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Simultaneous Identification of Tyrosine Hydroxylase and Choline Acetyltransferase Neurons

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Abstract: Tyrosine Hydroxylase (TH) and Choline Acetyltransferase (ChAT) immunoreactivity were examined in the adult mouse brain by a modified double labelling immunofluorescence method. Mice were transcardially perfused with saline followed by fixative (4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4). Frozen brain was cut at 30μm thickness for light microscopy. All staining procedures were conducted under room temperature. Dopaminergic neurons were identified from Cy2-coupled-donkey-anti-rabbit antibody and cholinergic neurons by Cy3-coupleddonkey-anti-goat for ChAT. The cell bodies of both TH- and ChAT-positive neurons with their respective axonal and dendritic processes were easily visible. This method proved reliable for simultaneous demonstration, in the same brain tissue sections, of TH- and ChAT-containing neurons.

Key words: ChAT, Tyrosine hydroxylase, brain, immunoflurescence, identification, neurons

INTRODUCTION

Immunofluoresence is a biological assay combining the use of antibodies and fluorescent molecules for the detection of specific targets in cells and tissues. This is based on the high selectivity and affinity of antibodies for their antigens as specific cellular constituents, notably proteins (Fritschy and Hartig, 2001). Cholinergic and dopaminergic neurons belong to two neuronal pathways which are associated with neurodegenerative diseases. There is extensive evidence that several neurotransmitter systems within the Basolateral Amygdala (BLA) influence memory consolidation (Lalumiere *et al*., 2004). Basal ganglia functions are said to rely on a homeostatic dopamine-acetylcholine interaction (Chang, 1988). Maintenance of balance between cholinergic and dopaminergic tone within the basal ganglia has long been appreciated as being central to the clinical management of many extrapyramidal motor disorders (Weiner *et al*., 1990). The anatomical evidence for identity of the receptor subtypes which mediate the diverse effects of muscarinic and dopaminegic drugs on basal ganglia function has been reported by Weiner *et al*. (1990). Activity in the cerebral cortex is modulated by both cholinergic cells of the forebrain and monoaminergic axons which have axons in the brainstem (Mesulam *et al*., 1983). Lalumiere *et al*. (2004) reported that dopaminergic activation within the BLA modulates memory consolidation and that the modulation involves activation of both D1 and D2 receptors and concurrent activation of beta-adrenergic and cholinergic influences within the BLA.

Pharmacological approach to cholinergic disorders in Alzheimer Disease (AD) and dopaminergic disorder in Parkinsion's Disease (PD) are considering the interaction between cholinesterase and Monoamine Oxidase (MAO-B) activities (Weinstock *et al*., 2000). Tyrosine Hydroxylase (TH), the rate limiting enzyme in the conversion of tyrosine to DOPA, is a reliable marker for dopaminergic neurons while Choline Acetyltransferase (ChAT) is for cholinergic neurons. Usually the goal of most immunocytochemical study is to identify an immunoreactive neuron. This study was therefore designed to concurrently visualise dopaminergic and cholinergic neurons in the same brain tissue sections using a double labelling immunofluorescence technique.

MATERIALS AND METHODS

Animals: Twenty-four month old normal male mice were used for this study. The animal treatment was according

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to the rules approved by the Laboratory Animal Care and Use Committee at the University of Leipzig, Germany.

Perfusion fixation: Under deep anaesthesia transcardial perfusion was carried out with saline, followed by 50 mL of fixative (4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4) using a peristaltic pump. The brains were carefully removed from the skull and postfixed in the same fixative overnight at 4°C. Cryoprotection was carried out by putting the brain tissue in a 30% sucrose solution before sectioning.

Sectioning: This was done using a freezing microtome (Leica SM2000R model, Germany). The brain was frozen at -40°C. The frozen brain was cut in the coronal plane (usually in the rostral caudal direction) at 30 μm thickness for light microscopy. Five serial sections were collected in each vial containing 0.1M phosphate buffer. Sections were then stored at 4°C prior to staining.

Staining: Free-floating sections with areas of interest were selected for staining using the rat brain atlas of Paximos and Waston (1986).

Double labelling immunofluorescence: All procedures were carried out with constant agitation. Free floating brain sections were extensively rinsed with Tris-Buffered Saline (TBS, pH 7.4) and blocked with 5% normal donkey serum in TBS also containing 0.3% Triton X-100 (NDS-TBS-T) for 1 h at room temperature. Subsequently the sections were incubated with a cocktail of primary antibodies using goat-anti-choline acetyltransferase (ChAT) AB 144 (Chemicon, Hofheim/Taunus, Germany, 1:50) and rabbit-anti-tyrosine Hydroxylase (TH) AB 152 (Chemicon, Hofheim/Taunus, Germany, 1:70) in blocking solution (NDS-TBS-T) overnight at room temperature.

