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A new cytochemical method for *in situ* detection of cholinergic synaptic transmission by staining of Cu^{2+} incorporated in frog neuromuscular junction during nerve stimulation

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ABSTRACT

A new cytochemical method was devised in order to visualize Cu^{2+} ions in the synaptic area after their intracellular penetration during nerve stimulation of the frog neuromuscular junction (NMJ). The motor nerves were stimulated in presence of Cu^{2+}. After total blockade of the neuromuscular junction, the tissue was treated by ferrocyanide, a precipitating agent of Cu^{2+}, and fixed for optical and electron microscopic observation. The oxidoreductase-like catalytic activity of the copper ferrocyanide precipitate was used to amplify the cytochemical staining by a treatment with diaminobenzidine and H_2O_2, after permeabilization of cell membranes by Triton X-100. At optical level, an intense staining was observed in the synaptic area. Application of d-tubocurarine (d-TC), a selective inhibitor of nicotinic acetylcholine receptors (nAChRs), markedly reduced the staining. No reaction could be observed in absence of membrane permeabilization. These results suggest that Cu^{2+} was localized in the cytoplasm of muscle cells after its penetration through nAChRs. At electron microscopic level, cytochemical reaction was found in the cytoplasm of muscle cells near the postsynaptic membrane, and in a few synaptic vesicles in the vicinity of the active zone. This method may be used for the identification of cholinergic inputs in central and peripheral nerve systems and, generally speaking, for the detection of synaptic activity elicited by specific nerve stimulation.

In situ visualization of synaptic activity during nerve transmission remains a difficult challenge for neuroscientists. Histochemical staining of sensory neurons stimulated by capsaicin (7, 10, 16, 18) and of cerebral neurons stimulated by kainate (11, 19) was performed after uptake of Co^{2+}. These authors used a "Co^{2+} uptake buffer solution" with pharmacological agonists. The intraneuronal staining was obtained after the penetration of Co^{2+} and its precipitation in cobalt sulfide, followed by silver amplification. However, these experiments were lacking in physiological control of Co^{2+} uptake. In another way, histochemical staining of cytochrome oxidase activity has been proposed as a reliable clue of neuronal activity (17). Nevertheless, this method is suitable for the detection of the activity at the cellular level rather than at the synaptic level. Morphological features of postsynaptic structures were proved to be accurate markers for the real time detection of in-

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crease or decrease in synaptic activity in the central nervous system (4, 8). However, this method does not provide precise information at ultrasructural level on the activity occurring in the limited synaptic area (20).

The aim of the present study was to propose the visualization, at both optical and electron microscopical levels, of a specific synaptic activity in the cholinergic nerve transmission. We, thus, devised a new cytochemical method based on *in situ* localization of Cu^{2+} after its intracellular penetration in the synaptic area during cholinergic nerve stimulation.

The divalent metallic cations, such as Co^{2+}, are known as potent blocking agents of the cholinergic synaptic transmission. It is thought that they hinder the release of the neurotransmitter by blocking presynaptic Ca^{2+} channels. Moreover, we have recently demonstrated that Co^{2+} may also interfere with the postsynaptic ligand-gated channels (13). We, thus, suppose that another divalent metallic cation, Cu^{2+} cupric ion, may block the synaptic transmission in a similar way. An additional property of these divalent metallic cations is a non-specific penetration into the postsynaptic cells through the ligand-gated channels (6). Since we recently demonstrated the penetration of Co^{2+} through the postsynaptic membrane into the muscle cells (1), it may be assumed that Cu^{2+} could also penetrate into the postsynaptic cells.

The staining of these divalent metallic cations after their penetration in postsynaptic cells during the stimulation of the presynaptic pathways may be a valuable method to visualize the activity of synaptic transmission. Moreover, combined with the use of selective inhibitors of synaptic transmission, such a staining may allow the elucidation of the type of neurotransmitter involved in the transmission. In such conditions, the divalent cations penetrate in extremely small quantity. Though Co^{2+} could be visualized directly under transmission electron microscope (1, 13), these cations are hardly visible under the optical microscope.

In the present work, we propose to visualize cholinergic synaptic transmission by means of intracellular penetration of Cu^{2+} ions in frog neuromuscular junction (NMJ) during nerve stimulation. A selective inhibitor of cholinergic transmission, d-tubocurarine (d-TC), was used to ensure the specificity of the staining. After penetration into the cells, Cu^{2+} was precipitated with potassium ferrocyanide, which results in a minute precipitate of copper ferrocyanide. The oxidoreductase-like catalytic activity of the precipitate (14) allowed us to amplify the staining by the use of a mixture of diaminobenzidine (DAB) and H_2O_2, a known cytochemical detector of peroxidase activity. In these conditions, precise localization of the cytochemical precipitate, including copper ferrocyanide and DAB, could be observed both at optical and electron microscopic level.

