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AXONAL TRANSPORT OF RECEPTORS: COEXISTENCE WITH NEUROTRANSMITTER AND RECYCLING

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Abstract—Receptor sites move along the axon of neurones by means of fast transport mechanisms. In rat sciatic nerves, the accumulation of muscarinic receptors above a ligature was linear up to 24 hr after the operation and exactly paralleled that of dopamine-β-hydroxylase. After 6-hydroxydopamine treatment, there was a marked reduction of these receptor sites in the rat sciatic nerve and the spleen. Fractionation of dog splenic nerves by differential and isopycnic centrifugation enabled us to show that the muscarinic receptors in sympathetic nerves are associated with noradrenaline and dopamine-β-hydroxylase in synaptic vesicles. Receptor and neurotransmitter may thus coexist in the same subcellular organelle. Opiate receptors measured *in vitro* and *in vivo* with ^{3}H-lofentanil in the rat vagus nerve were found to accumulate on both sides of the ligature. After capsaicin treatment this accumulation was markedly reduced suggesting that the opiate receptors in the vagus are mainly associated with substance P neurones. Double ligature experiments suggest the existence of a recycling phenomenon in the perikaryon for vesicles containing muscarinic receptors and dopamine-β-hydroxylase. This may represent a pathway to convey informational molecules from the extracellular to the intracellular compartment of a neurone.

The perikaryon, the cell body of neurones, is the main site of protein synthesis; indeed macromolecules are not synthesized in nerve axons or terminals. Consequently, the soma has to export a considerable number of macromolecules, but also of smaller molecules like neurotransmitters, which flow down towards the nerve endings by axoplasmic transport mechanisms.

The existence of orthograde axoplasmic transport is widely accepted; various transmitters, enzymes, labelled proteins and peptides are known to move rapidly along the axon of different nerve fibres [1–7]. Recently, muscarinic receptors were found to rapidly accumulate on both sides of a ligature in dog splenic nerves, demonstrating a bidirectional axonal transport for these receptors [8, 9]. Identical results were then obtained in cat hypogastric nerve [10] and in rat nerve [11]. Similarly a large build-up of opiate receptors was found proximal to the ligature in rat vagus when *in vitro* labelling techniques were used [12, 13]; but the accumulation appeared on both sides of the ligature when opiate receptors were labelled *in vivo* with ^{3}H-lofentanil [13]. More recently, axonal transport was also demonstrated for nicotinic receptors [14].

At the nerve terminals, most of the receptors are involved in the regulation or modulation of neurotransmitter release; so, the stimulation by acetylcholine of muscarinic receptors in sympathetic nerve endings, elicits an inhibitory effect on noradrenaline release [15]. However, macromolecules coming from outside of the neurone can be specifically taken up by receptors located at the nerve terminals, and then move retrogradely to the perikaryon. A good example of this is the nerve growth factor (NGF) which is taken up selectively by peripheral sensory and adrenergic nerve terminals and which, following retrograde transport, elicits a selective induction of tyrosine hydroxylase and dopamine-β-hydroxylase in the perikaryon [16]. Hence, in addition to the anterograde transport of informational molecules (neurotransmitter) the neurones also are capable of transferring trophic information from the effector organ to the innervating neurone.

We now present evidence that muscarinic receptors in sympathetic nerves are transported in synaptic vesicles containing noradrenaline and dopamine-β-hydroxylase. Double ligature experiments indicate a possible recycling mechanism in the perikaryon. Finally, opiate receptors in the vagus were found to be mainly associated with substance P neurones.

AXONAL TRANSPORT OF MUSCARINIC RECEPTORS IN VESICLES CONTAINING NORADRENALINE AND DOPAMINE-β-HYDROXYLASE

Since the accumulation of muscarinic receptors can be more easily measured in rat sciatic nerves than in dog splenic nerves, we decided to study the time course of the receptor accumulation on both sides of a ligature in the rat. A silk-thread was tied around both sciatic nerves of rats anesthesized with ether. At different times after the operation, 3 mm segments were removed two above (proximal to) and below (distal to) the ligature. ^{3}H-QNB was selected as ligand because of its very low non-specific binding and low absorption on the glass fibre filters.

