Accumulation of Acetylcholine at Synapse Increases the Amount of Retrograde Transport of HRP in the Masseter Motoneuron of Rat

Shigenori Kawagishi, Kenichi Yoshino, Takatoshi Murata and Niichiro Amano
Department of Oral Neuroscience, Kyushu Dental College, Kitakyushu, Japan

Received June 15, 2004.
Accepted July 21, 2004.

Abstract

In view of the role of retrograde transport for delivery of information about the nerve terminal environment, there is the possibility that the transmitter accumulation at synapse may cause some changes of retrograde transport in order to inform the neuronal cell body about the changes in the terminal environmental situation. To examine this possibility, we measured the amounts of retrograde transport of HRP in the masseter motoneurons of rats with or without administration of acetylcholinesterase inhibitors. Three kinds of inhibitors were used: diisopropyl fluorophosphate, eserin, neostigmine.

The rats were administered one of inhibitors with atropine. At the same time, HRP was injected into the bilateral masseter muscles. In the brain section with HRP reaction, the amount of HRP reaction products in the masseter motoneuron was quantitatively measured using an image processing system. The amounts of HRP reaction products in the masseter motoneurons of rats with administration of any inhibitors were significantly higher than those in the control animals. These results suggested that accumulation of the transmitter acetylcholine at synapse of neuromuscular junction increased retrograde transport of HRP in the masseter motoneuron of rat.

Key words: Acetylcholinesterase inhibitor/Transmitter/Neuromuscular junction

Introduction

Various materials are transported within the axon and such transport is divided into anterograde axonal transport from the cell body toward the synaptic terminal and retrograde axonal transport in the reverse direction.

When horseradish peroxidase (HRP) is injected into muscles, it is taken up at the motor nerve axons and retrogradely transported to their cell bodies. HRP accumulated in the cell bodies is hydrolyzed soon. The studies on uptake, transport and metabolism of HRP may
Acetylcholine accumulation increases HRP retrograde transport in motoneuron (Kawagishi et al.) — 105 —

be helpful for evaluation of physiological functions of retrograde transport. We have been studying factors which affect the retrograde transport by measuring the amount of retrograde transport of HRP injected into the rat masseter muscles under various conditions\(^6\).\(^5\)

Retrograde transport plays an important role for delivery of information about the nerve terminal environment\(^6\).\(^9\). In view of this role, there is the possibility that transmitter accumulation at synapse may cause some changes of retrograde transport in order to inform the neuronal cell body about changes in the terminal environmental situation. In the present study, to examine this possibility, we measured the amounts of retrogradely transported HRP in the masseter motoneurons of rats with or without administration of three kinds of acetylcholinesterase (AChE) inhibitor which causes the accumulation of acetylcholine (ACh) in the vicinity of cholinergic nerve terminals\(^9\).

**Materials and Methods**

Male 8-week-old Wistar rats were used in this study. Animals were equally divided into experimental and control groups. The experimental group was given an AChE inhibitor and the control group given the corresponding vehicle only. Three kinds of AChE inhibitor (Sigma, USA) were used: diisopropyl fluorophosphate (DFP, an irreversible inhibitor, blood–brain barrier (BBB) permeable), eserine (a reversible inhibitor, BBB permeable), neostigmine (a reversible inhibitor, BBB impermeable). The concentration of each inhibitor was determined from the other works in which the inhibitors effectively acted\(^6\).\(^1\)

In the experiment with DFP, five rats were examined in each of experimental and control groups. DFP dissolved in peanut oil was injected at the dose of 2.0 mg/kg into the gluteus maximus muscles. At the same time atropine (1.5 mg/kg) was intraperitoneally administered in order to block the actions of AChE inhibitors on autonomic effector organs with muscarinic ACh receptor, cortical and subcortical site in the CNS and autonomic ganglia. Then, under anesthetization with ether, a 10 \(\mu\)l volume of 7.5 % HRP (type IV, Sigma, USA) solution was injected bilaterally into the prescribed locus, 1 mm above the intersection of the parotid duct and the perpendicular from a given point 5 mm posterior to the eye fissure, in the anterior superficial part of the masseter muscles using a 26-gauge needle connected by a short length of polyethylene tubing to a Hamilton microsyringe as described previously\(^6\). The needle was vertically inserted into the muscles to a depth of 1.5 mm.

In the experiment of eserine, three rats were examined in each of experimental and control groups. A 10 \(\mu\)l of the mixture of 7.5 % HRP and 10 \(\mu\)M eserine in water was injected into the masseter muscles with intraperitoneal administration of atropine as described in the DFP experiment.

