THESES OF DOCTORAL (Ph.D.) DISSERTATION

THE ROLE OF HYDROXYL FREE RADICALS IN THE INDUCTION OF DIFFERENTIATION IN NEUROBLASTOMA CELL LINES

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1. INTRODUCTION

The membrane hypothesis of aging (MHA) assumes that there is a continuous flux of hydroxyl free radicals ($OH^{\bullet}$) in the living organisms *(Zs.-Nagy, 1994)*, which plays an important biological role. This hypothesis explains the phenomena of differentiation and aging and the role of $OH^{\bullet}$-free radicals in these processes. According to MHA aging is just due to an „overdriven" maturation, i.e., differentiation and aging have the same causes. MHA accepts some basic issues of the „Free radical theory of aging" (Harman, 1956, 1981, 2001) namely that the continuous presence of $OH^{\bullet}$-free radicals in the living cells may also damage the cell components.

According to MHA $OH^{\bullet}$-free radicals can much more efficiently cross-link the dense structures, e.g., the plasma membranes than the diluted ones. In addition to this, the cell membrane is exposed to another serious damaging factor called residual heat of the action potentials. These damaging effects can gradually alter the physicochemical state of living cells, and these processes lead to aging. The alterations of the membrane physichochemistry reflect a considerable rigidization of the membrane structure, which is certainly able to negatively influence the transport functions of the membranes, including the monovalent ion permeability, condensing the colloid structures of the living cells.

Specifically, the main outcome of membrane damages caused by free radicals is the decrease of the passive K^+-permeability, which causes an increase in the intracellular K^+ ion content of the cells. On the one hand this helps to maintain the polarized state and the excitability of the cell membranes while on the other hand, the increased intracellular monovalent ionic strength of the cells causes an increasing condensation (aggregation) of the colloids, and this phenomenon has multiple consequences. First, because of the density dependence of this process, it leads to increased cross-linking and damaging efficiency of $OH^{\bullet}$-free radicals in the cytosol. Furthermore, the increasingly condensed colloidal system loses its water content due to decreased colloid osmotic pressure. This way a continuous dehydration process takes place during the life, parallel with the relative increase of intracellular dry mass content.

According to the MHA the above features are not only of old-age phenomena, they start with the fertilization. The MHA assumes that the age-dependent water loss is useful in the development of the optimum performance of the individual, i.e., it is obviously necessary in the first part of maturation, in order to reach a sufficient level of dry mass concentration in the bones, muscle and other tissues. But because of the ever on-going character of this process, it becomes rate limiting for the further growth and self-destroying during the late phases of life. One can draw the conclusion that the driving „force" of maturation and aging is the same.

Starting from the above considerations, our laboratory studied the possible role of $OH^{\bullet}$-free radicals in the induction of cell differentiation. In the present series of experiments I worked with two neuroblastoma cell lines. The nervous system has to play a very important role during the maturation and aging caused by $OH^{\bullet}$-free radicals, because of its very high oxygen consumption. It is known that the central nervous system consumes much more oxygen than any other tissues. Further interesting experimental fact is that the oxygen availability in mammals increases after birth, and nerve cells differentiate within a short time *(Jones et al., 1982)* under increased tissue oxygen concentration. Moreover, young individuals consume more oxygen per unit of mass and time, than the old ones. Under these

conditions the young cells and organisms are able to grow and differentiate, while the old ones (in spite of the less free radical production) decline their functions. We also know that the increased ambient oxygen pressure in vitro decreases the cellular growth rate in several cell types *(Grant et al., 1992; Absher et al., 1994)*, including neuroblastoma cells (PC-12), where hyperoxia induces differentiated neuronal phenotype *(Katoh et al., 1997).* On the basis of all these facts, the following question arises: Do the $OH^{\bullet}$-free radicals play a role in the induction of differentiation processes?

Based on the above-mentioned facts, the results of investigations on other cell cultures in our laboratory *(Nagy et al., 1993, 1995)* and setting out from the demand on generalization, I have started my experiments with two neuroblastoma models (PC-12 and SK-N-MC cell lines). My investigations concerned the question, whether the single or/and repeated $OH^{\bullet}$-free radical treatments produce an effect on the differentiation properties of these cell lines. There were two issues in our concrete experiments:

First, we investigated the changes of the activities of enzymes, which produce and eliminate the reactive oxygen species, deriving from the oxygen metabolism. Specifically, the activities of superoxide dismutase (SOD), catalase and glutathione peroxidase after $OH^{\bullet}$-free radical treatments were investigated.

