SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

ALTERATION OF DOPAMINE AND ENDOCANNABINOID SYSTEM IN NEURODEGENERATIVE DISEASES: POSTMORTEM HUMAN AUTORADIOGRAPHIC STUDY ON PARKINSON’S AND ALZHEIMER’S DISEASE BRAIN SAMPLES

by Szabolcs Farkas MD

Supervisor: László Csiba MD, PhD, DSc

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Head of the Examination Committee: Miklós Antal MD, PhD, DSc
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   Klára Matesz MD, PhD, DSc
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The Examination takes place at 11:00 on the 13th of June 2014, in the Library of Department of Neurology, Faculty of Medicine, University of Debrecen

Head of the Defense Committee: Miklós Antal MD, PhD, DSc
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The PhD Defense takes place at 13:00 on the 13th of June 2014, in the Lecture Hall of Building “A”, Department of Internal Medicine, Faculty of Medicine, University of Debrecen
1. INTRODUCTION

Investigation of receptors in central nervous system is extremely important for brain research and clinical medicine. Neuroreceptors can be examined with in vivo (positron emission tomography – PET, single-photon emission computed tomography – SPECT) and in vitro (autoradiography, in situ hybridization, immunohistochemistry) techniques.

In our study we applied traditional receptor autoradiography and functional autoradiography. These techniques are based on the detection of radioactively labelled ligands binded to target molecules with films or photographic emulsion. The receptor or radioligand autoradiography reveals the localization of radioactively labelled ligands in the studied tissue, while functional or [\(^{35}\)S]GTP\(\gamma\)S binding autoradiography is used for detection of active receptors coupled to G-protein. We investigated dopamine D\(_2/D_3\) and cannabinoid type-1 receptor (CB\(_1\)R) density, as well as dopamine D\(_2/D_3\) receptor – G-protein signal transduction on postmortem parkinsonian brain samples. Furthermore, CB\(_1\)R density in prefrontal cortex of Alzheimer’s disease (AD) patients with different Braak stages was also studied.

Alteration of dopaminergic neurotransmission plays crucial role in pathogenesis of Parkinson’s disease (PD). However, to date it is not clarified whether alterations of PD dopamine receptor density associates intracellular signal transduction changes or whether functional compensation appears together with receptor number reduction. It was reported that among striatal structures the caudate nucleus, through its strong connections with frontal cortex, participates in cognitive, executive functions. At the same time caudate nucleus shows coactivation with cingulate regions as well. There is lack of knowledge about dopaminergic signal transduction activity in the previously mentioned brain regions. Exploring the details of the pathophysiological process could help us to better understand the effects and side-effects of chronic levodopa treatment upon cognitive functions in patients with Parkinson’s disease.
In the central nervous system the main function of the endocannabinoid (EC) system is the retrograde neuromodulation through presynaptic CB₁Rs. The most studied form is the endocannabinoid-mediated long-term depression (eCB-LTD). In indirect striatal pathway cooperation of CB₁ and D₂ receptors is necessary to induce eCB-LTD. This phenomenon could have key role in extrapyramidal movement organization, as well as in pathophysiology of PD. Similar mechanisms had also been described in the prefrontal cortex. However, to date common alterations of dopamine and cannabinoid system in human PD are not yet clarified. To reveal these processes the first step should be parallel investigation of D₂ dopamine receptor and CB₁R densities, namely clarifying the question whether in PD D₂ dopamine receptor downregulation associates with CB₁R density changes.

The EC system regulates glutamatergic, GABAergic and, likely cholinergic signal transductions in hippocampus and prefrontal cortex through presynaptic CB₁R. In this way it plays a crucial role in the modulation of synaptic plasticity and cognitive processes. In AD alteration of synaptic plasticity precedes the appearance of β-amyloid deposits, and it is augmented with progression of neuropathological changes. Thus, it can be supposed that EC system reacts to alteration of synaptic plasticity, as well as to progression of neurodegeneration, which could manifest in CB₁R density changes. Detecting such receptor density alterations in the early stages of AD could show us direction for future studies in methodological classification of neuropathologically intact regions of AD brains, and could reveal unknown details of pathophysiology of AD. Furthermore, presence of early CB₁R density alterations could provide help in diagnosis of AD through novel, receptor specific radioligands and sophisticated imagistic techniques.
2. AIMS

The fact that lack of striatal dopamine results in dopamine receptor upregulation and altered dopaminergic signal transduction had been confirmed in PD patients and animal models of the disease. It is well known that dopaminergic treatment results in remarkable clinical improvement in the early stages of the disease. This type of medication remains effective during the whole progression of PD, however, severe side-effects could appear as well. In the present study we examined dopamine D_2/D_3 receptor density and signal transduction on brain samples originated from PD patients who received chronic dopaminergic substitution therapy. To the best of our knowledge, the agonist stimulated dopamine D_2/D_3 receptor–G-protein signal transduction has never been investigated in late stages of human Parkinson’s disease. We related the dopamine D_2/D_3 receptor density to receptor–G-protein signal transduction activity in case of long-term treated PD. Our aim was to support the hypothesis, that dopaminergic depletion could be compensated functionally by increased intracellular signal transduction activity.

