**28. A Combined Method of Immunocytochemistry
of Choline-acetyltransferase Detection
and of Autoradiography of [³H]choline
after Retrograde Axonal Transport**

***Study of Cholinergic Pathways in the Central Nervous System
at Light and Electron Microscopic Level***

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Abstract: Immunocytochemistry of choline-acetyltransferase (ChAT) has been combined with autoradiography of [³H]choline after retrograde axonal transport on the same tissue section. This combination was performed with efficient preservation, in situ, of [³H]choline or its derivatives by ionic fixation with silicotungstic acid. The cholinergic neuronal pathway from the diagonal band of Broca (DBB) of basal forebrain to the olfactory bulb (OB) was investigated with this method. After the injection of [³H]choline into the OB, radiolabelled neurons were identified in the DBB. Most of them were ChAT immunoreactive confirming the specificity of [³H]choline retrograde labelling for cholinergic neurons. At the electron microscopic level, silver grains could be observed only on the cytoplasm. This method will be useful to study the coexistence of transmitters in the cholinergic pathways and at the same time to determine the chemical nature of the afferences making contact with a defined soma of a cholinergic pathway.

Key words: [³H]choline; retrograde axonal transport; specific tracing method; autoradiography; choline-acetyltransferase; immunocytochemistry; double labelling; electron microscopy.

In the last two decades, the cholinergic neurons in the central nervous system have been identified either by histochemistry of acetylcholinesterase (AChE)¹⁾ or by immunocytochemistry (ICC) of choline acetyltransferase (ChAT).^{2),3)} ChAT, the enzyme of acetylcholine (ACh) synthesis, is the most specific marker for the labelling of cholinergic neurons.⁴⁾ On the other hand, the identification of cholinergic neurons by the use of antibodies against ACh⁵⁾ was limited by the difficulty to fix ACh in situ.

Light microscopic autoradiography using neurotransmitter specific retrograde labelling⁶⁾ is a specific method for tracing neuronal pathways. Such a selective [³H]choline ([³H]Ch) retrograde labelling was observed in cell bodies when [³H]Ch was injected near the terminal area and then transported by axons to perikarya.^{7),8)} The tracing of cholinergic pathways was also attempted by combination of AChE histochemistry or

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ChAT ICC with non-specific tracer such as horseradish peroxidase.⁹⁾ Furthermore, Jones and Beaudet¹⁰⁾ studied cholinergic neurons by combination of [³H]Ch labelling with ChAT ICC on adjacent brain sections.

A combination of [³H]serotonin retrograde labelling and immunocytochemistry on the same histological sections was realised for tracing serotonergic pathways containing others neurotransmitters,¹¹⁾ and for identifying GABAergic afferences innervating cell bodies of spinal serotonergic pathway.¹²⁾ No similar combined method has yet been proposed for cholinergic pathways, probably because of choline diffusion. We earlier have proposed an ionic fixation procedure¹³⁻¹⁵⁾ to limit the diffusion of tissue acetylcholine for histological studies. Here we have applied this procedure to autoradiography of [³H]Ch detection¹⁰⁾ after retrograde transport, and have combined it to ChAT ICC¹⁶⁾ at the light and electron microscopic level.

The cholinergic neuronal pathway between the diagonal band of Broca (DBB) and olfactory bulb (OB)^{17,18)} was chosen to realise this specific tracing. Our results show that numerous cell bodies of the DBB are ChAT immunoreactive and [³H]Ch radiolabelled. Combination of both specific techniques represent a powerful tool to study cholinergic pathways.

Materials and methods. Eight male Wistar rats (Iffa Credo, 180-300 g) were used. The commercial ethanol solution of [methyl-³H]choline chloride 2.9 Bq/mmol (Amersham) was evaporated and redissolved in 0.9% saline to the concentration of $2-5 \times 10^{-4}$ M. Rats anaesthetized with pentobarbital (60 mg/kg) were stereotactically injected with 1 μ l of radioactive solution (40 μ Ci) into the OB as described by Araneda *et al.*¹¹⁾ Forty-eight h after [³H]Ch injection and 24 h after colchicine treatment (50 μ g) the brains were fixed (4% paraformaldehyde in 0.1 M phosphate buffer) by intracardiac perfusion. Vibratome sections (50 μ m) were taken from DBB, OB and midbrain raphe. Free-floating sections were incubated with anti-ChAT monoclonal antibodies (gift of Dr. B. H. Wainer), diluted 1:200, overnight at 4°C. Then, they were treated with biotinylated goat antirabbit immunoglobulin (1 h, diluted 1:200) and finally incubated with peroxidase labelled avidin-biotin complex (Vectastain) for 1 h. The reaction was revealed by conventional immunoperoxidase. Brain sections were then fixed in 5% aqueous solution of silicotungstic acid for 15 min (in order to fix [³H]Ch)¹³⁾ and washed. Autoradiography was carried out according to the method of Droz¹⁹⁾ with slight modifications as described by Araneda *et al.*¹¹⁾ All tissue sections were examined with a Leitz microscope equipped with bright and dark field illumination.

Figs. 1a, b and c. Combined autoradiography of [³H] choline after retrograde transport and immunocytochemistry of choline acetyltransferase (ChAT) on the same histological section of the horizontal band of Broca (DBB), 48 h after injection of [³H] choline in the olfactory bulb and 24 h after colchicine treatment.