Second, we were curious to know, which markers of differentiation appear in the cells. In this issue we measured the differences between the activities of acetylcholinesterase (AchE) and ganglioside sialidase (GS) enzymes before and after the free radical treatments.

2. AIMS

My experimental work is connected to the wider research concept called MHA. According to this hypothesis $OH^{\bullet}$-free radicals deriving from the heterolysis of H_2O_2 during the whole life play a more complex role in cell physiology, than simply causing oxidative damages in the cells. This hypothesis suggests that the $OH^{\bullet}$-free radicals are physiologically useful, important factors of growth and maturation of the organisms.

My specific aims were the following:

1. to prove the effect on the differentiation induction of $OH^{\bullet}$-free radicals in the neuroblastoma cell lines (PC-12 and SK-N-MC),

2. to measure the changes in the activities of SOD, catalase and glutathione peroxidase (the enzymes which form and eliminate the reactive oxygen species deriving from the oxygen metabolism) after free radical treatments in PC-12 cells,

3. to measure the changes of enzymes after treatments with Fenton reaction, which are markers of differentiation, like AchE and GS in SK-N-MC cells,

4. to get acquainted with the hypotheses of aging with special regard to the MHA, and the biological role of free radicals.

3. METHODS

Cell cultures

The applied models in our experiments were the PC-12 (ATCC CRL 1721) rat phaeochromocytoma and the SK-N-MC (ATCC HTB 10) human neuroblastoma cell lines. The cell cultures were maintained under standard conditions.

Treatments with Fenton reaction

The cell cultures were treated with Fenton reaction *(Fenton, 1894)*, which means the simultaneous addition of ADP-Fe^{2+} complex and H_2O_2. In the culture medium the final concentration of ADP was always 20-times higher than the concentration of iron; this molar ratio inhibits safely the auto-oxidation of ferrous iron even at nearly neutral pH.

The culture medium was renewed every second day on the PC-12 cells, and every third day on the SK-N-MC cells, in agreement with the literary data and our own experimental findings. At the same time, when the culture media were changed, we carried out our free radical treatments, too. In compliance with these, we treated the PC-12 cells in 1x48 and 2x48, or the SK-N-MC cells 1x72 and 2x72 hours incubation periods. All the enzyme activities were measured at the end of these treatment periods.

Control cultures without any treatment were also studied. Parallel to these, 5-bromodeoxyuridine (BrdU) treatments were also carried out in the cultures of SC-N-MC cells; these cultures were considered as „positive controls".

Measurement of enzyme activities

Superoxide dismutase

SOD activity was measured according to the method of *Flohé and Ötting (1984)*. The method involves the generation of superoxide anion ($O_2^{-\cdot}$) radicals by means of xanthine-xanthine oxidase system. These radicals reduce cytochrome-c, which can be detected spectrophotometrically. The SOD content of the cell extracts dismutating the $O_2^{-\cdot}$ radicals decreases this reduction level in a quantitative way.

Catalase

Catalase activity was determined by using the method of *Gaunt and De Duve (1976)*. This method is based on the detection of the decrease of known concentration of buffered H_2O_2, decomposed by the catalase content of cell extracts. After a given time interval, $TiOSO_4$ was added to stop the reaction, forming a yellow complex with the remaining fraction of H_2O_2. The absorbance of the solution was measured spectrophotometrically. The enzyme activity was calculated from the difference between the samples with and without cell extract (blank).

Glutathione peroxidase

Glutathione peroxidase measurement was carried out according to *Flohé and Günzler (1984)*. Essential steps of the method are the following: glutathione peroxidase produces oxidized glutathione (GSH→GSSG), which is reduced by glutathione reductase (GR) quantitatively. This latter compound is added in excess to the assay mixture. During the above reactions NADPH is consumed quantitatively, the extent of which can be monitored spectrophotometrically.

Acetylcholinesterase

AchE activity was determined according to *Blume et al. (1970).* The radio-labeled substrate, 1-[^{14}C]acetylcholine was incubated with the cell homogenate. The product was 1-[^{14}C]acetate, it was separated from undegraded substrate by ion-exchange chromatography, than quantified in a liquid scintillation counter (LSC).