After rinsing with TBS 3 times for 10 min, the sections were incubated for 1 h at room temperature with secondary antibodies using Cy3-coupled-donkey-antigoat (1:50) for ChAT and Cy2-coupled-donkey-anti-rabbit (1:70) for TH. (Each 20μg mL^{-1} TBS+2% bovine serum albumin; Jackson ImmunoResearch, WestGrove, PA, USA-German Supplier: Dianova, Hamburg). Thereafter, the sections were rinsed 3 times for 10 min in TBS (pH 7.4) and once for 1 min in distilled water. Sections were mounted on glycerol-albuminized slides, air dried, passed through toluene for a minute and coverslipped with Entellan? (Merck, Germany).

Control sections were blocked with 5% normal donkey serum + 0.3% Triton X-100 in

TBS (+ Sodium azide for stability) and the primary antibodies omitted.

Evaluation: Analysis of stained brain sections and photography were carried out using a Zeiss Axioplan 2 light microscope equipped with fluorescence filters (Oberkochen, Germany) and fitted with a Sony DXC-930P colour video camera system.

RESULTS AND DISCUSSION

Tyrosine Hydroxylase (TH) containing neurons were visualised as green fluorescence under blue light while Choline Acetyltransferase (ChAT) containing neurons were identified as red fluorescence under green light. The combination of Cy2 green fluorescent dye excited by blue light and Cy3 red fluorescent dye excited by green light produced clearly visible neuron cell bodies and axonal and dendritic processes of both dopaminergic and cholinergic neurons in the same brain tissue sections. Distinct neuronal populations were stained. Dopaminergic neurons immunoreactive for TH were observed predominantly in thethalamus, periventricular and substantia nigra while cholinergic

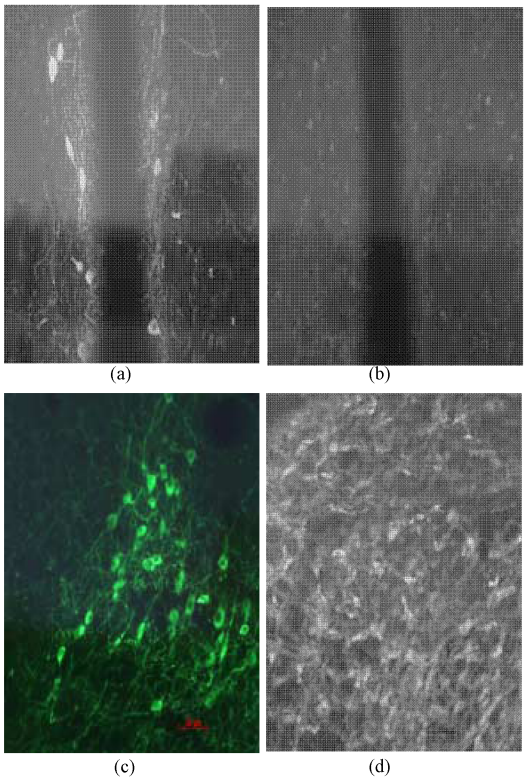

Fig. 1: In the periventricular region, substantia nigra and ventral tegmental area, dopaminergic neurons with axonal and dendritic processes were visible as green fluorescence (A, C and D). No ChAT positive neurons was visualised in the periventricular region under red fluorescence (B)

