

**Thesis for the degree of doctor of philosophy (Ph.D.)**

**Studying the effects of the cholinergic modulation on giant cells and astrocytes of the rat cochlear nucleus**

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## **Aims**

The cochlear nucleus (CN) is the first area of auditory information processing at brain stem level. Previous results have proved the amplifier role of cholinergic modulation in the CN, but the cellular processes of this mechanism remain unknown. The experimental work in this thesis is a part of the efforts of our laboratory to get information about the structure and function of rat CN. In connection with this theme my main aim was to study the phenomena related to the cholinergic modulation of the nucleus.

In the first part of the work I have studied the electrophysiologic characteristics of giant cells using the patch-clamp technique on slices prepared from the dorsal part of the nucleus (DCN). During the experiments I tried to fulfil the following tasks:

1. to study the expression of the muscarinic acetylcholine (ACh) receptors (mAChR) in the CN, at mRNA level in different ages;
2. to identify giant cells and analyze the effect of the cholinergic agonist, carbamylcholine (CCh) on their electrophysiological properties;
3. to investigate the inhibitory and stimulatory postsynaptic currents on giant cells evoked by electrical stimulation from the superficial and deep layer of teh DCN, respectively, and to analyze the effects of CCh on these postsynaptic currents;
4. to explore the postsynaptic consequences of using CCh.

Recently the astrocytes have been shown to be a third active participant in chemical synapses between neurons that can influence the transmission taking place here. Thus, during the examination of a brain area it is important to investigate the astrocytes located here, too. Consequently, in the second part of this work I have studied the mechanism of the cholinergic modulation in primary astrocyte cultures isolated from the CN with the investigation of the CCh-induced cytoplasmatic calcium concentration changes. My tasks were:

1. to produce primary astrocyte cell cultures and to indentify the cells;

2. to detect the expression of the different mAChR subtypes in astrocytes at mRNA and protein level;

3. to analyze the pharmacological modulation and kinetic characteristics of CCh induced cytoplasmatic calcium transients.

## **Introduction**

### **CN and the giant cells**

The CN is the first brain stem area where decoding of the auditory information takes place. The nucleus can be divided into dorsal (DCN) and ventral (VCN) parts. The electrophysiological experiments presented in this work report about the cholinergic modulation of the giant cells of DCN. These neurons are truly big, they have cell bodies with some 50  $\mu\text{m}$  in diameter, and their nuclei and nucleoli are similarly oversized. The dendritic arborization of the giant cells covers a huge area in the CN, often extending to 500-600  $\mu\text{m}$ . Thus these cells can penetrate both the DCN and the VCN. As a result of the large area covered by their dendrites they have a huge receptor field, hence they are capable of integrating inputs from a wide band of the acoustic nerve fibers. On the basis of the information yielded by investigating the responses of the giant cells to the stimulation of either the acoustic nerve root or the anterior VCN (AVCN), it seems that the giant cells are activated both directly by the acoustic nerve fibers and by excitatory interneurons of the VCN. There are several lines of evidence indicating that cholinergic modulation is present in the CN, and it is involved in adjusting the activity of the CN neurones.

### **The muscarinic cholinergic receptors**

The muscarinic cholinergic receptors (mAChR) are metabotropic receptors and have five subtypes (later on are mentioned as M1-M5). The M1, M5 and M3 receptors can activate the phospholipase-C (PLC)-inositol-trisphosphate ( $\text{IP}_3$ ) pathway through G proteins,

resulting in intracellular calcium concentration increases. In the body, including the central nervous system all mAChR subtypes show specific expression patterns and take place in the modulation of several cholinergic effects.

### **Glial cells in the nervous system; the structural and functional characteristics of the astrocytes**

In previous decades it has been confirmed that astrocytes exert effects on synaptic transmission between neurons. Glial cells have two main groups, the peripheral and central populations. One subgroup of the central glial cells is the macroglia, named also as astrocytes. These cells have the most general occurrence and the most complex function. Their functions are supporting the neuronal units by their intermediate filaments (eg. Glial Fibrillary Acidic Protein; GFAP), their branches ensheath the synapses and help to eliminate neurotransmitters and potassium ions from extracellular space that is very important to retain the physiological function of neurons.

In the last decades it has been noted that the astrocytes express several types of neurotransmitter receptors (e.g. glutamate, catecholamines, GABA, ATP and ACh) on their surface membrane. Most of these receptors are metabotropic ones which can modulate cell functions mainly through the modulation of intracellular calcium changes. Moreover, astrocytes release neurotransmitter-like substances (eg. glutamate, TNF $\alpha$  or ATP). These substances are the so-called gliotransmitters, which can influence neuronal synaptic transmission. As a consequence of the above findings, a new concept of the synaptic machinery has been created, in which the synapse is formed by three functional elements. Two of these are the pre- and postsynaptic membranes of the neurons while the third part is given by the surrounding astrocyte.