Ganglioside sialidase

GS activity was measured according to *Lieser et al. (1989).* The tritiated substrate, [^{3}H]GM3 ganglioside was incubated with the cell homogenate, than the product, namely lactosyl-[^{3}H]ceramide, was separated from undegraded substrate by ion-exchange chromatography, quantified in an LSC analyzer.

Measurement of protein and DNA contents

Protein contents of the cell homogenates were determined spectrophoto-metrically by using the Folin phenol reagent.

We determined the DNA content of our samples in two different ways. First the methods of *West et al. (1985)* and *Teixeira et al. (1995)* were followed. DNA content of cell homogenates treated by Triton-X-100 were measured by means of a fluorescent dye (Hoechst 33258). DNA was precipitated with alcohol then resolved in EDTA. This first method was precise and very sensitive. Secondly the method of *Bashford and Harris (1987)* was performed. DNA was measured also in this case by means of the fluorescent Hoechst dye (33258), however, after only a short incubation time and without precipitation. This measuring process was very quick and simple.

4. RESULTS

Results of the investigations in the PC-12 cell line

In the PC-12 cell line we investigated how the activities of enzymes forming and eliminating oxygen free radicals had changed after single or repeated treatments with $OH^{\bullet}$-free radicals of the cell cultures. We measured the activities of SOD, catalase and glutathione peroxidase enzymes. We established first the Fe^{2+} and H_2O_2 concentrations, the simultaneous addition of which were not toxic for the cells, but inhibited the cell proliferation. 100 μM Fe^{2+} (in complex of Fe-ADP) and 25 μM H_2O_2 proved to be the most suitable concentrations for this purpose. SOD activity (expressed in μg SOD/mg total protein) was 4.29 ± 0.259 (± SEM) in the untreated PC-12 cells. The first treatment affected a small (not significant, $p < 0.19$) increase in this enzyme activity, it was 4.87 ± 0.308. After the second treatment, SOD activity increased up 5.32 ± 0.373. The difference between this last value (two-times treated) and the value measured in control cells was significant ($p < 0.05$). We did not find a significant difference ($p < 0.36$) between the activities after one or two repeated treatments So, in the case of SOD enzyme we have found a slow and gradual increase of activity during the treatments, which was altogether a 24 % increase compared to the value in the untreated cells.

The catalase activity (expressed in unit catalase/mg total protein) were 14.89 ± 0.209 (± SEM) in the untreated (control) PC-12 cells. The catalase activity showed an extensive increase after the first treatment, it was 21.81 ± 0.765 (46% increase, $p < 0.001$). After the repeated treatment this value increased no more (21.05 ± 1.086), the difference between the single and repeated treatments remained insignificant ($p < 0.65$).

The glutathione peroxidase activity (as mmol NADPH consumption/min/mg total protein) was 0.50 ± 0.029 in the untreated (control) cultures. Increases of 68% (0.84 ± 0.020) and 156% (1.28 ± 0.058) were found in this enzyme activity after single or repeated treatments, respectively. The difference between the single and double treatments proved to be strongly significant in statistical terms ($p < 0.001$).

All the measured parameters (SOD, catalase and glutathione peroxidase activities) showed an increase after our single and double treatments with Fenton reactants.

Results of the investigations in the SK-N-MC cell line

In the SK-N-MC cell line we measured the changes of enzymes after either $OH^{\bullet}$-free radicals or BrdU treatments, which are markers of differentiation, like AchE and GS sialidase. BrdU is a well-known agent to induce differentiation in SK-N-MC and other neuroblatoma cell lines *(Mühl, 1992, 1996; Kopitz et al., 1994)*, so we could explain these samples as „positive controls".

We established first the Fe^{2+} and H_2O_2 concentrations, the simultaneous addition of which were not toxic for the cells, but inhibited the cell proliferation. 100 μM Fe^{2+} (in complex of Fe-ADP) and 10 μM H_2O_2 proved to be the most suitable concentrations for this purpose.

In our experiments with BrdU, the AchE activity increased 4.5 and 6.4 times after 72h and 2x72h, respectively, as compared to the control cultures of the same age. The Fenton treatment of 72h caused a 3.2-fold increase of this enzyme activity, whereas after 2x72h no further increase could be observed. Parallel with the decrease of added iron concentration (50 or 25μM), the enzyme activity decreased as well.