MATERIAL AND METHODS

Neuromuscular preparation. Frog (*Rana catesbiana* L.) cutaneous pectoris muscles were taken out together with the motor nerve bundles and pinned on the bottom of an experimental chamber containing 5 mL Ringer solution (112 mM NaCl, 2 mM KCl and 1.8 mM $CaCl_2$) buffered with 25 mM Tris (hydroxymethyl) amino-methane-HCl (Tris-HCl) buffer solution at pH 7.8.

Blockade of the neuromuscular transmission by $CuCl_2$ (Fig. 1A). The bundle of motor nerves was stimulated electrically with a pair of silver chloride electrodes (supra-maximal voltage: 3–7 V, duration: 0.3 ms) at 1/3 Hz and the muscle contractions were monitored on a chart recorder (Recti-Horiz; San-ei,

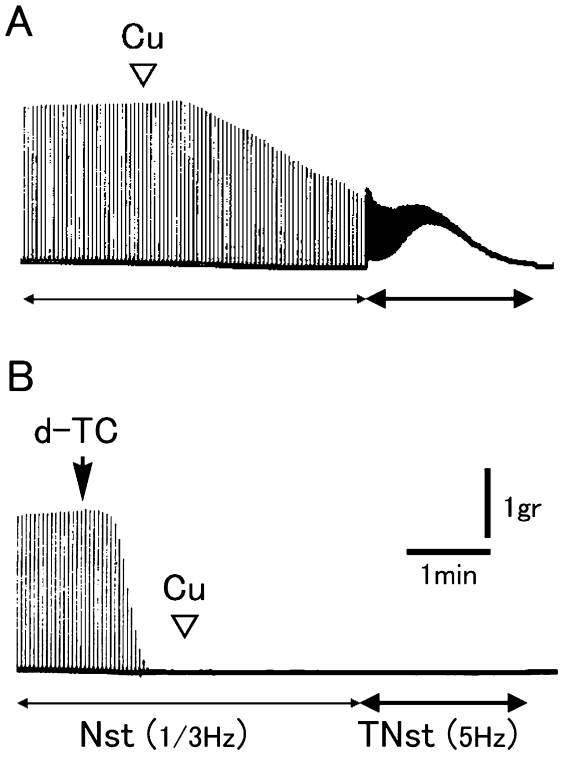

Fig. 1 Electrical stimulations of the motor nerves in the presence of Cu^{2+}, without and with d-TC.
A: Blockade of the neuromuscular transmission by $CuCl_2$. B: Effect of d-tubocurarine (d-TC). (Explanation in Material and Methods)

Tokyo, Japan) by isometric mechano-electric transducer (TB-611T; Nihon Kohden, Tokyo, Japan). The peak tension of the muscular control twitch due to nerve stimulation was approximately 3 g. Then, $CuCl_2$ (90 µL of 100 mM) was added to the chamber (5 mL) providing a final concentration of 1.8 mM Cu^{2+} ions. When the synaptic transmission was inhibited to the half level (half amplitude of the original muscle twitches) after 2 min, the tetanic electrical stimulation was applied to the motor nerves (5 Hz, 600 pulses) during 2 min. The neuromuscular transmission was completely blocked within 2 min. The control muscle preparation, with the low frequency monitoring electrical stimulations and the tetanic electrical stimulation of the motor nerves in the absence of $CuCl_2$, provided no blockade of the muscle contraction.

Effect of d-tubocurarine chloride (d-TC) (Fig. 1B). d-TC at 0.1 mM was added to the chamber during the monitoring muscle twitches by low frequency electrical stimulation (1/3 Hz). The synaptic transmission was blocked within 1 min. Cu^{2+} was added in this blocked state, and, after 3 min, the tetanic electrical stimulation (5 Hz) was applied for 2 min, as it was in the experiment performed without d-TC.