In the experiment of neostigmine, four rats were examined in each of experimental and control groups. The methods for injection of HRP and neostigmine were the same as in the experiment of eserine besides the 100 \(\mu\)M neostigmine concentration.
In all experiments, at 8 hours after HRP injection, all animals were deeply anesthetized with Nembutal (100 mg/kg, i.p.), given a small intraventricular dose of heparin, and perfused transcardially with 200 ml of isotonic saline, followed by 350 ml of a fixative containing 9 % formalin in 0.1 M sodium phosphate buffer (pH 7.4). The fixative was then replaced by 200 ml of 10 % phosphate–buffered sucrose (pH 7.4). The brainstem was immediately removed and stored overnight in 30 % phosphate–buffered sucrose (pH 7.4, 4 °C). The brainstem was cut transversely into 60 μm serial sections using a freezing microtome. Sections were immediately processed for HRP activity according to a modification of Mesulam’s protocol using tetramethyl benzidine as the chromogen\(^{12,13}\). The matching series of serial sections from the experimental and control groups were processed together in the same large reaction bath under identical conditions. All sections were mounted on chrome alum–gelatin coated slides.

The amount of retrograde transport of HRP in the masseter motoneuron was quantitatively measured as described previously\(^{9}\). Briefly, the gray image of a labeled unilateral motoneuron pool in a section was fed to the image processing system (SPICCA, Nippon Avionics Co. Ltd., Japan). The gray image was changed to three binary images at the 78, 156 and 234 levels indicative of high, medium and low stain densities (the 234 level corresponds to white, the lack of HRP reaction product). The areas of labeled motoneurons in three binary images were measured. Such measurements were made of 10–12 consecutive coronal sections, each of which contained a number of labeled motoneurons in the trigeminal motor nucleus in question. All measured values were added and used as the amount of retrograde transport of HRP in masseter motoneurons located in a unilateral trigeminal motor nucleus.

All surgical procedures and subsequent maintenance of animals were carried out according to the guidelines directed by the Animal Care Facilities of the Kyushu Dental College, Kitakyushu, Japan.

Statistically significant differences between means obtained from the experimental and control groups were estimated by using the Student’s \(t\)-test or Welch’s \(t\)-test for unpaired observations.

**Results**

In the present study, the DFP-administered rats were observed to have trembled in every limb, this is the particular response to administration of large dose of AChE inhibitor. DFP was systemically administered because peanut oil was too viscous to be injected exactly with a very small volume. Local administration of each 10 μl of 10 μM eserine and 100 μM neostigmine did not cause trembling of the rats whose behavior was normal. Under these conditions, all three of AChE inhibitors examined significantly increased the amounts of retrograde transport of HRP (\(p < 0.05\) by Welch’s \(t\)-test in the cases of DEP and eserine, \(p < 0.01\) by Student’s \(t\)-test in the case of neostigmine). The accelerative effects of AChE inhibitors on the retrograde transport of HRP is shown in figure 1. The amounts (mean ±
Acetylcholine accumulation increases HRP retrograde transport in motoneuron (Kawagishi et al.) — 107 —

Fig. 1 Accelerative effects of acetylcholinesterase inhibitors on HRP retrograde transport.
A: Effects of DFP. Ten samples of the trigeminal motor nucleus from five rats were examined for retrograde transport of HRP in each of experimental (+DFP) and control (−DFP) groups. Open circle is the HRP amount in a trigeminal motor nucleus. Means±S.D. in the +DFP and −DFP groups are 434±169 (n=10), 258±72 (n=10), respectively. B: Effects of eserine. Means±S.D. in the +Eserine and −Eserine groups are 279±96 (n=6) and 192±31 (n=6), respectively. C: Effects of neostigmine. Means±S.D. in the +Neostigmine and −Neostigmine groups are 275±21 (n=8) and 211±30 (n=8), respectively. *, p<0.05 by Welch’s t-test. ††, p<0.01 by Student’s t-test.

S.D.) of retrograde transport of HRP in the experimental and control groups were as follows; 434 ± 169 (n=10) and 258 ± 72 (n=10) for DFP, 279 ± 96 (n=6) and 192 ± 31 (n=6) for eserine, 275 ± 21 (n=8) and 211 ± 30 (n=8) for neostigmine.

Discussion

In the present study, all three of AChE inhibitors examined significantly increased the amounts of retrograde transport of HRP. The mean values of retrograde transport of HRP in the experimental groups were about 1.7, 1.5 and 1.3 folds higher than those in the control groups of DFP, eserine and neostigmine, respectively. These results indicated the acceleration of retrograde transport of HRP by AChE inhibitor in the masseter motoneuron of rat.