The interaction between dopaminergic and cannabinoid system has been confirmed by numerous studies using animal models of PD and some examinations on human postmortem parkinsonian brain samples. However, to date dopamine D_2/D_3 receptor and CB_1R densities have never been studied parallel on postmortem brain specimens of long-term L-DOPA treated PD patients. Based on the well known functional interaction between these two receptor types as well as on the existence of CB_1R and D_2 receptor coexpression on glutamatergic and GABAergic neurons we wished to reveal whether in PD the dopamine D_2/D_3 receptor downregulation associates with CB_1R density alteration.

It is accepted that in AD glutamatergic synaptic plasticity becomes affected in the earliest stages of disease, which precedes the appearance of neuropathological changes. The
next part of our study was based on the fact that CB₁R contributes to modulation of this altered glutamatergic synaptic plasticity. To investigate this a brain region was needed which presents no neuropathological changes, only altered synaptic plasticity in early (I-II) Braak stages, but becomes affected by neurofibrillary tangles and amyloid plaques in Braak stage III-IV, and where a serious degeneration in latest (V-VI) Braak stages is observed. For these reasons prefrontal cortex was chosen. **We wanted to answer the questions whether CB₁R density alteration associates with synaptic plasticity affection in the early stages of AD; whether there are specific CB₁R density changes in different neuropathological stages of disease.**

### 3. MATERIAL AND METHODS

#### 3.1. Brain tissues

Experiments were carried out on frozen brain samples. The study material included caudate nucleus, cingulate and middle frontal gyrus specimens of six patients with clinically and neuropathologically justified PD and six control patients. Dopamine D₂/D₃ receptor density and stimulated receptor–G-protein coupling were investigated on these specimens. In addition CB₁R was studied on three control and three parkinsonian middle frontal gyrus and caudate nucleus samples. Five of the six PD patients were under dopamine replacement therapy with L-DOPA for 7.25±2.21 years (mean ± SEM). In one case there was no information about antiparkinsonian treatment. PD cases showed advanced Lewy body disease pathology with severe neocortical involvement, consistent with Braak alpha-synuclein stage V or VI.
In our further studies CB₁R density was examined on eleven prefrontal cortex specimens (Brodmann area 10) from patients with AD-related neurofibrillary degeneration and six control patients. Three samples of AD Braak stage I–II (early stages, no clinical symptoms – intact prefrontal cortex), four samples of III–IV (mild cognitive impairment – starting prefrontal affection) and four samples of stage V–VI (final stages, dementia – severely affected prefrontal cortex) were studied.

Control patients had no documented history of neurological or psychiatric disorders and showed no signs of neurodegeneration at neuropathological examinations.

### 3.2. Dopamine D₂/D₃ receptor autoradiography

D₂/D₃ receptor density was investigated with traditional receptor autoradiography using [³H]raclopride as radioligand. Readings were made in a Fujifilm BAS-500 phosphorimager (Fujifilm, Tokyo), using tritium sensitive phosphorimager plates (Fujifilm Plate BAS-TR2025). Quantitative densitometry was performed using Multi Gauge 3.2 phosphorimager software. Nonspecific binding was determined in the presence of 10 µM cold raclopride. Autoradiographic [³H]microscales (RPA510, Batch 18, Amersham) – placed alongside the brain tissue sections – were used to quantify the data and render the quantitative values in pmol/gram tissue. Optical densities measured on scale units were matched to known radioactive concentrations (nCi/mg), which were later transformed to [³H] pmol/gram tissue concentrations. Graph and formula of quantification was created converting optical densities to pmol/gram tissue. Measurements were made in duplicates and average values of total and blocked (non-specific) binding of the same specimens were calculated. The difference between mean total and blocked (non-specific) binding represented the specific binding of the radioligand on a specimen. Finally, for both control and disease specimens mean specific binding values of the same brain regions were calculated and used for final evaluation.
3.3. Dopamine receptor stimulated $[^{35}\text{S}]$GTPγS binding autoradiography