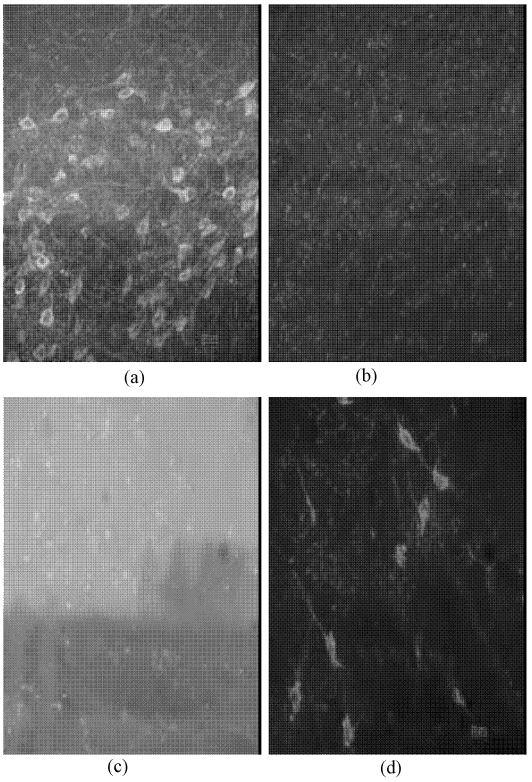

Fig. 2: In the thalamus, dopaminergic neurons were easily identified (A) under green fluorescence while immunofluorescence reactivity for ChAT neurons under red fluorescence was negative (B). In the striatum, no TH -containing (dopaminergic) neurons was observed in the striatum under green fluorescence (C). On the contrary ChAT-positive neurons were readily visible under red fluorescence (D)

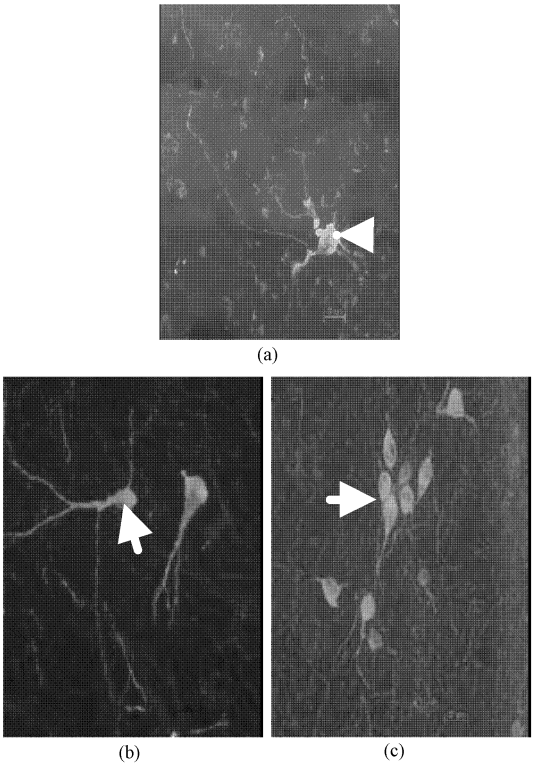

Fig. 3: Tyrosine Hydroxylase (TH) neurons in selected regions of the brain using alternate dyes and red fluorescence in mouse (A and B) and in rat (C). Arrows show the perikaryon

neurons immunoreactive for ChAT were visible in the striatum (Fig. 1 and 2). Overnight incubation with the primary antibodies at room temperature also proved ideal for this method. A comparison in the mouse and rat with reversal of the fluorescence dye also showed distinct neuronal structure (Fig. 3).

The results of this study confirm the suitability of immunoflorescence staining for simultaneous labelling of multiple targets, taking advantage of the spectral selectivity of most modern fluorochomes that allows different signals to be discriminated by using appropriate filter combinations (Hartig and Fritschy, 2001). The immunofluorescence of both TH and ChAT neurons can be described as strong.

The double labelling immunofluorescence protocol adopted did not seem to have any negative influence on the staining of either of the antigens in the same brain sections. Simultaneous use of Fluorescein Isothiocyanate (FTC)-conjugated antibodies enabled a rapid confirmation that the primary antibodies were properly bound within the tissue sections (Chang, 1988), thereby eliminating time needed to labelling these neurons on individual basis. Morphological appearance of the ChAT-immunoreactive axons were generally thicker than those of TH-immunoreactive axons. Cell bodies of the ChAT positive neurons were larger in size than those of TH positive neurons. This technique provides a simple protocol for co-localisation of ChAT and TH immunoreactive neurons.

Our findings did also fulfill the requirement that there should be minimal interference of the conjugate dye with the antibody binding properties (Hartig and Fritschy, 2001). Varying the dyes in both rat and mouse section indicate that species differences within rodents did not seem to negatively influence the result when this technique was adopted. Although, convergence of cholinergic and dopaminergic neurotransmission within

the basal ganglia occur in the striatum (Weiner *et al*., 1990) and in the substantia nigra (Bolam *et al*., 1991), synaptic interactions between both ChAT and TH could not be visualised with this technique. This may require further exploration of co-localisation techniques in addition to the physiological properties of the synapses.

Double labelling immunofluorescence proved to be very reliable for co-localisation of TH- and ChAT-containing neurons in the same brain tissue sections. This technique will be useful in analysis of brain sections in neurodegenerative diseases of Parkinson's Disease (PD) and Alzheimer's Disease (AD).

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