The characteristic pharmacological action of AChE inhibitors has been reported to prevent hydrolysis of ACh at sites of cholinergic transmission, causing the accumulation of ACh in the vicinity of cholinergic nerve terminals⁹. The AChE inhibitors are effective at junctions of the various cholinergic nerve endings with their following effector organs; autonomic
effector organs with muscarinic ACh receptor, all autonomic ganglia and skeletal muscle with nicotinic ACh receptor, cortical and subcortical site in the CNS where the receptors are largely of the muscarinic type.

In our examination, changes in the amounts of retrograde transport of HRP could be brought by actions of AChE inhibitors on the neuromuscular junction, because atropine, which blocks the actions of AChE inhibitors on autonomic effector organs with muscarinic ACh receptor, cortical and subcortical site in the CNS and autonomic ganglia, was administrated in every experiments. In addition, all three inhibitors with different characters accelerated retrograde transport of HRP. It was strongly suggested that the accumulation of transmitter at synapse was one of factors facilitating of retrograde transport of HRP in the motoneuron.

The accumulation of ACh at synapse may depolarize the presynaptic site3). It could activate endocytosis by which HRP is uptaken at the synapse of motoneuron terminal4,14,15), resulting on the increase of the amount of retrogradely HRP transport. Although the physiological significance of the change of HRP transport is not clear, the speculation may be interesting that changes in the amounts of materials including HRP conveyed in retrograde transport can influence cell body metabolism.

Conclusion

In order to examine whether or not the accumulation of transmitter at synapse affect retrograde transport, we measured the amount of retrograde transport of HRP in the masseter motoneuron of the rat administered AChE inhibitor. The inhibitors used in this study were as follows: diisopropyl fluorophosphates, (an irreversible inhibitor, BBB permeable), eserine (a reversible inhibitor, BBB permeable), neostigmine (a reversible inhibitor, BBB impermeable).

The rats were administered one of inhibitors with atropine. At the same time, HRP was injected into the bilateral masseter muscles. In the brain section with HRP reaction, the amount of HRP reaction products in the masseter motoneuron was quantitatively measured using an image processing system. The amounts of HRP reaction products in the masseter motoneurons of rats with administration of any inhibitors were significantly higher than those in the control animals. These results suggested that accumulation of the transmitter acetylcholine at synapse of neuromuscular junction increased retrograde transport of HRP in the masseter motoneuron of rat.

References

3) Krinstenson, K. and Olsson, Y.: Uptake and retrograde axonal transport of exogenous protein
Acetylcholine accumulation increases HRP retrograde transport in motoneuron (Kawagishi et al.) — 109 —

シナプスにおけるアセチルコリン蓄積によるラット咬筋運動ニューロンのHRP 逆行性軸索輸送量の増加

河岸重則・吉野賢一・村田貴俊
天野仁一朗

九州歯科大学口腔科学講座

逆行性軸索輸送は神経終末周辺の環境情報を細胞体に伝えていると示唆されていることから、シナプスに神経伝達物質が蓄積したとき逆行性輸送に変化が現れることが予想される。本研究ではこの可能性を検討するため、ラットにアセチルコリンコリンエステラーゼ阻害剤を投与し、咬筋運動ニューロンのHRP 逆行性軸索輸送量に変化が現れるか否かを観察した。使用した阻害剤はdiisopropyl fluorophosphates（血液脳関門透過性非可逆的阻害剤）、eserine（血液脳関門透過性可逆的阻害剤）、neostigmine（血液脳関門非透過性可逆的阻害剤）の3種である。8週齢のラットに阻害剤のいずれかを投与し実験群とした。対照群には阻害剤の溶媒を投与した。その際中枢神経系や自律神経系を介した影響を除くため、アトロピンを投与した。次いで両側の咬筋の一定部位に、HRPを注入し、8時間生存させた。心灌流固定後、厚さ60μmの脳幹の凍結連続切片を作製し、HRP反応に供した。画像解析装置を用いて咬筋運動ニューロン細胞内のHRP反応産物量を測定し、HRP逆行性輸送量とした。輸送量はいずれの阻害剤の場合も、阻害剤投与ラット群の方が非投与群より有意に多く、神経筋接合部のシナプスでの神経伝達物質アセチルコリンの蓄積が咬筋運動ニューロンのHRP逆行性軸索輸送を促進することが示唆された。