Fig. 1a. ChAT immunoreactive neurons in horizontal DBB. Because of the low magnification ($\times 75$), simultaneous radiolabelling of the neurons is not visible (two examples are marked by arrows with letters b and c, referring to the Figs. 1b and 1c. Figs. 1b and 1c. Parts of Fig. 1 at higher magnifications ($\times 360$ and $\times 470$, respectively). Arrows in each figure indicate neurons which are double-labelled by immunoreactivity and radioactivity.

Fig. 2. Electron microscopic autoradiography of retrograde [³H]choline labelled neurons of DBB. The silver grains are localised in the perikarya (arrows) but not in the nucleus, (N); Afferent nerve terminals (A) in contact with this cell body ($\times 18,000$).

Fig. 3. Radiolabelled (arrows) and ChAT immunoreactive (diaminobenzidine precipitate) perikarya. Note that a non immunoreactive nerve terminal (A) is in contact with this cell body ($\times 8,000$).

Electron microscopy. Immunostained sections of the DBB were fixed in 5% silicotungstic acid for 30 min, osmified in a solution containing 2.5% silicotungstic acid and 2% OsO₄ for 30 min, washed in distilled water, dehydrated in a series of graded ethanols and embedded in Araldite.¹⁴⁾ Ultrathin sections (70 nm) were counterstained with lead citrate, coated with Ilford L4 emulsion diluted 1:4, exposed for two months, developed and transferred on copper grids. Electron microscopic observations were carried out with JEOL 1200 EX electron microscope at 80 kV.

Results. [³H]Ch injections were confined to the rostral part of the rat OB. In the vicinity of the point of injection, an intense autoradiographic reaction was observed within granular, plexiform and glomerular layers. Silver grains were observed in the neuropile but not in the neuronal cell bodies.

Two types of labelling were observed on the cell bodies localized in the DBB: brown cytoplasmic ChAT immunoperoxidase precipitates and silver grain accumulation. An important number of neurons were ChAT immunoreactive (Fig. 1 a) and some of them were also radiolabelled (Figs. 1 b,c). Among 292 [³H]Ch radiolabelled and ChAT immunoreactive neurons observed, 60% were distributed in the horizontal part of the DBB and 40% in the vertical one. Although a slight background was observed on the midbrain raphe, the serotonergic neurons were never radiolabelled. The electron microscopic autoradiography showed that most of the silver grains were localized in the perikarya of the radiolabelled neurons; they were present on the cytoplasm but not on the nucleus. No diffusion of [³H]Ch was observed in the extracellular space (Fig. 2). Some neurons were both [³H]Ch radiolabelled and ChAT immunoreactive (Fig. 3). Double labelled neurons (ChAT immunoreactive and radiolabelled) were observed more frequently at light microscopic level than at electron microscopic level. This seems to be due to unsatisfactory penetration of antibodies into the Vibratome section, while radiolabelling could be found at any depth of the section.

Discussion. Ionic fixation of ACh-like cations^{13)–15)} was performed in order to avoid the diffusion of [³H]Ch or its derivatives accumulated in the cell body after selective retrograde axonal transport. This ionic fixation allowed us to combine the autoradiography with ChAT ICC on the same tissue sections at both the optic and ultrastructural level. Thus, the autoradiography of [³H]Ch performed with silicotungstic acid (ionic fixation) provides an important advantage over conventional autoradiography of [³H]Ch without ionic fixation realised by others investigators.¹⁰⁾

The specificity of the retrograde axonal transport of [³H]Ch was proved by the presence of radiolabelled neurons in the DBB and their absence in the midbrain raphe. The cell bodies of the serotonergic neurons localised in the midbrain raphe, send their projections to the OB and can be selectively radiolabelled following [³H]5-HT injections into the OB.¹¹⁾ Most of the [³H]Ch radiolabelled neurons in the DBB were ChAT immunoreactive, confirming the previously described cholinergic projections to the OB.^{17),18)} Some ChAT immunoreactive neurons of the DBB^{20),21)} were not [³H]Ch radiolabelled, indicating that their projections sites are not the OB.

At the electron microscopic level, the silver grains were concentrated mainly in the perikarya (Figs. 2, 3). The [³H] choline or its derivatives observed in our experiments, are believed to be different from the membrane incorporated lipid compounds,²²⁾ since in our case its preservation was not possible without ionic fixation. The fact that some radiolabelled neurons were not ChAT immunoreactive could be explained by insufficient accumulation of ChAT after colchicine treatment or lack of penetration of ChAT antibodies. However, both ChAT immunoreactivity and [³H]Ch radiolabelling were often observed on the same neurons (Fig. 3). This finding presents an important technical advantage for chemical identification of afferences to cholinergic soma, as it was recently

described for the serotonergic neurons receiving GABA immunoreactive terminals.¹²⁾

In conclusion, the method we described in this paper provides the following advantages: 1) identification of cholinergic neuronal pathways by sensitive autoradiography of [³H]Ch or its derivatives after retrograde axonal transport in perikarya, and by simultaneous ChAT ICC performed on the same neuron at light and electron microscopic level; 2) possibility to study the co-localization of the neurotransmitters, peptides or enzymes in cholinergic pathways and 3) possibility to identify the chemical nature of afferences on cell bodies of a defined cholinergic pathway.

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