Tissue preparations for cytochemical detection of traces of Cu^{2+} at optical microscopic level. The tissue was quickly washed in isotonic saline solution (NaCl, 120 mM) containing 2 mM EDTA. It was, then, immersed, during one hour, in a mixture of 2.5% glutaraldehyde (tissue fixative), 10 mM potassium ferrocyanide (precipitating agent of Cu^{2+} ion and donor of catalytic activity to Cu^{2+}) and 100 mM NaCl (osmotic pressure compensator). After fixation, the tissue was treated with 0.5% Triton X-100 dissolved in isotonic saline solution during 30 min or by repetitive freezing and thawing. Finally, the tissue was processed during 20 min with a conventional 3,3'-diaminobenzidine tetrahydrochloride (DAB, 4 mg/10 mL isotonic saline solution) with 0.01% H_2O_2. After washing in saline solution, the muscle fibers were preserved in 2.5% glutaraldehyde dissolved in saline solution and kept at 4°C. One part of the muscle fibers were separated and mounted in Aquatex (Merck, Germany), for optical microscopic observation.

Electron microscopic observation of the cytochemical reaction. The remaining of the muscle fibers, preserved in 2.5% glutaraldehyde, was washed in saline solution and post-fixed by 2% OsO_4 (4% OsO_4 diluted in saline solution) for 30 min. After washing in H_2O, small pieces of the neural zone of the preparation were cut out, dehydrated in alcohol, and embedded in Araldite. Ultra-thin sections were obtained with a diamond knife and observed without counterstaining under a JEOL 100 CX electron microscope (JEOL Ltd.) at 80 kV.

RESULTS

Compatibility of the buffer with Cu^{2+}

The addition of Cu^{2+} to the Ringer solution constrained us to find out a buffer solution which does not precipitate with the cation. Only Tris-HCl buffer was compatible with Cu^{2+}, though it combined with Cu^{2+} (appearance of blue color and drop of the pH from 7.8 to 6.1, in the present experimental conditions). However, Cu^{2+} was still able to block the synaptic transmission and to penetrate into the muscle cells.

Evidence for intracellular penetration of traces of Cu^{2+}

In absence of membrane permeabilization by Triton X-100 or of repetitive freezing and thawing, no staining could be observed. This indicates that the copper ferrocyanide stained in this work was located inside the cells, only when the penetration of DAB was made possible by a partial destruction of the cell membranes. Moreover, washing of the tissue with saline solution containing EDTA prevented from an artifactual staining of their surface.

Positive Cu^{2+} reaction limited to the neuromuscular junction

The first type of preparation was subjected to the tetanic electrical stimulation of the motor nerve in the presence of Cu^{2+} (see Fig. 1A). The staining was limited to the synaptic area of the neuromuscular preparation (Fig. 2A and A'). An intense red staining appeared in a restricted part of the synaptic area of the NMJ, while a weak specific staining was observed on the major part of the synaptic area. The typical lamellar structure of the subneural apparatus (junctional folds) was not stained. The second type of preparation was treated by d-TC prior to Cu^{2+} application and tetanic electrical stimulation of the motor nerve (see Fig. 1B). d-TC reduced markedly the staining of the synaptic area observed in the experiment performed without d-TC. Only a weak cytochemical reaction was visible after d-TC treatment (Fig. 2B).