Assuming that the protein content of the cells increases during the differentiation, which may influence the results expressed on a per mg protein basis, we expressed the AchE activities also as per μg DNA. The differences between the AchE activities become more accentuated between the 72 h and 2x72h periods for both the controls and the Fenton- or BrdU-treated cultures, respectively. The untreated cultures also displayed a significant increase of AchE activities between 72h and 2x72h culturing, which may be considered as a sign of some spontaneous differentiation tendency. This latter resulted in a 40 % or 80 % increase of AchE activity, if expressed per mg protein or μg DNA, respectively. However, the spontaneous differentiation remained always significantly lower than the increases found in any of the treated cultures.

The second biochemical marker of differentiation in this cell line we studied was the increase of GS activity. After 72h incubation period, GS activity displayed a 1.7-fold increase in the Fenton-treated cultures, and a 1.9-fold increase in the BrdU-treated ones, compared to the untreated control values. After 2x72h decreased enzyme activities were found in both the Fenton-treated and in the BrdU-treated cultures, while a weak increasing tendency was detected only in the untreated controls

5. DISCUSSION

1. Our experiments have revealed that the Fenton-treatments can be carried out only in a relatively narrow range of concentration and duration. Furthermore, in this range the effect of free radicals on differentiation depends on their concentration.

2. Our experiments have proven as well that it is worth to examine the radical influence on differentiation after only one treatment.

3. It was very interesting that the cells can maintain high levels of the measured enzyme activities for a relatively long time (a week) after a very short flux of hydroxyl free radicals.

4. On the other hand, we have to emphasize that the influence of free radical treatments quantitatively never reached the influence of differentiating agents (with the exception of GS enzyme).

5. The generally increased enzyme activities in PC-12 cells (SOD, catalase and glutathione peroxidase) can be interpreted by assuming that the cells maintain their own radical production level at a higher rate for a long time, which is their reply to the external, very short flux of free radicals. We suggest that the differentiated state is connected to the higher level of free radical production. Increased SOD activity in the cells may be considered as a source of further H_2O_2 production. Catalase and glutathione peroxidase eliminate partly the produced H_2O_2, but also Fenton reaction takes place very quickly (between Fe^{2+} and H_2O_2), so the result will be much more production of $OH^{\bullet}$-free radicals as well. In addition to this, we suppose that the cells consume more oxygen. There are experiments running at present in this regard.

6. We hypothesize that the increased activities of the above mentioned enzymes are connected to a more differentiated state of the cells.

7. The increased AchE enzyme activity in SK-N-MC cells suggests that the trends in changes of biochemical markers influenced by free radicals will be similar to the differentiated state, because this enzyme is a strong differentiation marker for the given cell line. The highest AchE levels are found in the definitively differentiated state of these cells, achieved after treatments of BrdU. The influence of the Fenton-treatment on cells is always smaller than that of BrdU. It was very interesting that the second radical treatment did not increase further this enzyme activity, confirming that it is worth to examine the radical influence on differentiation after only one treatment.

8. The investigations of the second biochemical marker called GS enzyme caused a surprising result. The enzyme activity after both BrdU and Fenton treatments was very similar. This means that the influence of free radicals on this parameter was quantitatively comparable to that of the known differentiation-inducing agent. This observation demonstrates again that it is worth to examine the radical influence on differentiation after only a single treatment. Moreover, this enzyme activity decreased after the second treatments with both BrdU and Fenton reactants.

9. We also followed the morphological appearance of both cell lines by means of an inverted microscope. The SK-N-MC cells were photographed after they were fixed on the bottom of the culture flasks. At the end of incubation periods (2x72h) both the control and BrdU-treated cells were confluent. Moreover, the BrdU-treated cultures contained more adherent, more transparent and larger cells. In the cultures treated with hydroxyl free radicals, the confluency was much less evident, there were some larger cells with more pronounced formation of cell processes, but the signs of nuclear condensation were also observed. This may be considered as manifestation of iron toxicity. The influence of Fenton treatment on morphology was not examined in details, because in this case the main line of our experiments was to measure the above mentioned biochemical markers

10. Summing up, we demonstrated the involvement of oxygen free radicals in the induction of differentiation of both neuronal cell lines (PC-12 and SK-N-MC), representing two independent experimental models. Our experimental work, therefore, contributed to a deeper knowledge about the aging hypotheses and the biological role of oxygen free radicals.