Figure 1 shows that specific ^{3}H-QNB binding and dopamine-β-hydroxylase (DBH) increased linearly in the proximal P_1 segment of rat sciatic nerves up to 24 hr after ligature but decreased after 2 days. It is noteworthy that the DBH profile resembled exactly that of the binding sites. By contrast, the binding did not change with time in the P_2 segment located

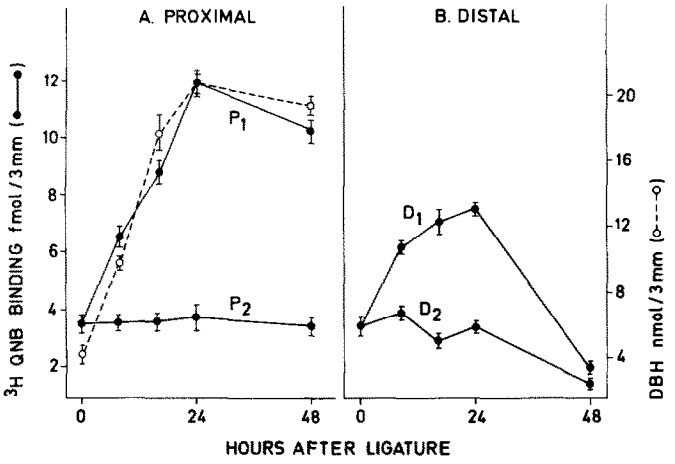

Fig. 1. Time course of the accumulation of specific ^{3}H-QNB binding on both sides of a ligature in rat sciatic nerves. At different times after the operation, 3 mm segments were removed from both sciatic nerves, two above (proximal P_1 and P_2) and two below (distal D_1 and D_2) the ligature. Note that P_1 and D_1 were directly adjacent to the silk-thread. The segments from 5 different nerves were pooled and homogenized in 3.5 ml of phosphate buffer. Binding was performed using 0.5 nM ^{3}H-QNB (spec. act. 31 Ci/mmol) as previously described [9, 17]. Specific binding was defined as the difference between total binding and binding in the presence of 2×10^{-7} M dexetimide. Dopamine-β-hydroxylase was measured according to the method of Kato *et al.* [18]. Each point represents the mean value obtained from five different pools of 5 segments (± S.E.M.).

Table 1. Inhibition of specific ^{3}H-QNB and ^{3}H-dexetimide binding in the nerves and brain

	IC_{50} (nM)			
	Rat sciatic nerves	Dog splenic nerves		Rat striatum
Substance	^{3}H-QNB	^{3}H-QNB	^{3}H-dexetimide	^{3}H-dexetin
Dexetimide	4.7	1.3	2.2	1.4
Atropine	18	2.5	6.3	6.8
Oxotremorine	1000	—	3200	6500
Carbachol	16,000	—	—	—
Levetimide	16,000	12,000	28,000	6500
Nicotine	—	—	> 100,000	> 100,000

Table 2. Effect of 6-hydroxydopamine on muscarinic receptors in the rat sciatic nerve and the spleen

	Control	6-OHDA-treated*	(%) Control
^{3}H-QNB binding fmoles/3 mm (± S.E.M., N = 5)			
Sciatic nerve (P_1 segment)			
16 hr after ligature	8.77 ± 0.45	6.67 ± 0.3	− 24
48 hr after ligature	10.25 ± 0.39	6.95 ± 0.65	− 34
^{3}H-dexetimide binding fmoles/mg protein (± S.E.M., N = 5)			
Spleen	0.145 ± 0.013	0.084 ± 0.0012	− 42

* Male Wistar rats (150–200 g) were injected i.v. with 6-hydroxydopamine bromide (200 mg/kg) the first day and 100 mg/kg the 2nd and 3rd day. Twelve days after the first injection, the sciatic nerves were ligated and the P_1 segment was removed with the spleen, 16 and 48 hr after the operation.

more proximally. In the distal D_1 segment, the ^{3}H-QNB binding increased linearly for a very short period of time and then decreased in a manner that, 2 days after ligature, the specific binding in both D_1 and D_2 segments was much lower than in unligated nerves. Interestingly, the accumulation of muscarinic receptors in the P_1 segment of dog splenic nerves was linear up to 2 days after ligature [8]. This point will be discussed further in terms of recycling phenomenon.