## **Materials and methods**

### **Preparation of the cochlear nucleus slices**

The experiments were conducted on 10- to 14-day-old ( $n = 193$ ). Wistar rats of both sexes. Briefly, the animals were killed by decapitation and the splitted brains transferred into ice-cold low-sodium aCSF. The cerebellum was removed from both tissue pieces, and 200- $\mu\text{m}$ -thick sagittal slices were cut using a vibratome. The slices were kept in the incubation chamber ( $37^{\circ}\text{C}$ ) and continuously oxygenated until the start of the experiments.

### **Data recording and analysis**

The slices were placed into the recording chamber, and they were continuously superfused with aCSF bubbled with a mixture of 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . Patch pipettes were filled with a solution containing biocytin. To prevent action potential firing of the investigated neurone, while leaving the firing of all other neurones unaffected, QX314-chloride (an inhibitor of the voltage-gated  $\text{Na}^+$  channels) was applied in the pipette solution. Whole-cell patch-clamp recordings were conducted using an Axopatch 200A amplifier. In the voltage-clamp configuration, the holding potential was usually  $-60$  mV, and either spontaneous or evoked postsynaptic currents (PSCs) were recorded at this membrane potential. The evoked PSCs were induced by using a monopolar stimulating electrode that was connected to a stimulator device. During stimulation, the pulses were delivered with a frequency of 50 Hz and the amplitude of the stimuli was set to the minimum value which was just capable of eliciting PSCs with a minimal number of failures ('minimal stimulation'). In some experiments the current-clamp configuration of the patch-clamp technique was employed to detect spontaneous and CCh-induced membrane potential changes, including action potentials.

Inhibitory PSCs (IPSCs) were evoked when glutamatergic neurotransmission was blocked by the application of 10  $\mu$ M NBQX and 50  $\mu$ M D-AP5; whereas excitatory PSCs (EPSCs) were recorded in the presence of 1  $\mu$ M strychnine and 10  $\mu$ M bicuculline. During the analysis the paired-pulse ratio (PPR) was calculated as the ratio of the amplitude of the second to the first PSC. To establish the possible presynaptic target(s) of the CCh application, the coefficient of variation (CV) was calculated ( $CV = SD/mean$ ). A significant reduction in the  $1/CV^2$  value indicated that the drug administered had presynaptic target(s).

### **Biocytin labelling and visualisation of the labelled neurones**

During the electrophysiological recordings, the investigated neurones were filled with biocytin. After the measurements, the slices were fixed overnight (4% paraformaldehyde in 0.1 M phosphate buffer). Fixed slices were permeabilised in phosphate-buffered saline (PBS), supplemented with 0.1% Triton X-100. After permeabilisation, the slices were labelled with streptavidin conjugated Alexa488. At the end of the procedure the cells were visualised using a Zeiss LSM 510 confocal laser scanning microscope.

### **Enzymatic isolation and tissue culturing of astrocytes**

7–9- day-old Wistar rats were decapitated, their brain removed in ice-cold dissecting solution (DS). The enzymatic dissociation of the CN was achieved by employing DS containing trypsin. The feeding solution (FS) was Minimum Essential Medium (MEM) with 10% fetal bovine serum (FBS). Individual cells were obtained by applying very gentle mechanical agitation with fire-polished Pasteur pipettes. The cell suspension was diluted to a density of 100,000 cells/ml and 0.5 ml volumes of this suspension were transferred onto coverslips situated in a 24-well culturing plate. The FS was changed on the following day and every other day thereafter.

## **Immunocytochemistry**

Immunocytochemistry was usually conducted on 7–10-day-old astrocyte cultures by which time 70–80% confluence level was reached. The cells were fixed, then permeabilization was carried out by bathing the cells in PBS containing Triton X-100. Aspecific binding was prevented then the astrocytes were incubated with the primary antibodies. For investigation of the specificity of primary antibodies preadsorption control measurements were performed. In the next day the cells were rinsed in PBS, then incubated with the appropriate secondary antibody, and mounted by DAPI. The immunoreactions usually were investigated employing a fluorescence microscope. In some cases confocal microscopy images were acquired using a Zeiss LSM 510 microscope.

## **Intracellular calcium imaging**

### Theoretical background

For the measurement of intracellular  $[Ca^{2+}]_i$ , the acetoxymethylester form of the fluorescent dye Fluo-4 (Fluo-4 AM) was employed. The  $Ca^{2+}$  indicator dye, Fluo-4 after binding the  $Ca^{2+}$ , increase its fluorescence emission in the whole spectrum (the excitation and emission maxima of Fluo-4 are 494 and 515 nm, respectively), thus this dye is not suitable for ratiometric measurements. In our experiments we did not make effort to determine the concentration of the ic.  $Ca^{2+}$ , only the fluorescence intensity, proportional to the  $Ca^{2+}$  concentration, was given in arbitrary units (AU).

### Mechanisms of calcium imaging, and data analysis

The stage of the microscope held an experimental chamber, in which continuous flow of the incubating HaCSF was applied. At room temperature the coverslips with the astrocytes were placed into this chamber. Before starting the recordings using the software, several “regions of interest” (ROIs) were defined. One of the ROIs was cell-free, and the fluorescence intensity measured here was considered as “background fluorescence” (Fbg).

