distribution of neurotensin in human brain. *J Neurochem* **38**: 1777–1780, 1982.
16. Goedert M and Emson PC, The regional distribution of NT-like immunoreactivity in centarl and peripheral tissues of the cat. *Brain Res* **272**: 291–297, 1983.
17. Kataoka K, Mizuno N and Frohman LA, Regional distribution of immunoreactive neurotensin in monkey brain. *Brain Res Bull* **4**: 57–60, 1979.
18. Kitabgi P, Neurotensin modulates dopamine neurotransmission at several levels along brain dopaminergic pathway. *Neurochem Int* **14**: 111–119, 1989.
19. Goedert M, Pittaway K and Emson PC, Neurotensin receptors in the rat striatum. *Brain Res* **299**: 164–168, 1984.
20. Quirion R, Chiueh CC and Pert A, Comparative localization of neurotensin receptors on nigrostriatal and mesolimbic dopaminergic terminals. *Brain Res* **327**: 385–389, 1985.
21. Hervé D, Tassin JP, Studler JM, Dana C, Kitabgi P, Vincent JP, Glowinski JP and Rostène W, Dopaminergic control of ^{125}I-labeled neurotensin binding site density in corticolimbic structures of rat brain. *Proc Natl Acad Sci USA* **83**: 6203–6207, 1986.
22. Schotte A, Rostène W and Laduron PM, Different subcellular localization of neurotensin-receptor and neurotensin-acceptor sites in the rat brain dopaminergic system. *J Neurochem* **50**: 1026–1031, 1988.
23. Castel M-N, Malgouris C, Blanchard J-C and Laduron PM, Retrograde axonal transport of neurotensin in the dopaminergic nigrostriatal pathway in the rat. *Neuroscience* **36**: 425–430, 1990.
24. Kitabgi P, Carraway R, Van Rietschoten J, Granier C, Morgat JL, Mendez A, Leeman S and Freychet P, Neurotensin: specific binding to synaptic membranes from rat brain. *Proc Natl Acad Sci USA* **74**: 1846–1850, 1977.
25. Rivier JE, Lazarus LH, Perrin MH and Brown MR, Neurotensin analogues: structure–activity relationships. *J Med Chem* **20**: 1409–1412, 1977.
26. Rioux F, Quirion R, Regoli D, Leblanc MA and St-Pierre S, Pharmacological characterization of neurotensin receptors in therat portal vein using analogues and fragments of neurotensin. *Eur J Pharmacol* **66**: 273–279, 1980.
27. Granier C, Van Rietschoten J, Kitabgi P, Poustis C and Freychet P, Synthesis and characterization of neurotensin analogues for structure–activity relationship studies. Acetyl-neurotensin-(8-13) is the shortest analogue with full binding and pharmacological activities. *Eur J Biochem* **124**: 117–125, 1982.
28. Mazella J, Poustis C, Labbe C, Checler F, Kitabgi P, Granier C, Von Rietschoten J and Vincent JP, Monoiodo-(Trp11) neurotensin, a highly radioactive ligand of neurotensin receptors. Preparation, biological activity and binding properties to rat brain synaptic membranes. *J Biol Chem* **258**: 3476–3481, 1983.
29. Baldino FJ, Davis LG and Wolfson B, Structure–activity studies with carboxy- and amino-terminals fragments of neurotensin on hypothalamic neurons *in vitro*. *Brain Res* **342**: 266–272, 1985.
30. Kreutzberg GW, Neuronal dynamics and axonal flow. IV. Blockade of intra-axonal enzyme transport by colchicine. *Proc Natl Acad Sci USA* **62**: 722–728, 1969.
31. Castel M-N, Faucher D, Cuiné F, Dubédat P, Boireau A and Laduron PM, Identification of intact neurotensin in the substantia nigra after its retrograde axonal transport in dopaminergic neurons. *J Neurochem* **56**: 1816–1818, 1991a.
32. Mazella J, Leonard K, Chabry J, Kitabgi P, Vincent JP and Beaudet A, Binding and internalization of iodinated neurotensin in neuronal cultures from embryonic mouse brain. *Brain Res* **564**: 249–255, 1991.
33. Vanisberg MA, Maloteaux JM, Octave JN and Laduron PM, Rapid agonist-induced decrease of neurotensin receptors from the cell surface in rat cultured neurons. *Biochem Pharmacol* **42**: 2265–2274, 1991.
34. Castel MN, Woulfe J, Wang X, Laduron PM and Beaudet A, Light and electron microscopic localization of retrogradely transported neurotensin in rat nigrostriatal dopaminergic neurons. *Neuroscience* **50**: 269–282, 1992.
35. Deutch AY, Goldstein M, Baldino FJ and Roth RH, Telencephalic projections of the A8 dopamine cell group. *Ann NY Acad Sci* **537**: 27–50, 1988.
36. Fallon JH, Leslie FM and Cone RI, Dynorphin-containing pathways in the substantia nigra and ventral tegmentum: double labeling study using combined immunofluorescence and retrograde tracing. *Neuropeptides* **5**: 457–460, 1985.
37. Rakowicz-Szulczynska EM, Rodeck U, Herlyn M and Koprowski H, Chromatin binding of epidermal growth factor, nerve growth factor and platelet-derived factor in cells bearing the appropriate surface receptors. *Proc Natl Acad Sci USA* **83**: 3728–3732, 1986.