To characterize the ^{3}H-QNB binding in rat sciatic nerves, various drugs were tested *in vitro* using a total homogenate. Table 1 gives the IC_{50}-values of ^{3}H-QNB and ^{3}H-dexetimide binding in the rat sciatic and dog splenic nerves and in rat striatum. Dexetimide and atropine competed with both ^{3}H-ligands in the nanomolar range while levetimide and nicotine were practically inactive in the micromolar range. From these data we conclude that the ^{3}H-QNB binding sites in rat sciatic nerves are of muscarinic nature.

Now the question arises: in which types of neurones are the muscarinic receptors in the sciatic nerve located? Table 2 shows that a treatment with 6-hydroxydopamine markedly decreased the accumulation of specific ^{3}H-QNB binding in the proximal P_1 segment of rat sciatic nerves ligated 16 or 48 hr previously. Similarly, there was a marked drop in specific ^{3}H-dexetimide binding in the spleen. This suggests that a large amount of muscarinic receptors are associated with noradrenergic fibers in the rat sciatic nerve and the spleen, indicating thus a presynaptic localization. Double ligature experiments revealed nevertheless that a certain number of receptor sites did not migrate in the sciatic nerve suggesting also a postsynaptic or non-neuronal localization.

In dog splenic nerves, the rate of transport of muscarinic receptors was about 1.2 mm/hr, a value which corresponds to that previously found for noradrenaline in the same experimental conditions [19]. Our first calculations in the rat sciatic nerve, apparently provided a lower value of about 0.44 mm/hr but without taking into account the fraction of muscarinic receptors which did not migrate (see above). After correction, the rate of transport in the rat sciatic nerve became 2.7 ± 0.2 mm/hr, a value which is very close to that found for DBH (3.1 ± 0.32 mm/hr) in the same conditions. The later value is in good agreement with those reported previously by Brimijoin *et al.* [20] 4.6 mm/hr and by Oesch *et al.* [21] 1.94 mm/hr. The fact that noradrenaline and DBH flow down in the axons at the same rate as the muscarinic receptors, could suggest that both receptor and neurotransmitter are associated in the same subcellular organelle, the synaptic vesicles.

In order to determine, the intracellular localization of muscarinic receptors in sympathetic nerves, dog splenic nerves were fractionated by differential and isopycnic centrifugation. Figure 2 shows the distribution pattern of specific ^{3}H-dexetimide and ^{3}H-QNB binding in different fractions obtained by differential centrifugation; they were found to be mainly enriched in the P_3, P_4 and P_4 fractions. Interestingly, the distribution profiles of DBH, noradrenaline and 5′-nucleotidase were quite similar to that of ^{3}H-QNB and ^{3}H-dexetimide binding. In contrast, the