The program calculated the average fluorescence intensities of the ROIs. To calculate the changes of the average Ca<sup>2+</sup>-related fluorescence intensities in the “kinetic view” mode, first the Fbg value was determined from the cell-free ROI, then the resting fluorescence intensities (Frest) of the cell-containing ROIs were obtained as the average of the data recorded during a period of 10 s prior to adding any drugs. The peaks of the fluorescence transients were found by calculating the average of three consecutive points and identifying those points that gave the highest average value (Fmax). The amplitudes of the Ca<sup>2+</sup>-related fluorescence transients were expressed relative to the resting fluorescence ( $\Delta F/F$ ) and were calculated by the following formula:

$$\Delta F/F = (F_{\max} - F_{\text{rest}}) / (F_{\text{rest}} - F_{\text{bg}})$$

where the various symbols have the meanings defined above. For comparison of the consecutive transients of the same astrocyte, the relative amplitudes were also determined. For calculating  $\Delta F/F_{\text{rel}}$ , the following formula was applied:

$$\Delta F/F_{\text{rel}} = (\Delta F/F_{\text{second}}) / (\Delta F/F_{\text{first}})$$

where  $\Delta F/F_{\text{first}}$  is the peak  $\Delta F/F$  value during the first application of CCh, and  $\Delta F/F_{\text{second}}$  is the maximal  $\Delta F/F$  value during the second application of CCh, alone or together with another compound.

### **Quantitative polymerase chain reaction (Q-PCR)**

In some of the experiments Total RNA was isolated from the cochlear nuclei of rats aged between 3 and 80 postnatal days. In other experiments Total RNA, arised from confluent cultures of astrocytes was isolated by using RNeasy Plus Mini Kit. The volume and purity of the isolated RNA were verified with a Nano-Drop 1000 Spectrophotometer. Prior to the reverse transcription DNase treatment had to be performed. A 1 $\mu$ g sample of the total RNA was reverse transcribed into cDNA by using AMV reverse transcriptase and random primers. Q-PCR was carried out on an ABI PRISM 7000 Sequence Detection System, using

the 51-nuclease assay. The PCR amplification was carried out using by TaqMan primers and probes. Transcripts of  $\beta$ -actin were used as internal control when applying relative quantization technique ( $\Delta$ CT) to determine the relative amounts of the target cDNAs.

## **Statistics**

The averages are given as mean  $\pm$  S.E.M. The averages were compared using the appropriate forms of Student's t-test. The level of significance was set at  $p < 0.05$ .

## **Results**

### **I. Effects of cholinergic neuromodulation on giant neurones of the rat dorsal cochlear nucleus**

#### Identification of the giant cells

The morphology of the giant cells has been described earlier. For ensuring correct and unambiguous identification of the investigated neurones, each cell was labelled with biocytin (only a single neurone was investigated in every slice prepared). The morphology of the cells was assessed on the basis of the reconstructed confocal images. Giant neurones were identified on the basis of their large (30–50  $\mu$ m in diameter), polygonal soma, which gave rise to several (four or more) major dendritic branches that projected to all layers of the DCN, and showed extensive arborisation in the superficial layer. Altogether 294 DCN neurones were investigated. On the basis of the above morphological criteria, 184 of them were identified as giant cells.

#### Cholinergic modulation increased firing frequency of giant neurones

Current-clamp measurements were conducted on 36 positively identified giant neurones, whose resting membrane potential was  $51 \pm 2$  mV. Three of these cells produced action potentials even in the absence of stimulation. When 50  $\mu$ M CCh was applied the resting membrane potential of the giant cells depolarized and all cells produced high-frequency action potential firing. Our observation suggests that the CCh-induced

depolarization was only partly responsible for the increased activity of the giant cells. The presence and physiological relevance of the cholinergic modulation of the giant cells of the cochlear nucleus was confirmed also by investigating the effects of neostigmine. Neostigmine application (50  $\mu$ M) induced a depolarization of  $8.5 \pm 2.7$  mV in the investigated cells (n = 5).

#### IPSCs evoked from the superficial and the deep layers had different sensitivities to cholinergic stimuli

To record IPSCs from the giant neurones, excitatory inputs were blocked by the simultaneous application of the glutamate receptor antagonists NBQX and D-AP5. Spontaneous IPSCs (sIPSCs) were observed in 146 out of the 162 giant neurones. In the presence of 1  $\mu$ M strichnine and 10  $\mu$ M bicuculline, the sIPSCs were completely abolished (n = 93). While strychnine alone abolished the sIPSCs completely, bicuculline reduced both the frequency and the amplitude of the sIPSCs but it did not inhibit them entirely. These observations indicate that the inhibitory fibres that terminated on the giant cells, were mostly glycinergic with a smaller contribution of GABAergic projections.

Differences had been detected when IPSC trains were evoked by stimulating the superficial and the deep layers of the DCN, respectively. Stimulation of the superficial layer resulted in an IPSC train that showed pronounced short-term depression (STD), unlike the IPSCs evoked from the deep layer, where neither short-term depression nor short-term facilitation (STF) could be observed.