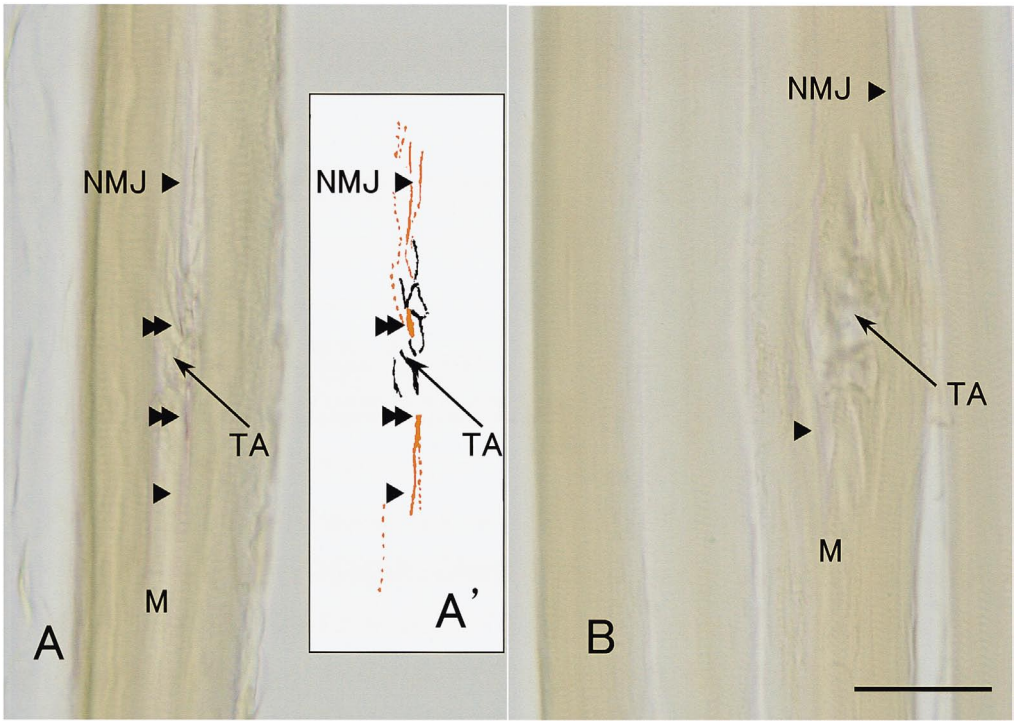

Fig. 2 Optical microscopic observation of the cytochemical staining of Cu^{2+} in the frog neuromuscular preparations treated in two different conditions (Fig. 1 A, B).
A: Motor nerve stimulation and addition of Cu^{2+} in the absence of d-TC. The terminal axon (TA) is on the middle of the muscle fiber (M). After tetanic electrical stimulation of the motor nerve in the presence of Cu^{2+} and cytochemical staining of DAB, long pre- and postsynaptic areas of the neuromuscular junction (NMJ) are faintly stained (arrowheads). A restricted region of the NMJ is intensely stained (double arrowheads). A': Tracing of reacted part of the neuromuscular junction. The bold, fine and dotted red lines correspond respectively to the intense, faint and traceable staining obtained on the frog neuromuscular junction. The reticular structure (black) around the terminal axon (TA) corresponds to the nuclei of teloglia and muscle cell. B: Treatment with d-TC followed by addition of Cu^{2+} and motor nerve stimulation. The terminal axon (TA) is on the middle of the muscle fiber (M). After d-TC pretreatment, Cu^{2+} addition and tetanic motor nerve stimulation, the long synaptic areas of the neuromuscular junction (NMJ) are faintly stained by DAB precipitate (arrowheads). Scale bar 50 µm.

Ultrastructural localization of the cytochemical precipitate

The cytochemical reaction was found in the cytoplasmic side of the postsynaptic membrane of the NMJ (Fig. 3A) and in some synaptic vesicles in the vicinity of the active zone of the presynaptic membrane (Fig. 3B).

DISCUSSION

Cytochemical detection of traces of Cu^{2+} by means of a catalytic activity of ferrocyanide complex

In the present work, we propose a new cytochemical method to stain traces of Cu^{2+} after its penetration inside the cells at the level of the synaptic region. For this purpose, we used the catalytic activity of Cu^{2+} combined with ferrocyanide $[Fe^{2+}(CN)_6]$-4 ion, which results in a copper ferrocyanide precipitate. The "peroxidase-like" catalytic activity of this precipitate (12) was interpreted in a general term as an oxidoreductase-like catalytic activity (14). The mechanism of this catalytic activity was already supposed to be an oscillating intramolecular oxidoreduction between Cu^{2+} and Fe^{2+} fluctuating alternatively with the states of Cu^{1+} and Fe^{3+} (15).

Potassium ferrocyanide is used, instead of potassium ferricyanide, in order to obtain catalytic activity with Cu^{2+}

Another histochemical precipitate, the copper ferricyanide precipitate, may be obtained with potassium ferricyanide. However, this precipitate of copper ferricyanide is not endowed with the catalytic activity, since alternative oscillation of electrons is not possible between Cu^{2+} and Fe^{3+}. Thus, potassium ferricyanide may be used only when the amount of intracellular Cu^{2+} is sufficient to be detected without amplification by DAB-H_2O_2 treatment. In the pres-