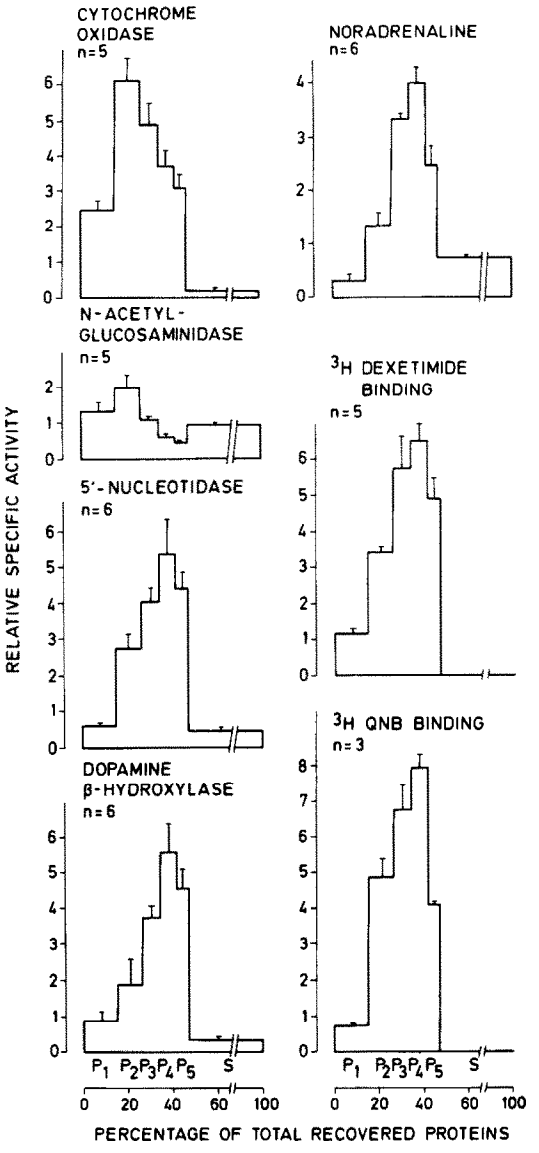

Fig. 2. Distribution patterns of ^{3}H-QNB and ^{3}H-dexetimide binding and various constituents in subcellular fractions obtained by differential centrifugation of dog splenic nerve homogenates according to Chubb *et al.* [22]. The five pellets were obtained as follows: P_1 at 6596 g_{av} for 8 min; P_2 and P_3 at 20,203 g_{av} for 15 and 30 min, respectively; P_4 and P_5 at 55,364 g_{av} for 22 and 35 min respectively. The results are presented graphically in histograms and the enzymes measured as described elsewhere [23].

cytochrome oxidase, a mitochondrial enzyme and the *N*-acetylglucosaminidase, a lysosomal enzyme, revealed a quite different pattern with a main enrichment in the first fractions. Therefore, one may certainly rule out a mitochondrial or lysosomal localization for muscarinic receptors.

Subfractionation of muscarinic receptors was carried out by submitting a particulate fraction to equilibration in sucrose density gradients. Figure 3 reveals two peaks of ^{3}H-QNB binding after isopycnic equilibration: a small one equilibrated in zones of high density (1.17), and paralleled the distribution of

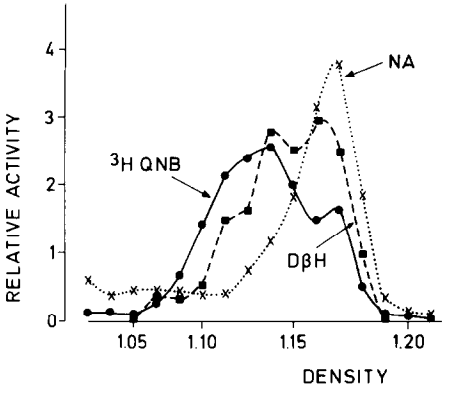

Fig. 3. Distribution of ^{3}H-QNB binding, dopamine-β-hydroxylase (DBH) and noradrenaline (NA) after isopycnic equilibration in density gradient, of a particulate fraction from dog splenic nerves. Four milliliters of a P_1-P_5 fraction (cf. Fig. 2) was layered on a 36 ml continuous sucrose gradient (0.4 → 2 M) and allowed to equilibrate in a vertical VTI rotor (Spinco centrifuge) for 1 hr at 30,000 RPM. Then 16 fractions of 2.5 ml were collected, diluted with distilled water, and ^{3}H-QNB binding dopamine-β-hydroxylase (DBH) and noradrenaline (NA) were determined in all the fractions.

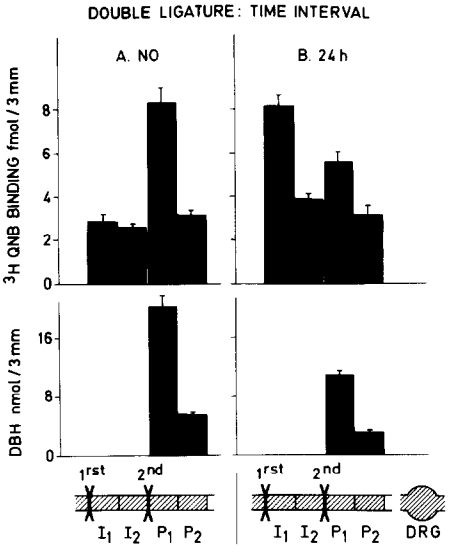

Fig. 4. Accumulation of specific ^{3}H-QNB binding and DBH in double ligature experiments performed in rat sciatic nerves. In (A) the two ligatures were placed at the same time. In (B) the time interval between the first and the second ligature was 24 hr. All the nerve segments were removed 16 hr after the last ligature. ^{3}H-QNB binding and DBH were measured as in Fig. 1. Each point is the mean of five different pools of 5 segments (± S.E.M.). DRG, dorsal root ganglion.

noradrenaline and DHB. A second peak of ^{3}H-QNB binding was recovered in areas of lower density (1.14) where only DBH was present. 5′-Nucleotidase (not shown) appeared at a lower density (1.13). These results thus reveal two types of organelles with muscarinic receptors; the peak 1.17 which also contains noradrenaline and DBH and then the peak 1.14 which is devoid of noradrenaline. The first type of particles might correspond to the "dense core vesicles" of morphologists with noradrenaline and dopamine-β-hydroxylase whereas the second ones would be the "empty vesicles" but where membrane-bound DBH is present. Therefore these results indicate that muscarinic receptors could coexist with noradrenaline and/or dopamine-β-hydroxylase in the same synaptic vesicles.