The two types of the evoked IPSCs showed clearly different sensitivities to the application of CCh, too. The presence of the cholinergic agonist did not affect either the amplitude of the first IPSC, or the PPR value if the train was evoked from the superficial layer. In the case of deep-layer stimulation, however, CCh application markedly decreased the amplitude of all IPSCs in a train. The effect of CCh on the evoked IPSC amplitudes proved to be reversible.

In the next step of the experiments, the identity of the cholinergic receptors involved in the mediation of the CCh action on the IPSCs evoked by the stimulation of the deep parts of the CN was investigated using various muscarinic receptor antagonists. The amplitude of the CCh-sensitive component of the first IPSC was calculated as the difference between the amplitudes of the IPSCs measured in the control solution and in the presence of CCh. This difference was expressed as a percentage of the first amplitudes of the IPSCs measured in the control solution (in further; control).

When the effects of the various muscarinic (M-type) receptor antagonists were compared the same procedure was employed, and the percentage values of the CCh-sensitive IPSC magnitudes were determined. 10  $\mu\text{M}$  atropine was capable of almost completely preventing the effects of CCh on the IPSCs so it was concluded that the cholinergic modulation of the IPSCs was mediated mostly via muscarinic receptors. Pirenzepine, AF-DX 116 and tropicamide did not significantly modify the effect of CCh. These observations indicated that M1, M2 and M4 receptor activations had no significant roles in mediating the CCh effect. Application of the M3-specific antagonist 4-DAMP, however, effectively opposed the CCh action similarly to that when CCh was applied in combination with atropine.

#### EPSCs were modulated by cholinergic stimulation via a presynaptic mechanism

In the next step of the experiments, the effects of CCh application on the EPSCs recorded from the giant cells were determined. In these cases the IPSCs were eliminated by the combined application of strychnine and bicuculline. It was shown that CCh (50  $\mu\text{M}$ ) reduced the amplitude of the first EPSCs by 28% and it effectively abolished the STD when EPSCs were evoked by stimulating the superficial layer. To determine whether the effect of CCh decreased the release probability (indicating a presynaptic mechanism of action) or reduced the amplitude of the EPSCs by utilising a postsynaptic mechanism the value of  $1/CV^2$  was calculated. The  $1/CV^2$  value was significantly reduced ( $P = 0.007037$ ) in the

presence of CCh, indicating that the cholinergic effect on the EPSCs was mainly mediated via presynaptic mechanism(s).

EPSCs evoked by the activation of the deep layer were more sensitive to CCh: in these cases the amplitude of the first EPSC was reduced by 42%. Similarly to the superficial layer stimulation, the relative amplitudes of the EPSCs in a train showed statistically significant increases when CCh was applied. The PPR statistically decreased during this treatment. As the  $1/CV^2$  value significantly decreased ( $P = 0.00362$ ) it was concluded that the effects of CCh on the EPSCs evoked by deep layer stimulation were also mediated via presynaptic mechanisms. The involvement of individual muscarinic acetylcholine receptors in mediating CCh action on EPSCs was tested using the same strategy as in the cases of the IPSCs. Atropine application could completely prevent the onset of the CCh action. It was shown that the use of the subtype-specific muscarinic receptor antagonists Pirenzepine, AF-DX 116 and tropicamide did not significantly modify the effects of CCh whereas the M3-specific 4-DAMP exerted an action that was very similar to that of atropine. These results indicate that the cholinergic modulation acting on the EPSCs evoked by the stimulation of the superficial layers of the DCN was chiefly mediated via M3 receptors.

We used the same procedure to determine which muscarinic acetylcholine receptor were activated as a consequence of deep-layer stimulation. In these cases atropine application could completely prevent the onset of the CCh action, too. Pirenzepine had a negligible effect on the CCh induced alterations, whereas the actions of the other three subtype-specific antagonists resembled those of atropine.

#### Cholinergic stimulation acted via postsynaptic mechanisms as well

The results presented so far clearly indicate that cholinergic modulation influenced both the inhibitory and excitatory synaptic inputs reaching the giant cells. Moreover, the alterations found in the  $1/CV^2$  values suggested that the modulation of the excitatory synaptic activity was mediated via presynaptic mechanisms. However, it was noted during the

experiments that the holding current recorded from the giant cells also showed a marked decrease in the presence of CCh, strongly suggesting the contribution of postsynaptic mechanisms. In the next step of the experiments, therefore, the postsynaptic actions of the CCh application were studied. Our hypothesis was that cholinergic modulation exerted its postsynaptic effects by interfering with a carbachol-sensitive  $K^+$  current, and we presumed that this current component could have corresponded to the M-type  $K^+$  current. To confirm or reject this possibility, the membrane potential of the giant cells was set to and maintained at -40 mV, at which potential most of the voltage-gated  $K^+$  channels are inactivated, whereas the M-type channels (if present) generate an outward  $K^+$  current. During these experiments all synaptic activities were blocked by the combined application of 10  $\mu$ M bicuculline, 1  $\mu$ M strychnine, 10  $\mu$ M NBQX and 50  $\mu$ M D-AP5. In the presence of 10  $\mu$ M atropine the reduction in the holding current was significantly smaller indicating that the effects of CCh on the holding current were mediated via muscarinic receptors. Moreover, application of 10 mM tetraethylammonium ( $TEA^+$ ), a well-known inhibitor of most  $K^+$  channels, induced similar changes in the holding current as did CCh application. In addition, no further reduction in the holding current could be seen when CCh was added to the extracellular solution already containing  $TEA^+$ . These experiments indicate that the reduction in the holding current seen in the presence of CCh at -40 mV was indeed the consequence of the reduction in a  $K^+$  conductance.