6. PUBLICATIONS AND PRESENTATIONS

Publications used in the Ph.D. theses:

1. Oravecz, K., Bazsó-Dombi, E., Jeney, F., Nagy, K., Gecse, M., Zs.-Nagy, I. (2001): The involvement of hydroxyl free radicals in differentiation of the PC-12 rat phaeochromocytoma cell line. Arch. Gerontol. Geriatr., 33, 61-69. *(IF: 0.681)*

2. Oravecz, K., Kalka, D., Jeney, F., Cantz, M., Zs.-Nagy, I. (2002): Hydroxyl free radicals induce cell differentiation in SK-N-MC neuroblastoma cells. Tissue and Cell, 34 (1), 33-38. *(IF: 0.808)*

Further publications:

3. Szabó, J., Jeney, F., Bazsó-Dombi, E., Oravecz, K., Nagy, K., Zs.-Nagy, I. (1999): Szabadgyökök lehetséges szerepe az endocrin ophthalmopathia kialakulásában. Magyar Belorvosi Archivum, 52, 277-280. (in Hungarian)

4. Bazsó-Dombi, E., Oravecz, K., Jeney, F., Nagy., K., Zs.-Nagy, I. (2000): On the useful role of $OH^{\cdot}$ free radicals in differentiation of cultured human fibroblasts. Arch. Gerontol., Geriatr. 31, 233-242.*(IF: 0.681)*

5. Jeney, F., Bazsó-Dombi, E., Oravecz, K., Szabó, J., Zs.-Nagy, I. (2000): Cytochemical studies on the fibroblast-preadipocyte relationship in cultured fibroblast cell lines. Acta Histochem., 102, 381-389.*(IF: 0.867)*

Presentation used in the Ph.D. theses

6. Oravecz, K., Jeney, F., Bazsó-Dombi, E., Zs.-Nagy I. (1998): A Fenton reakció által indukált oxigéneredetű szabadgyökök hatása a PC-12 neuroblasztóma sejtvonal differenciálódási sajátságaira. Magyar Gerontológiai Társaság 1998. évi kongresszusa, Miskolc-Lillafüred, Palotaszálló, 1998. május 21-23. (in Hungarian)

Further presentations:

7. Bazsó-Dombi, E., Nagy, K., Jeney, F., Oravecz, K., Zs.-Nagy, I. (1998): A Fenton reakció által indukált hidroxil ($OH^{\cdot}$) szabadgyökök hatása a SOD és a kataláz enzimek génexpressziójára a sejtdifferenciálódás során. Magyar Gerontológiai Társaság 1998. évi kongresszusa, Miskolc-Lillafüred, Palotaszálló, 1998. május 21-23. (in Hungarian)

8. Jeney, F., Nagy, K., Oravecz, K., Bazsó-Dombi, E., Zs.-Nagy, I. (1998): Az oxigéneredetű szabadgyökök hatása a sejtdifferenciálódásra – in vitro. Magyar Gerontológiai Társaság 1998. évi kongresszusa, Miskolc-Lillafüred, Palotaszálló, 1998. május 21-23. (in Hungarian)

9. Jeney, F., Szabó, J., Nagy, K., Oravecz, K., Zs.-Nagy, I. (1997): The effect of $OH^{\cdot}$ free radicals deriving from Fenton reaction on the growth characteristics of human retrobulbar fibroblast cultures. AESF Meeting, München, 1997. december 4-6.

10. Jeney, F., Szabó, J., Bazsó-Dombi, E., Oravecz, K., Zs.-Nagy, I. (1998): The role of free radicals on the growth characteristics of human retrobulbar fibroblasts from EOP patients and normal individuals. 5. Leipziger Allerlei, Lipcse, 1998. október 1-3.

11. Jeney, F., Szabó, J., Bazsó-Dombi, E., Oravecz, K., Zs.-Nagy, I. (1998): VI^{th} International symposium on Graves' Ophthalmopathy, Amszterdam, 1998. november 27-28.

7. REFERENCES

In order of mentioning:

Zs.-Nagy, I. (1994): *The Membrane Hypothesis of Aging*, CRC Press, Boca Raton.

Harman, D. (1956): Aging: a theory based on free radical and radiation chemistry*, J. Gerontol*., 11, 298-300.

Jones, M.D., Jr., Rosenberg, A.A., Simmons, M.A., Molteni, R.A., Koehler, R.C. and Traystman, R.J. (1982): Oxygen delivery to the brain before and after birth, *Science,* 216, 324-325.