POSSIBLE RECYCLING OF MUSCARINIC RECEPTORS

Previous experiments have shown that the accumulation of opiate receptors above a ligature in the rat vagus nerve markedly decreased when the time interval between ligature and injection of labelled drug increased [13]. This was compatible with a recycling phenomenon of opiate receptors; indeed the ligature also interrupts the retrograde transport of receptors so that less receptor sites become available for recycling in the perikaryon; obviously this decrease is dependent on the rate of transport and on the distance between ligature and perikaryon. However, such a kind of experiment was not feasible for muscarinic receptors because they could not be labelled in *in vivo* conditions. To overcome this difficulty we decided to perform double ligature experiments. Figure 4 shows that the accumulation of muscarinic receptors labelled *in vitro* with ^{3}H-QNB and of DBH in the P_1 segment, thus above the second ligature, was much lower when the time interval between the two ligatures increased from zero to 24 hr. To note that DBH in the P_2 segment also was significantly decreased after a 24 hr interval when compared to the corresponding segment at time zero. Figure 5 shows the time course for the decrease of muscarinic receptor accumulation in the P_1 segment. For a time interval up to 8 hr there was no change. However, when the time interval between both ligatures exceeded 8 hr, there was a marked drop in the number of muscarinic receptors which accumulate above the second ligature. These results can be interpreted in terms of receptor recycling in the perikaryon.

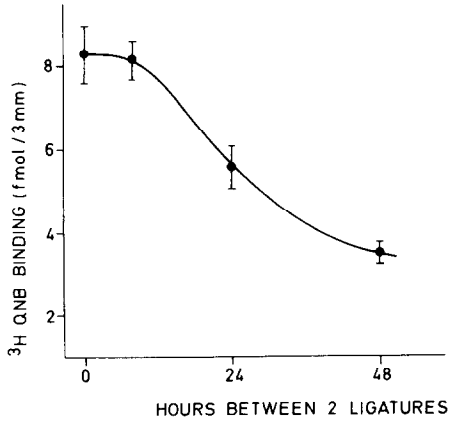

Fig. 5. Specific ^{3}H-QNB binding in the P_1 segment of ligated rat sciatic nerves (cf. Fig. 2) 16 hr after the second ligature which was placed more proximally at different time intervals after the first ligature (cf. Fig. 4).

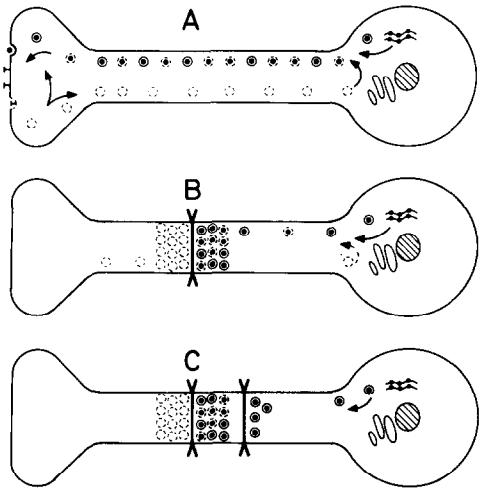

Fig. 6. Model (A) illustrating in a neurone the antero (◉) and the retrograde (○) axonal transport of vesicles bearing receptors and neurotransmitters. In order to make the model more clear, a distinction was made between newly synthesized vesicles (solid line) and vesicles recycled or to be recycled (stippled line) presumably in the perikaryon. Parts (B) and (C) illustrate the disposition and the proportion of newly synthesized and recycled receptors when one (B) or two (C) ligatures are applied on the nerve; for the latter, the time interval between each ligature should exceed 1 day. Recycling or reusing of vesicles in the nerve terminal itself cannot be ruled out.