To determine the type of muscarinic acetylcholine receptors that may be responsible for mediating the effect of CCh resulting in a reduced holding current, subtype-specific muscarinic receptor antagonists were employed. The M4-specific tropicamide almost completely abolished the CCh-induced reduction in the holding current. The M3-specific antagonist 4-DAMP was also capable of reducing the effect of CCh, but it was less effective in achieving this than tropicamide. The other two subtype-specific blockers (pirenzepine and AF-DX 116) did not exert significant effects on the effect of CCh.

The pronounced effects of the cholinergic stimulation on the holding current raised the possibility that there must be a carbachol-sensitive  $K^+$  current component expressed by the giant neurones. It was reasonable, therefore, to investigate further the effect of cholinergic activation on  $K^+$  currents expressed by the giant cells. In these sets of experiments a holding potential of -60 mV was applied, from where 400-ms-long voltage steps were applied ranging between -100 and +40 mV in 10 mV increments. To inhibit the voltage-gated  $Na^+$  currents the extracellular solution contained 1  $\mu$ M tetrodotoxin, and the  $Ca^{2+}$  currents were reduced by using 0.5 mM  $Ca^{2+}$  and 2.5 mM  $Mg^{2+}$  in the bathing medium. The effect of CCh was assessed by determining the CCh-sensitive current component. Our results demonstrated that the effect of CCh on the  $K^+$  current was abolished in the presence of atropine and tropicamide indicating that cholinergic stimulation was coupled to the  $K^+$  currents via M4 muscarinic receptors. Moreover our results obtained in the presence of TEA confirm the previous statement, that the postsynaptic effect of CCh was exerted on  $K^+$  channels. However, the precise identity of the CCh-sensitive current component was not sought in the present work.

#### Q-PCR data supporting the functional results

As the functional studies were conducted using 10- to 14-day-old animals, they could not reveal whether the same receptors are also present in younger or older animals. Q-PCR experiments were performed, therefore, and the relative expressions of the relevant mRNA molecules were determined in animals of various ages (3-80 days). At the age of 15 days, the quantity of the M3-specific mRNA was the highest, followed by the amount of the M2-specific mRNA. The presence of the M1- and M4-specific mRNAs could also be detected, whereas the amount of the M5-specific mRNA barely reached detection level. Moreover, it could also be seen that, at the age of 15 days and in older animals, the amounts of the M3- and M2-specific mRNAs were significantly higher than the quantities of the mRNAs encoding the other muscarinic receptors. It was also noticed that, at the age of 15 days, the amounts of the M4- and M1-specific mRNAs were somewhat higher than in more

mature animals, whereas the M5 receptor expression was not prominent in animals older than 5 days.

## **II. Cytoplasmic $\text{Ca}^{2+}$ concentration changes evoked by cholinergic stimulation in primary astrocyte cultures prepared from the rat cochlear nucleus**

### Characterisation of the carbachol-induced $\text{Ca}^{2+}$ concentration changes

The experiments to be reported on were carried out using primary astrocyte cell cultures. Although the culturing conditions were chosen to grow the glial cells preferentially, the identity of the cells in the cultures was regularly checked by detecting the expression of glial fibrillary acidic protein (GFAP), a widely accepted astrocyte-specific marker with GFAP-specific immunolabelling. In all cases more than 90% of the cells were GFAP-positive. As for the morphological appearance of the astrocytes, the cells showed certain degree of variability. Some of them had the well-known star-like morphology with several long, thin appendages, while other astrocytes did not possess such appendages, and they presented a more rotund shape. These variations of the GFAP positive cells were present after the consecutive passages, too.

For finding the appropriate concentration, several doses of CCh were applied in the preliminary experiments. Although the heterogeneity of the CCh-evoked responses was pronounced in these experiments, 50–100  $\mu\text{M}$  CCh regularly evoked  $\text{Ca}^{2+}$  transients in the responding cells. In the present experiments the certainly supramaximal 1 mM CCh was chosen in order to ensure well-detectable cytoplasmic  $\text{Ca}^{2+}$  concentration increases.

It became evident in the very early phase of the experiments that a relatively low proportion of the cultured astrocytes responded with  $\text{Ca}^{2+}$  transients to the CCh treatment. Several efforts were made to clarify whether the experimental circumstances were responsible for this low responsiveness, and the small number of the responding cells raised

the necessity of testing whether a generally poor condition of the astrocytes could be an explanation. Earlier reports about the  $\text{Ca}^{2+}$  signalling in astrocytes indicated that ATP was able to evoke  $\text{Ca}^{2+}$  transients in a high percentage of these cells so we employed, therefore, ATP as a possible positive control agent. In the course of our experiments, 0.1 mM ATP was applied in 660 astrocytes and 611 (92.6%) out of these cells responded with cytoplasmic  $[\text{Ca}^{2+}]$  increase. These findings suggest that only a part of the cultured astrocytes are sensitive to CCh treatment.