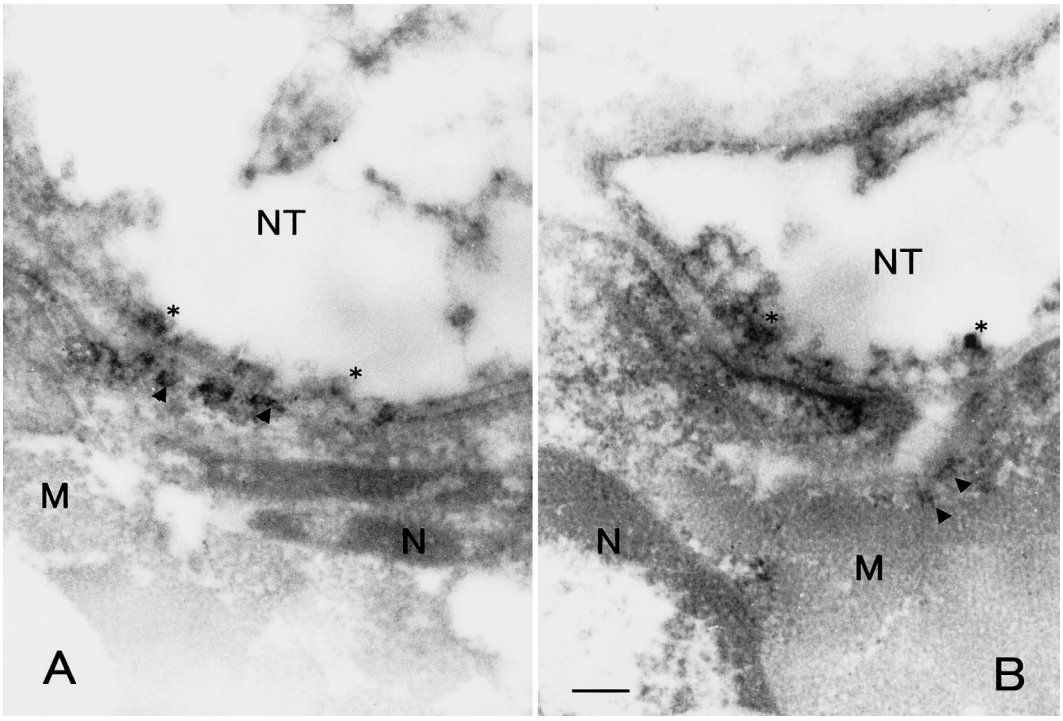

Fig. 3 Electron microscopic observation of the cytochemical staining after motor nerve stimulation and addition of Cu^{2+} in the absence of d-TC. A poor preservation of the ultrastructure is observed after membrane permeabilization. The cytochemical staining is found in the cytoplasmic side of the postsynaptic membrane (arrowheads) and in a few synaptic vesicles (*) attached to the presynaptic membrane. NT: nerve terminal, M: muscle cell, N: nucleus. Scale bar 250 nm.

ent case, the minute quantity of Cu^{2+} that penetrated in the cells needs the use of potassium ferrocyanide in order to obtain the catalytic activity of copper ferrocyanide.

Cytochemical detection of Cu^{2+} at optical microscopic level

The cytochemical reaction was observed only after membrane permeabilization. Moreover, d-TC treatment caused a remarkable reduction of the cytochemical staining. These two observations indicate that most of the staining corresponded to the penetration of Cu^{2+} in the cytoplasm of muscle cells through the nicotinic ACh receptor (nAChR) located in the postsynaptic membrane. The postsynaptic membrane of frog NMJ is characterized by regular foldings, described as lamellar subneural apparatus (2). Recent researches showed that nAChRs were concentrated on the part of the postsynaptic membrane situated near the nerve terminals, whereas the sarcoplasmic membrane was devoid of nAChR in the depth of the folds (5, 9). This may explain why Cu^{2+} staining was observed in the postsynaptic area but not in the lamellar subneural apparatus. It is worth to note that histochemical localization of acetylcholinesterase was obtained in the lamellar subneural apparatus associated with the synaptic gutter (3).

Cytochemical detection of Cu^{2+} at electron microscopic level

Under electron microscope, the staining was detected in the cytoplasm of muscle cells close to the postsynaptic membrane. This confirms the observations made at optical microscopic level. Cytochemical reaction was not observed on the presynaptic membrane, while the reaction was found in a few synaptic vesicles attached to the presynaptic membrane. The staining in the synaptic vesicles may be due to vesicular uptake of copper ions. This vesicular staining of a very small quantity of Cu^{2+} is probably the result of the amplification of Cu^{2+} by DAB.

Perspective of Cu^{2+} penetration method

Our new method is based on the penetration of Cu^{2+} in postsynaptic cells and on the localization of this cation by precipitation with ferrocyanide and amplification with DAB and H_2O_2. It allows the visualization of synaptic activity during nerve stimulation. Blockade of cation penetration in the postsynaptic

cells by specific inhibitors of membrane receptors may provide information on the type of neurotransmitter involved in the presynaptic nerve input. The present work indicates that cholinergic input can be characterized by such method, using d-TC, a selective inhibitors of nAChR. This method remains to be tested on diverse nervous tissues in different physiological and pharmacological conditions.