The model presented in Fig. 6 is based on the assumption that the largest amount of vesicles bearing receptors and moving to the nerve terminal or accumulating above a ligature, originate from the receptor pool coming back from the nerve endings before being recycled in the Golgi apparatus of the perikaryon. The marked drop in muscarinic receptors that we observed above a second ligature when the time interval between the two ligatures exceeded 8 hr is compatible with such a model. As the distance between the ligature placed on the rat sciatic nerve and the perikaryon (dorsal root ganglion or spinal cord) is about 2.5–3 cm, it is obvious that 8 hr is not enough for a complete return of the vesicles flowing retrogradely above the ligature at a rate of 3 mm/hr. In contrast a time interval of 24 hr and mostly of 48 hr allows a progressive depletion in vesicles which flow back or undergo recycling in the perikaryon. Consequently if the time interval is high enough, for instance one or two days, the vesicles that are still able to accumulate above a second ligature, must be synthesized *de novo*. Supporting this view is the fact that the accumulation of muscarinic receptors in the proximal segment of dog splenic nerves was linear up to 48 hr after ligation [8] which, thus, differ from our results obtained in rat sciatic nerves (cf. Fig. 1). The reason for such a difference may be that the distance between the perikaryon (coeliac ganglion) and the ligature is much higher in dog splenic nerves than in rat sciatic nerves. In fact more time is thus available in the dog before seeing a depletion of recycled receptors.

The present results also demonstrate that the vesicles containing dopamine-β-hydroxylase in rat sciatic nerves behave in the same way as those of muscarinic receptors, giving thus more support to the idea of a coexistence of both entities in the same vesicles.

AXONAL TRANSPORT OF OPIATE RECEPTORS IN THE VAGUS NERVE

In rat vagus, an accumulation of opiate receptors was only found above the ligature when *in vitro* labelling techniques were used [12, 13] whereas the accumulation appeared on both sides when the receptors were labelled *in vivo* with ^{3}H-lofentanil [13]. The reason for such a discrepancy is not clear and was ascribed either to a degradation of the receptor sites or to its inhibition by endogenous substances.

The previous results in rat sciatic nerves give more support to the first hypothesis; indeed the accumulation of muscarinic receptors in the distal part of the ligated sciatic nerve was only detectable at short time intervals after the operation but not after two days.

Opiate receptors in the rat vagus can be labelled *in vivo* with ^{3}H-lofentanil. Figure 7(A) shows the accumulation of labelling above and below a ligature placed 2 days previously on vagus nerves of rat injected with ^{3}H-lofentanil. In order to explain the higher *in vivo* accumulation of ^{3}H-lofentanil in the D_1 than in the P_1 segment, a bivagotomy was performed at the level of the stomach. In these conditions, the labelling was reduced by 63% in the D_1

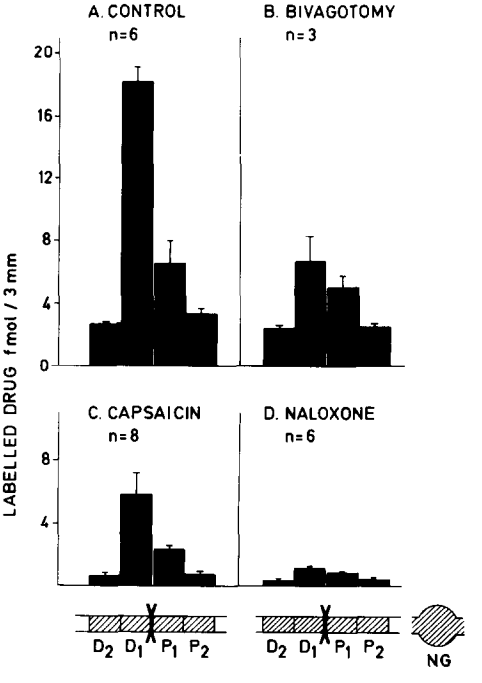

Fig. 7. Effect of bivagotomy, capsaicin and naloxone on *in vivo* ^{3}H-lofentanil binding in various segments of rat vagus nerves 48 hr after ligature. Ten microcuries of ^{3}H-lofentanil was injected i.v. immediately after the ligature [13]. Bivagotomy was performed at the level of the lower oesophagus through abdominal incision just before ligation. Naloxone 10 mg/kg was given i.v. 30 min before and after ligature.

segment but was not significantly changed in the P_1 segment (Fig. 7B). The reason for this is that the nerve terminals from the gut could not contribute to the accumulation of labelling below the ligature while the terminals from the heart and lung still operated.

