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Colocalization of muscarinic and nicotinic receptors in cholinceptive neurons of the suprachiasmatic region in young and aged rats

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In the present study muscarinic and nicotinic cholinergic receptors in the SCN region were demonstrated and analyzed, employing monoclonal antibodies to purified muscarinic and nicotinic cholinergic receptor proteins. A near-total colocalization of the two acetylcholine receptor subclasses in cholinceptive neurons of the SCN area was found. The antibodies applied to aging rat brain (at 30–34 months) revealed a clear decrease in immunoreactivity in senescence albeit with a high level of individual variability. Furthermore, in 8 out of 10 aged animals examined a considerable increase of astrocytes possessing muscarinic cholinergic receptors was observed.

The suprachiasmatic nucleus (SCN) of the hypothalamus has been identified as a primary pacemaker for circadian rhythms in mammals and has been subject to extensive investigation¹². Several studies point at a role of the neurotransmitter acetylcholine (ACh) in the time-keeping system of the SCN. It was shown that both the ACh-degrading enzyme acetylcholinesterase (AChE) and the ACh-synthesizing enzyme choline acetyltransferase (ChAT) are present in the SCN and its surrounding neuropil^{1,6,10,17}. Furthermore, the cholinceptive character of the SCN is indicated by the electrophysiological responsiveness of neurons to ACh in both in vivo and in vitro preparations^{9,15}. Also, application of the non-selective cholinergic agonist carbachol shifts the circadian activity rhythms of rodents in a phase-dependent manner^{3,13,25}.

It is obvious that neurons in the SCN that receive a cholinergic innervation should possess cholinergic receptors of the muscarinic (mAChR) or nicotinic (nAChR) type, or both. Autoradiographic ligand binding studies utilizing tritiated quinuclidinylbenzilate (QNB)⁸ or propylbenzilylcholine mustard (PrBCM)¹⁹ and iodinated α -bungarotoxin (α -BTX)^{4,16} revealed both muscarinic and nicotinic receptors in the SCN.

The present study was carried out to analyze the cholinceptive characteristics of the SCN by immunocytochemical single and double labeling experiments employing monoclonal antibodies raised against purified

mAChR-proteins and nAChR-proteins in the rat. As part of our general interest in the effect of aging on the cholinergic system⁵ we also studied its receptors in the SCN in senescence. A preliminary report of this study was presented elsewhere²³.

The findings presented here were obtained from 10 young adult (3 months) and 10 aged (30–34 months) male Wistar rats. The animals had been housed individually or in small groups at a light/dark schedule of 12/12 h, with lights on at 8.00 h. Fixation of the brain was carried out in the early light period by transcardial perfusion with 300 ml fixative composed of 3% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer (PB) (pH 7.4). Immediately upon fixation the fixative was removed by a perfusion with 150 ml 10% sucrose in 0.1 M PB. The brains were cryoprotected by overnight storage at 4 °C in 30% sucrose in 0.1 M PB and subsequently coronally sectioned on a cryostat microtome at a thickness of 20 μ m.

Muscarinic and nicotinic receptor proteins were visualized by means of the monoclonal antibodies M35 and WF6, respectively. Extensive descriptions of production, characterization and immunocytochemical applications of these antibodies have been reported previously^{20–22}. For single labeling, the brain sections were incubated 24–48 h at 4 °C in the primary antibody solution in phosphate-buffered saline (PBS) containing mouse IgM anti-mAChR (M35, 1:2000) or mouse IgG anti-nAChR

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(WF6, 1:10). After rinsing, the sections were exposed to biotinylated rabbit anti-mouse IgM (Zymed) or biotinylated sheep anti-mouse IgG (Amersham), respectively, followed by incubation in Streptavidin-HRP (Zymed). Double-labeling experiments for the study of colocalization of both receptor types were carried out with fluorescence techniques. For dual labeling, the sections were exposed to one of the primary antibodies as for single labeling. For WF6 the primary antibody step was followed by goat anti-mouse Phycoerythrin (Tago). After completion of the WF6 staining, the sections were incubated with M35 followed by biotinylated rabbit anti-mouse IgM and FITC-conjugated Streptavidin (Zymed). Standard control experiments for single labeling were performed by omission of the primary antibody step, or by replacing the primary antibody by normal mouse serum. In all cases the controls yielded negative results, i.e. absence of any detectable labeling.

Single labeling for muscarinic (M35) and nicotinic (WF6) acetylcholine receptors demonstrated the presence of both receptor types in cholinceptive neurons in the SCN region (Fig. 1). In this area loosely scattered immunoreactive cells with their dendritic processes were found, however, without a clear rostrocaudal distribution pattern. The muscarinic and nicotinic cholinceptive neurons were not restricted to the SCN boundaries, but were spread over the entire suprachiasmatic area. The number of immunoreactive cells was somewhat higher in the dorsolateral part of the region. Some immunolabeled cells were present within the optic chiasm. The highest density of cholinceptive neurons was found in the middle portion of the SCN. In this region of the SCN, Cresyl violet-counterstained sections indicated that in single-labeling experiments approximately one out of 10 cells were immunoreactive for M35 or WF6. The cells showing immunoreactivity for M35 or WF6 predominantly had a somewhat elongated, bipolar morphology, although some round, multipolar cells have been found as well (Fig. 1).