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REFERENCES

1. Akagi T, Hashikawa T, Hirai K, Motelica-Heino I and Tsuji S (2000) Electron spectroscopic imaging (ESI) of cobalt ions responsible for the blockade of synaptic transmission and excitability of muscle cells in frog neuromuscular preparations. *Proc Jpn Acad* **76**, Ser B, 7–11.
2. Couteaux R (1947) Contribution à l'étude de la synapse myoneurale. *Rev Canad Biol* **6**, 563–711.
3. Couteaux R and Taxi J (1952) Recherches histochimiques sur la distribution des activités cholinestérasiques au niveau de la synapse myoneurale. *Arch Anat Micr* **41**, 352–392.
4. Dunaevsky A and Mason CA (2003) Spine motility: a means towards an end? *Trends Neurosci* **26**, 155–160.
5. Fertuck HC and Salpeter MM (1976) Quantitation of junctional and extrajunctional acetylcholine receptors by electron microscope autoradiography after ^{125}I-α-bungarotoxin binding at mouse neuromuscular junctions. *J Cell Biol* **69**, 144–158.
6. Hille B (2001) *Ion Channels of Excitable Membrane* 3rd ed, pp 187–188, Sinauer, Massachusetts, USA.
7. Hogan PG (1983) Expression of markers for pain sensory neurons in cell culture. Ph. D. thesis, Harvard University, Cambridge, Massachustts, USA.
8. Kasai H, Matsuzaki M, Noguchi J, Yasumatsu N and Nakahara H (2003) Structure-stability-function relationships of dendritic spines. *Trends Neurosci* **26**, 360–368.
9. Lester HA (1977) The response to acetylcholine. *Sci Am* **236**, 106–118.
10. Nagy I, Pabla R, Matesz C, Dray A, Woolf CJ and Urban L (1993) Cobalt uptake enables identification of capsaisin- and bradykinin-sensitive subpopulations of rat dorsal root ganglion cells in vitro. *Neuroscience* **56**, 241–246.
11. Pruss RM, Akeson RL, Racke MM and Wilburn JL (1991) Agonist-activated cobalt uptake identifies divalent cation-permeable kainate receptors on neurons and glial cells. *Neuron* **7**, 509–518
12. Tago H, Kimura H and Maeda T (1986) Visualization of detailed acetylcholinesterase fiber and neuron staining in rat brain by a sensitive histochemical procedure. *J Histochem Cytochem* **34**, 1431–1438.
13. Tanaka E, Ishii K, Akagi T, Hirai K, Motelica-Heino I, Katayama Y, Higashi H, Hashikawa T and Tsuji S (2004) A new cytochemical method for ultrastructural localization of Co^{2+} in rat hippocampal CA1 pyramidal neurons in vitro. *J Neurosci Method* **135**, 1–8.
14. Tsuji S (1998) Electron microscopic localization of acetylcholinesterase activity in the central nervous system: chemical basis of a catalytic activity of Hatchett's brown (cupric ferrocyanide) precipitate revealed by 3,3'-diaminobenzidine. *Folia Histochem Cytobiol* **36**, 67–70.
15. Tsuji S and Larabi Y (1983) A modification of thiocholine-ferricyanide method of Karnovsky and Roots for localization of acetylcholinesterase activity without interference by Koelle's copper thiocholine iodide precipitate. *Histochemistry* **78**, 317–323.
16. Winter J (1987) Characterization of capsaicin-sensitive neurons in adult dorsal root ganglion cultures. *Neurosci Lett* **80**, 134–140.
17. Wong-Riley MTT (1989) Cytochrome oxidase: an endogeneous metabolic marker for neuronal activity. *Trends Neurosci* **12**, 94–101.
18. Wood JN, Winter J, James IF, Rang HP, Yeats J and Bevan S (1988) Capsaicin-induced ion fluxes in dorsal root ganglion cells in culture. *J Neurosci* **8**, 3208–3220.
19. Yin HZ, Sensi SL, Carriedo SG and Weiss JH (1999) Dendritic localization of Ca^{2+}-permeable AMP/kainate channels in hippocampal pyramidal neurons. *J Comp Neurol* **409**, 250–260.
20. Yuste R and Bonhoeffer T (2001) Morphological changes in dendritic spines associated with long-term synaptic plasticity. *Annu Rev Neurosci* **24**, 1071–1089.