To look further for the possible explanation of the low proportion of the CCh-sensitive astrocytes, correlation was sought between the responsiveness of the cells and the duration of the culturing (number of passages), density of the cells (confluence level) as well as the age of the animals (in the 7–9 days range), but no decisive results were established. Moreover, the possible significance of cell morphology was also analysed in a group of the astrocytes. Out of the 198 cells used for this purpose, 161 astrocytes had the starlike appearance, whereas 37 possessed the more rotund shape. The ratio of CCh-sensitive astrocytes was rather similar in both cell populations; 38.8% and 27.6% in the former and latter group, respectively. As none of the conditions tested showed definite correlation with the CCh-sensitivity, all data yielded in the present work were pooled, resulting in the final conclusion that out of the 611 astrocytes investigated 222 (36.3%) produced intracellular  $\text{Ca}^{2+}$  transients in response to the CCh treatment.

As in the forthcoming experiments pharmacological modifications of the CCh effect were carried out, it seemed inevitable to test the effects of the repeated cholinergic stimulations on the amplitudes of the  $\text{Ca}^{2+}$  transients. In the cases of repetitive CCh treatments, the amplitude of the second transient was 51.7% of that of the first one, while the third transient decreased further (to 13.2% of the amplitude of the first response). However, the smaller  $\text{Ca}^{2+}$  transients could not be attributed to the deterioration of the cells or to any damage affecting their  $\text{Ca}^{2+}$  homeostasis, as the subsequent application of ATP was able to

evoke  $\text{Ca}^{2+}$  transients whose amplitude was similar to those of the first CCh-evoked responses.

#### Heterogeneity of the time course of the carbachol-induced $\text{Ca}^{2+}$ transients

The astrocytes showed a great diversity regarding the time course of the CCh-induced  $\text{Ca}^{2+}$  transients. In some cases a rapid increase of the cytoplasmic  $[\text{Ca}^{2+}]$  developed which decayed even in the presence of the CCh. In other cases the development of the transients was similarly fast, the falling phase, however, produced a secondary slow rise which decayed only when the CCh was removed from the extracellular solution. In some cases this secondary increase was not present, but the cells showed a plateau or a slowly declining falling phase. Moreover, in the astrocytes possessing the slow component, the appearance of the early rapid phase was variable.

On analyzing the response patterns of all CCh-positive astrocytes, altogether 100 (45.0%) cells produced rapid transients, whereas 112 (50.5%) astrocytes showed the plateau-type behaviour. Nevertheless, a small minority of the astrocytes produced  $[\text{Ca}^{2+}]$  oscillations (10 cells, corresponding to 4.5% of all investigated astrocytes). The occurrence of this behaviour, however, was so rare that its systematic investigation could not be performed.

The presence of the two variable components of the  $\text{Ca}^{2+}$  transients raised the possibility that several mechanisms might be responsible for the CCh-induced increase of the cytoplasmic  $\text{Ca}^{2+}$  concentration. A plausible question to be answered was whether the extracellular  $\text{Ca}^{2+}$  was involved in the development of these responses. First CCh induced plateau-type  $\text{Ca}^{2+}$  transients were evoked by CCh in normal aCSF ( $[\text{Ca}^{2+}] = 2\text{mM}$ ). On decreasing the external  $[\text{Ca}^{2+}]$  from 2 to 0.2 mM, the resting fluorescence intensity (hence the level of the cytoplasmic  $\text{Ca}^{2+}$ ) decreased, and 1 mM CCh evoked transient type signals that possessed the early component only. After elevating the external  $[\text{Ca}^{2+}]$  to the control value (2 mM), the resting intracellular  $[\text{Ca}^{2+}]$  recovered to the initial baseline value. When the CCh challenge was repeated again in the control solution, the duration of the  $\text{Ca}^{2+}$  responses

increased, although the original plateau-like shape of the signals was only partially restored. It has to be mentioned that in the 5 astrocytes that produced rapid transients neither the time course, nor the peak value of the  $\text{Ca}^{2+}$  responses was modified significantly by the  $\text{Ca}^{2+}$  withdrawal. In the cases of the 9 plateau-type  $\text{Ca}^{2+}$  responses CCh did not modify the amplitude of the transients, only the duration of these transients decreased.

#### Mechanism of the cholinergic effect on the astrocytes

Several approaches were tried to identify the pathway(s) responsible for the development of the CCh-induced cytoplasmic  $[\text{Ca}^{2+}]$  elevations in the cochlear astrocytes. It has been shown that the 1 mM CCh-induced  $\text{Ca}^{2+}$  transients were blocked by the application of 10  $\mu\text{M}$  atropine. When interpreting the atropine effect, however, the complication arising from the decreasing amplitudes of the consecutive CCh-induced transients had to be taken into account. To do so, when repeated CCh challenges were applied, the amplitudes of the transients obtained during atropine treatment were compared to the values recorded in the cases of the second transients without any pharmacological intervention. Our finding showed that atropine abolished the effect of CCh in a reversible manner.