38. Walicke PA and Baird A, Internalization and processing of basic fibroblast growth factor by neurons and astrocytes. *J Neurosci* **11**: 2249–2258, 1991.
39. Schwab M and Thoenen H, Selective trans-synaptic migration of tetanus toxin after retrograde axonal transport in peripheral sympathetic nerves: a comparison with nerve growth factor. *Brain Res* **122**: 459–474, 1977.
40. Burwen SJ and Jones AL, The association of polypeptide hormones and growth factors within the nuclei of target cells. *Trends Biochem Sci* **12**: 159–162, 1987.
41. Shepherd VL, Intracellular pathways and mechanisms of sorting in receptor-mediated endocytosis. *Trends Pharmacol Sci* **10**: 458–462, 1989.

42. Burgevin M-C, Castel M-N, Quarteronet D, Chevet T and Laduron PM, Neurotensin increases tyrosine hydroxylase messenger RNA-positive neurons in substantia nigra after retrograde axonal transport. *Neuroscience* **49**: 627–633, 1992.
43. Horellou P, Guibert B, Leviel V and Mallet J, A single RNA species injected in Xenopus oocyte directs the synthesis of active tyrosine hydroxylase. *FEBS Lett* **205**: 6–10, 1986.
44. Swanson LW, The projections of the ventral tegmental area and adjacent regions: a combined fluorescent retrograde tracer and immunofluorescence study in the rat. *Brain Res Bull* **9**: 321–353, 1982.
45. Dana C, Vial M, Leonard K, Beauregard A, Kitabgi P, Vincent JP, Rostène W and Beaudet A, Electron microscopic localization of neurotensin binding sites in the midbrain tegmentum of the rat. I. Ventral tegmental area and interfascicular nucleus. *J Neurosci* **9**: 2247–2257, 1989.
46. Szigethy E and Beaudet A, Correspondence between high affinity ^{125}I-neurotensin binding sites and dopaminergic neurons in the rat substantia nigra and ventral tegmental area: a combined radioautographic and immunohistochemical light microscopic study. *J Comp Neurol* **279**: 128–137, 1989.
47. Laduron PM, Genomic pharmacology: more intracellular sites for drug action. *Biochem Pharmacol* **44**: 1233–1247, 1992.
48. Podlecki DA, Smith RM, Kao M, Tsai P, Huecksteadt T, Brandenburg D, Lasher RS, Jarett L and Olefsky JM, Nuclear translocation of the insulin receptor. A possible mediator of insulin's long term effects. *J Biol Chem* **262**: 3362–3368, 1987.
49. Sheng M and Greenberg ME, The regulation and function of c-fos and other immediate early genes in the nervous system. *Neuron* **4**: 477–485, 1990.
50. Castel MN, Lechardeur D, Blanchard JC and Laduron PM, Reduction of the amount of neurotensin retrogradely transported in dopaminergic neurons of senescent rats. *Neurosci Lett* **124**: 111–114, 1991b.
51. Brunetti M, Miscena A, Salviati A and Gaiti A, Effect of ageing on the rate of axonal transport of cholinephosphoglycerides. *Neurochem Res* **12**: 61–65, 1987.
52. Goemaere-Vanneste J, Couraud JY, Hassig R, Di Giamberardino L and Van den Bosch de Aguilar P, Reduced axonal transport of the G4 molecular form of acetylcholinesterase in the rat sciatic nerve during aging. *J Neurochem* **51**: 1746–1754, 1988.
53. Geinisman Y, Bondareff W and Tesler A, Transport of (3H) fucose labeled glycoproteins in the septo-hippocampal pathway of young and senescent rats. *Brain Res* **125**: 182–186, 1977.
54. McMartin D and O'Connor JA, Jr, Effect of age on axoplasmic transport of cholinesterase in rat sciatic nerves. *Mech Ageing Dev* **10**: 241–248, 1979.
55. Stromska DP and Ochs S, Axoplasmic transport in aged rats. *Exp Neurol* **77**: 215–224, 1982.
56. Govoni S, Hong HS, Yang T and Costa E, Increase of neurotensin content elicited by neuroleptics in nucleus accumbens. *J Pharmacol Exp Ther* **215**: 413–417, 1980.
57. Scherman D, Denos C, Darchen F, Pollak P, Javoy-Agid F and Agid Y, Striatal dopamine deficiency in Parkinson's disease: role of aging. *Ann Neurol* **26**: 551–557, 1989.
58. Lechardeur D, Castel MN, Reibaud M, Scherman D and Laduron PM, Axonal transport of dopamine-containing vesicles labelled *in vivo* with (3H) reserpine. *Eur J Neurosci* **5**: 449–453, 1993.
59. Kessler JP and Beaudet A, Association of neurotensin binding sites with sensory and visceromotor components of the vagus nerve. *J Neurosci* **9**: 466–472, 1989.
60. Beaudet A, Mazella J, Nouel D, Chabry J, Castel M-N, Laduron P, Kitabgi P and Faure M-P, Internalization and intracellular mobilization of neurotensin in neuronal cells. *Biochem Pharmacol* **47**: 43–52, 1994.