Grant, M.M., Koo, H.C. and Rosenfeld, W. (1992): Oxygen affects human endothelial cell proliferation by inactivation of fibroblast growth factors. *Am. J. Physiol.* 263, L370-L375.

Absher, M., Makrides, W., Shapiro, P. and Evans, J.N. (1994): Hyperoxia inhibits proliferation of cultured rat tracheal smooth muscle cells, *Am. J. Physiol.,* 267, L101-105.

Katoh, S., Mitsui, Y., Kitani, K. and Suzuki, T. (1997): Hyperoxia induces the differentiated neuronal phenotype of PC-12 cells by producing reactive oxygen species, *Biochem. Biophys. Res. Commun*., 241, 347-351.

Nagy, K., Pásti, G., Bene, L. and Zs.-Nagy, I. (1993): Induction of granulocytic maturation of HL-60 human leukemia cells by free radicals: a hypothesis of cell differentiation involving hydroxyl radicals, *Free Rad. Res. Comm*., 19,1-15.

Nagy, K., Pásti, G., Bene, L. and Zs.-Nagy, I. (1995): Involvement of Fenton reaction products in differentuation induction of K562 human leukemia cells, *Leukemia Res*., 19, 203-212.

Fenton, H.J.H. (1894): Oxidation of tartaric acid in presence of iron, *J. Chem. Soc*., 65, 899-910.

Flohé, L. and Ötting, F. (1984): Superoxide dismutase assays, *Meth. Enzymol*., 105, 93-104.

Gaunt, G. L. and De Duve, C. (1976): Subcellular distribution of D-amino acid oxidase and catalase in rat brain, *J. Neurochem*., 26, 749-759.

Flohé, L. and Günzler, W.A. (1984): Assays of glutathione peroxidase, *Methods Enzymol.*, 105, 93-104.

Blume, A., Gilbert, F., Wilson, S., Farber, J., Rosenberg, R. and Nirenberg, M. (1970): Regulation of acetylcholinesterase in neuroblastoma cells, *Proc. Natl. Acad. Sci. USA*, 67, 786-792.

Lieser, M., Harms, E., Kern, H., Bach, G. and Cantz, M. (1989): Ganglioside GM3 sialidase activity in fibroblasts of normal individuals and of patients with sialidosis and mucolipidosis IV, *Biochem. J*., 260, 69-74.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951): Protein measurement with the Folin phenol reagent, *J. Biol. Chem*., 193, 265-275.

West, D.C., Satar, A. and Kumar, S. (1985): A simplified in situ solubilisation procedure for the determination of DNA and cell number in tissue-cultured mammalian cells, *Anal. Biochem*., 147, 289-295.

Teixeira, C.C., Hatori, M, Leboy, P.S., Pacifici, M. and Shapiro, J.M. (1995): A rapid and ultrasensitive method for measurement of DNA, calcium and protein content, and alkaline phosphatase activity of chondrocyte cultures, *Calcif. Tissue Int*., 56, 252-256.

Bashford, C.L. and Harris, D.A. (1987): *Spectrophotometry and Spectrofluorimetry – a Practical Approach*, IRL Press Limited, Oxford, Washington DC, pp. 64-65.

Mühl, C. (1992): Gangliosid GM3-Sialidasen menschlicher Neuroblastomzellen der Zellinie SK-N-MC: Einfluss spezifischer inhibitoren auf Zellproliferation und Differenzierung. *Diplomarbeit an der Fakultät für Biologie der Ruprecht-Karls-Universität*, Heidelberg, 1992.

Mühl C. (1996): Einfluβ der Hemmung der Ganglioside-Sialidase der Zelloberfläche auf Wachstum und Differenzierung kultivierter menschlicher Neuroblastom-zellen, *Inaugural-dissertation zur Erlangung des Doctor scientiarum humanarum (Dr.sc.hum.) der Medizinischen Fakultät der Ruprecht-Karls-Universität*, Heidelberg, 1996.

Nagy, K. and Zs.-Nagy, I. (1984): Alterations in the molecular weight distribution of proteins in rat brain synaptosomes during aging and centrophenoxine treatment, *Mech. Ageing Dev.,* 28, 171-176.

Kopitz, J., Von Reitzenstein, C., Mühl, C., Cantz, M. (1994): Role of plasma membrane ganglioside sialidase of human neuroblastoma cells in growth control and differentiation, *Biochem. Biophys. Res. Commun*., 199, 1188-1193.