These findings indicated the decisive role of the muscarinic cholinergic receptors. This assumption was supported further by the results obtained on using muscarine. In these cases 10  $\mu\text{M}$  muscarine produced plateau-type  $\text{Ca}^{2+}$  transients that could be blocked by atropine in a reversible manner, too.

Besides the above findings it was important to check the possible involvement of the nicotinic ACh receptors, as 1 mM CCh was reported to activate these receptors, too. In the step the effects of a nicotinic receptor blocker (hexamethonium) have been studied. 0.1 mM hexamethonium did not modify significantly the CCh-induced  $\text{Ca}^{2+}$  transients.

The presence of the muscarinic ACh receptor subtypes was investigated first at the mRNA then at the protein level. M1 and M3 muscarinic receptor subtypes could be detected, using both Q-PCR and immunocytochemical methods. The functional relevance of the M1

and M3 receptors was also assessed. In these experiments subtype-specific antagonists (pirenzepine and 4-DAMP) were used. Both antagonists abolished the effect of CCh in a dose-dependent and reversible manner.

## **Discussion**

### **Cholinergic modulation in the cochlear nucleus**

In the present work the mechanisms underlying cholinergic modulation of the giant cells of the CN have been investigated. Besides pointing out that the activation of cholinergic inputs increased the activity of giant neurones and depolarized them, we have provided evidence that cholinergic modulation involved both post- and presynaptic mechanisms, and was mediated via M2, M3 and M4 receptors.

The increased activity of the giant cells is mediated via both pre- and postsynaptic mechanisms. The latter one causes depolarization of these neurones by decreasing the amplitude of certain  $K^+$  currents (such as the M-type current) via stimulating M4 and M3 receptors. The cholinergic neuromodulation affecting the excitatory synapses acts via presynaptic mechanisms. While the EPSCs originating from the parallel fibres are modulated by M3 receptors alone, the synaptic transmission taking place between the acoustic fibres and giant cells is modulated by a combined effort of M2, M3 and M4 receptors. Cholinergic neuromodulation is important also in altering the inhibitory actions reaching the giant cells. While the inhibitory inputs originating from the cartwheel cells do not seem to be affected, those originating from the deep layers are inhibited. The inhibitory actions that involve the activation of M3 receptors were effectively reduced by cholinergic activation, so their activation may also increase and prolong the activity of the giant cells.

Functionally, cholinergic modulation of the auditory system may have significance under both physiological and pathological conditions. In an *in vivo* study it was found that the

auditory system possessed a tonic cholinergic stimulation even at rest. Similar amplifier function of the cholinergic modulation has been noted in the DCN, especially after cochlear damage or high intensity noise exposure. Cholinergic modulation of the VCN has also been noted: when the ipsilateral cochlea was damaged, the contralateral sound stimulation caused the excitation of the VCN. It has been postulated that these modulatory mechanisms are mediated by (amongst others) collaterals of the olivocochlear bundle, causing a delayed-onset excitation that is observed when unilateral hearing damage occurs, and is evoked by contralateral sounds. On the basis of the experimental data presented in our work we propose that the effect of cholinergic modulation has a number of possible targets at both pre- and postsynaptic locations. Moreover, it seems justified to assume that cholinergic modulation of the giant neurones may be part of a system which may fine-tune the responsiveness of various cellular elements of the cochlear nuclei to the strength of the acoustic stimuli and to the level of the contralateral sound exposure.

### **Cholinergic mechanisms in primary astrocyte cultures prepared from the rat cochlear nucleus**

The experiments presented here are the first to show that astrocytes isolated from the rat CN and grown in primary cultures are capable of producing cytoplasmic  $\text{Ca}^{2+}$  transients on cholinergic stimulation. The ratio of the responding cells, however, was only approx. 36% and a considerable heterogeneity in the time course of the  $\text{Ca}^{2+}$  transients was found. A majority of the CCh-sensitive astrocytes showed responses that included a slow component whose development depended on  $\text{Ca}^{2+}$  entry from the extracellular solution. Moreover, it was shown that the effects of the cholinergic stimulation were attributable to the activation of M1 and M3 muscarinic receptors.

The obtained data proved the presence and functionality of M1 and M3 receptors in a subpopulation of the CN astrocytes. It is suggested, therefore, that some of the astrocytes of

the CN may have roles in the cholinergic modulation of the neuronal circuits of this nucleus, at least at the age investigated in the present work. It is worth of emphasizing, however, that in the case of the 7–9-day-old rats the maturation of the auditory pathway is not yet complete, so the characteristics of these more or less “immature” astrocytes may reflect an intermediate developmental stage. In fact, data about the differential expression of purinergic receptors in juvenile versus neonatal rats have already been published, and similar changes may occur in the cases of the muscarinic receptors as well.