Adjacent sections stained for M35 and WF6 revealed identical staining patterns and cell types, suggesting that the cholinceptive cells in the SCN contain both types of acetylcholine receptors. Employing double-labeling fluorescent methods it became clear that nearly all cholinceptive neurons in the SCN region possess both muscarinic and nicotinic receptors (Fig. 2). Only occasionally could single labeled neurons for M35 or WF6 be detected.

Immunoreactivity to muscarinic and nicotinic receptor proteins in single-labeling procedures was also investigated in 10 aged rats. In two aged animals we observed an almost complete lack of M35 and WF6 immunoreactivity in the SCN region, while in 3 other cases the

Fig. 1. Photomicrographs of the localization of cholinceptive neurons in the SCN region. A: immunoreactivity to muscarinic receptor protein, showing cell bodies (arrowheads) and dendritic profiles. B: immunoreactivity to nicotinic receptor protein, approximately at an identical level of the SCN region, showing cell bodies (arrowheads) and dendritic profiles. Asterisks: blood vessels; OC, optic chiasm; 3V, third ventricle. Scale bar = 50 μ m.

number and density of neuronal labeling was considerably decreased (Fig. 3). In 5 other animals, however, no obvious effect of aging on cholinergic receptor labeling could be detected with the currently used methods, indicating a high level of variation in aging. The observed decrease in immunoreactive cells in the SCN area in some of the senescent rats was not the result of technical failure such as reduced antibody penetration, since there was no difference in immunostaining in other cholinceptive regions in the same sections. From these observations it may be concluded that the cholinergic receptors in the SCN area display a high susceptibility to the aging process, however, with a considerable individual variation.

Apart from the influence of aging on labeling with M35 and WF6 in neurons of the SCN, there was also a striking aging effect on M35 immunoreactivity in astrocytes. In

Fig. 2. Fluorescence images of double-labeling for muscarinic and nicotinic receptor protein in the middle portion of the SCN. Muscarinic cholinergic neurons are visualized by FITC (A,C), and nicotinic cholinergic neurons by Phycoerythrin (B,D). Neurons clearly displaying both receptor types are indicated by arrowheads. Asterisks: blood vessel. Scale bar = 25 μ m.

young adult animals the staining of astrocytes with M35 is rare. In the aging brain, however, in 8 out of 10 animals examined, a considerable increase of M35-labeled astrocytes was observed (Fig. 3A).

The currently presented data obtained with immunocytochemical methods employing monoclonal antibodies to mAChR and nAChR proteins revealed both ACh receptor types to be present in neurons in the SCN area. These findings agree well with previous autoradiographic ligand binding studies of the SCN. Specific binding of muscarinic receptor ligands in the SCN and its surrounding neuropil was reported for tritiated QNB and PrBCM^{8,19}. A similar pattern of high binding in the SCN and the adjacent dorsolateral SCN area is described for the nicotinic receptor antagonist α -bungarotoxin (α -BTX)¹⁶. Besides the results obtained with α -BTX, Mason¹¹ found stained processes and immunopositive neurons in the SCN using a monoclonal antibody, raised

against the α -subunit of nicotinic receptors isolated from the electric eel. However, no detailed study was made of this nucleus.

Effects of cholinergic manipulation of the SCN acting through both types of ACh receptor subclasses have been reported. For example, the nicotinic antagonist mecamylamine blocks light-induced phase shifts in circadian activity rhythm in the golden hamster⁷, and intravenous administration of nicotine has an excitatory effect on SCN cells¹⁴. Studies in brain slice preparations of the SCN reveal inhibitory effects on ACh application by the selective muscarinic antagonist atropine⁹. Since ACh as the endogenous messenger is a non-selective agonist for nicotinic and muscarinic receptors, both receptor types which are colocalized within the same neurons, will differentially contribute to the SCN time-keeping system.

In the brain of senescent rats, a striking effect of the

Fig. 3. Photomicrographs of muscarinic cholinergic neurons in aged rats. A: immunoreactivity for muscarinic receptor protein in neurons (arrowheads) of the SCN region decreased slightly. Increased numbers of immunoreactive astrocytes (arrows) were observed in most aged rats. B: in 3 of 10 aged rats only some weak-immunopositive neurons could be found (arrowheads). 3V, third ventricle; OC, optic chiasm; asterisk: blood vessel. Scale bar = 50 μ m.

aging process on M35 and WF6 immunoreactivity in the SCN region could be observed in half of the animals studied. The effects, however, showed a high degree of individual variation ranging from a near total absence to only a minor deterioration of immunostaining. It remains unclear whether the decrease or lack of cholinergic receptor staining in part of the aging animals is the result of cell death or break-down of receptor protein. In agreement with earlier studies^{2,18}, there was no obvious decrease of total cell numbers in the SCN of our senescent rats, suggesting a specific aging-associated anomaly of cholinergic receptor expression. This tentative receptor break-down is clearly limited and thus characteristic of the SCN area, since it was not observed in other cholinergic regions in the same brain sections. The observed decrease in cholinergic receptor immunoreactivity in the SCN in part of the aged animals may be associated with the reported deterioration of the timekeeping system of the SCN leading to a deranged circadian rhythm²⁴.

In nearly all aged animals an increase of M35-immunoreactive astrocytes in the SCN was observed. Taking the number of immunoreactive astroglia cells into consideration it is evident, however, that only a minor proportion of astroglia, even in the aging condition, expresses immunoreactivity to muscarinic receptors. In young adult cases such astrocyte staining was mainly present in the superficial layers of cerebral cortex and hippocampus²². The function of cholinergic neurotransmitter receptors in astrocytes, however, remains unclear.

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