In order to determine what type of neurone is involved in the axonal transport of opiate receptors in the vagus, rats were treated with capsaicin according to the scheme proposed by Gamse *et al.* [24]. In the capsaicin-treated animals, the accumulation of labelling on both sides of the ligature decreased by 60–70% (Fig. 7C). Further experiments revealed only a 40–50% reduction when the labelled drug was injected two days after the first capsaicin injection instead of 8 days. These results provide evidence that the bidirectional transport in the vagus nerve of opiate receptors occurs in capsaicin-sensitive neurones; therefore one may conclude that the opiate receptors in the vagus are mainly associated with substance P neurones.

Finally, when rats were pretreated with naloxone (Fig. 7D) there was no accumulation of labelling on both sides of the ligature; this indicates that the labelling which accumulates on both sides of the ligature after injection of ^{3}H-lofentanil was really associated with opiate receptors.

CONCLUSION

There is now growing evidence that receptor sites can be transported antero- and retrogradely by means of fast axoplasmic transport mechanisms. The foregoing results give more support to this idea; presynaptic muscarinic receptors were found to accumulate on both sides of a ligature in noradrenergic fibers in the dog splenic nerve but also in the rat sciatic nerve. Similarly, the axoplasmic transport of opiate receptors in the vagus nerve mainly occurred in the substance P neurones. Therefore one may conclude that these presynaptic receptors are modulated by neurotransmitters or substances originating from outside the neurones where they are located. Until now, the peripheral nerves appear to be the most convenient tool for these studies because the axonal flow can be interrupted by a ligature. In fact, such an approach allows to prove definitively the presynaptic localization of receptors; in this regard three conditions must be fulfilled: (1) identification of receptor sites in the cell body and the axon of well-characterized neurones; (2) axoplasmic transport along the axon; (3) physiological response at the nerve terminal. This evidence has been obtained for muscarinic receptors of splenic nerves; they have been identified in the coeliac ganglion wherefrom they flow down along the axon [8, 9] towards the spleen where acetylcholine is known to control noradrenaline release [25]. Until now, such a demonstration has not been obtained for the so-called presynaptic α_2-adrenoceptor [25 and unpublished results].

The rate of transport of muscarinic receptors in dog splenic and rat sciatic nerves is fast about 1–3 mm/hr; this corresponds to the values previously reported for noradrenaline and dopamine-β-hydroxylase [1, 2, 19–21].

The most interesting finding here is the localization of muscarinic receptors in vesicles containing noradrenaline and dopamine-β-hydroxylase; receptor site and neurotransmitter may thus coexist in the same synaptic vesicles. There is no need to call them receptosomes [26].

Morphological analyses provided some evidence that synaptic vesicles recycle during the process of transmitter release [27–28]; however, this recycling phenomenon occurs in the nerve terminals. Our double ligature experiments suggest the existence of another mechanism whereby synaptic vesicles would recycle in the perikaryon presumably through the Golgi route [29]. The model presented in Fig. 6 illustrates the dynamic events which are assumed to occur within the neuron. Receptors and other synaptic constituents are assembled in vesicles in the Golgi apparatus and flow down along the axon to nerve terminals; there the neurotransmitter is released by exocytosis, a mechanism whereby the receptors become externalized, thus functional. After internalization of the receptors presumably by agonist-induced desensitization processes [30], empty vesicles return to perikaryon where they can be recycled through the Golgi complex. Consequently the recycling phenomenon should be regarded as the vesicle reloading its intravesicular constituents which were released by exocytosis. Furthermore such vesicle traffic provides a pathway for transit whereby informational molecules from effector cells or other neurones can be taken up by nerve terminals and then move to perikaryon where it can potentially influence intracellular events. Our *in vivo* binding experiments with ^{3}H-lofentanil are compatible with such a transfer of information; indeed ^{3}H-lofentanil is taken up by the nerve terminals of the vagus and flows back to the cell body. However, the biological effect of the opiate in the perikaryon, presumably the nodose ganglion remains unclear; this needs further experimental data which could be important in understanding the long-term effects of opiates.

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