## Publications providing the basis of the thesis

### Publications

1. **Pap, P.**, Kőszeghy, Á., Szűcs, G., Rusznák, Z. (2009) Heterogeneous cytoplasmic  $\text{Ca}^{2+}$  concentration changes evoked by cholinergic stimulation in primary astrocyte cultures from rat cochlear nucleus. *Hearing Research* 255: 73–83 IF: 2.177
2. Pál, B., Kőszeghy, Á., **Pap, P.**, Bakondi, G., Pocsai, K., Szűcs, G., Rusznák, Z. (2009) Targets, receptors and significance of muscarinic neuromodulation on giant neurones of the rat dorsal cochlear nucleus. *European Journal of Neuroscience* 30: 769–782 IF: 3.418

### Posters

1. **Pap Pál**, Nagy Dénes, Rusznák Zoltán, Szűcs Géza: Patkány és humán primer astrocyta tenyészetek karbakol által kiváltott kalcium-tranzienseinek vizsgálata. Magyar Élettani Társaság LXXI. Vándorgyűlés, Pécs, 2007.
2. **Pap Pál**, Nagy Dénes, Rusznák Zoltán, Szűcs Géza: Karbakollal kiváltott intracelluláris kalcium-események vizsgálata patkány nucleus cochlearisból izolált primer astrocyta tenyészetben. Magyar Élettani Társaság LXXII. Vándorgyűlés, Debrecen, 2008.
3. **Pap, P.**, Rusznák, Z., Szűcs, G.: Carbachol-induced calcium transients in cultured astrocytes isolated from the rat cochlear nucleus. Magyar Idegtudományi Társaság XII. Konferenciája, Budapest, 2009.
4. Pál, B., Kőszeghy, Á., **Pap, P.**, Bakondi, G., Pocsai, K., Szűcs, G., Rusznák, Z.: Targets, receptors and significance of muscarinic neuromodulation on giant neurones of the rat dorsal cochlear nucleus. Magyar Idegtudományi Társaság XII. Konferenciája, Budapest, 2009.

5. Kőszeghy Áron, Pál Balázs, **Pap Pál**, Szűcs Géza, Rusznák Zoltán: A muszkarinos kolinerg neuromoduláció célpontjai, receptorai és jelentősége patkány nucleus cochlearis dorsalis óriás-sejtjein. 39. Membrán Transzport Konferencia, Sümeg, 2009.
6. Kőszeghy Áron, Pál Balázs, **Pap Pál**, Szűcs Géza, Rusznák Zoltán: A muszkarinos kolinerg neuromoduláció funkcionális következményei patkány nucleus cochlearis dorsalisban. A Magyar Élettani Társaság LXXIII. Vándorgyűlése, Budapest, 2009.

## Other publications not used in the thesis

### Publications

1. Pocsai, K., Pál, B., **Pap, P.**, Bakondi, G., Kosztka, L., Rusznák, Z., Szűcs, G. (2007) Rhodamine backfilling and confocal microscopy as a tool for the unambiguous identification of neuronal cell types: a study of the neurones of the rat cochlear nucleus. *Brain Res. Bull.* 71(5): 529-38 IF: 2.193
2. Kőszeghy, Á., Pál, B., **Pap, P.**, Pocsai, K., Nagy, Z., Szűcs, G., Rusznák, Z. (2009) Purkinje-like cells of the rat cochlear nucleus: a combined functional and morphological study. *Brain Research* 1297: 57-69 IF: 2.463

### Publication, accepted for publication

1. Nagy, D., Kosztka, L., **Pap, P.**, Nagy, Zs., Rusznák, Z., Csernoch, L., Szűcs, G. (2010) Cytoplasmic Ca<sup>2+</sup> concentration changes evoked by muscarinic cholinergic stimulation in primary and metastatic melanoma cell lines. *Melanoma Research*.

### Posters

1. Rusznák, Z., Pál, B., Pór, Á., Pocsai, K., **Pap, P.**, Szűcs, G.: Voltage-gated K<sup>+</sup> channel subunits expressed by the projection cells of the cochlear nucleus. Is there a unique pattern enabling their characteristic behaviour? Magyar Idegtudományi Társaság XI. Konferenciája, Pécs, 2005.
2. **Pap Pál**, Pál Balázs, Pocsai Krisztina, Rusznák Zoltán, Szűcs Géza: A nucleus cochlearis projekciós neuronjai által expresszált kv-alegységek: összefüggésbe hozható az alegységek megoszlása a jellegzetes aktivitási mintázattal? Magyar Élettani Társaság LXIX. Vándorgyűlése, Budapest, 2005.

3. **Pap Pál**, Nagy Dénes, Rusznák Zoltán, Szűcs Géza: Egy új fejlesztésű Munc13-1 specifikus monoklonális antitest alkalmazásával kapott eredményeink neuronális és nem neuronális szövetekben. Magyar Élettani Társaság LXX. Vándorgyűlése, Szeged, 2006.
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5. Pocsai, K., Pál, B., **Pap, P.**, Szűcs, G., Rusznák, Z.: Retrograde labelling and confocal analysis of the rat cochlear nucleus. Joint Meeting of The Brazilian and British Physiological Societies, Rio de Janeiro, 2006.

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