Dopamine stimulated $[^{35}\text{S}]$GTPγS binding (functional) autoradiography has been applied to reveal dopamine D$_2$/D$_3$ receptor–G-protein coupling. The sections were exposed to β-radiation sensitive film (Kodak BiomaxMR) for 4 days. The autoradiograms were digitized using a high resolution scanner (Epson Perfection V750 Pro) and Adobe Photoshop CS2 was used for measurements and image processing. To quantify $[^{35}\text{S}]$GTPγS binding the optical density values of $[^{14}\text{C}]$-microscale units were suited to known radioactivity (nCi/mg). Considering the specific activity of $[^{35}\text{S}]$GTPγS, nCi/mg values of $[^{14}\text{C}]$ isotope were transformed into $[^{35}\text{S}]$ pmol/gram tissue. Using the resulted scale (units in $[^{35}\text{S}]$ pmol/g tissue) and the respective optical densities we formed diagrams, to transform measured optical densities into pmol/gram tissue. Measurements were made in duplicates, and the average of the duplicates was used in calculations. For both control and disease specimens mean values of the same brain regions were calculated and used for final evaluation.

3.4. CB$_1$R autoradiography

CB$_1$R autoradiography has been performed using the novel CB$_1$R specific $[^{125}\text{I}]$SD7015 radioligand. The sections were exposed to β-radiation sensitive film (Kodak Biomax MS) for 24 hours. The autoradiograms were processed as described earlier. For quantification $[^{14}\text{C}]$-calibration scales were used and radioactivity concentrations of the $[^{14}\text{C}]$ plastic standards – supplied by the manufacturer – were transformed in tissue equivalent concentrations of $[^{125}\text{I}]$ expressed as disintegrations per minute per mm$^2$ (DPM/mm$^2$). Non-specific binding was determined in the presence of 10 µM rimonabant. Measurements were made in duplicates at PD caudate nucleus and middle frontal gyrus, and in quadruplicates in control and AD prefrontal cortex samples on consecutive sections. Calculation of specific
binding and evaluation of results were made as previously described at dopamine D₂/D₃ receptor.

4. RESULTS

Our study has several limitations which are mainly due to its explorative character. Based on our results definitive conclusions cannot be drawn since the relatively low sample size makes statistical analysis impossible.

4.1. Dopamine D₂/D₃ receptor density in Parkinson’s disease

In the caudate nucleus [³H]raclopride binding revealed markedly higher dopamine D₂/D₃ receptor density in controls in comparison with PD (24.08 ± 2.06 fmol/g tissue and 18.43 ± 2.82 fmol/g tissue, respectively, mean ± SEM). Neither frontal cortex nor cingulate cortex showed notable difference in dopamine D₂/D₃ receptor densities between PD and control samples.

4.2. Dopamine receptor stimulated [³⁵S]GTPγS binding autoradiography in Parkinson’s disease

The mean values of basal binding in the caudate nucleus were almost identical in control and PD groups (199 ± 17 fmol/g and 198 ± 21 fmol/g, respectively, mean ± SEM) whereas in the prefrontal and cingulate cortex were approximately 15% higher in PD than those in control.

Looking into dopamine stimulated D₂/D₃ receptor–G-protein coupling in caudate nucleus samples mean values of controls and PD groups were almost on the same level (control: 210 ± 15 fmol/g, PD: 215 ± 25 fmol/g, mean ± SEM), whereas mean values of
stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding showed mild increase in case of PD frontal and cingulate cortex.

4.3. Comparison of dopamine $D_2/D_3$ receptor density and dopamine stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding

Even though PD caudate nucleus samples showed markedly decreased dopamine $D_2/D_3$ receptor density in comparison to controls (18.43 ± 2.82 fmol/g and 24.08 ± 2.06 fmol/g and, respectively, mean ± SEM), in signal transduction through $D_2/D_3$ receptor–G-protein coupling there was no difference between controls and patients in this brain region (control: 210 ± 15 fmol/g, PD: 215 ± 25 fmol/g, mean ± SEM). This suggests that in PD patients caudate nucleus, in spite of downregulated $D_2/D_3$ receptor density, the signal transduction activity remains the same.

The mild increase of dopamine stimulated $D_2/D_3$ receptor–G-protein coupling in case of PD frontal and cingulate cortex could only be apparent, because of low sample size and data inhomogeneity.

4.4. Dopamine $D_2/D_3$ receptor and $\text{CB}_1\text{R}$ density in Parkinson’s disease

On caudate nucleus specimens dopamine $D_2/D_3$ receptor density was clearly decreased in case of PD compared to controls (12.84 ± 5.49 fmol/g vs. 30.26 ± 2.48 fmol/g, mean ± SEM). Using the same specimens we found no difference regarding to $\text{CB}_1\text{R}$ density in caudate nucleus (control: 16.66 ± 2.69 DPM/mm$^2$, PD: 18.30 ± 3.60 DPM/mm$^2$, mean ± SEM). In middle frontal gyrus $\text{CB}_1$, as well as $D_2/D_3$ receptor densities of PD and control sample groups were approximately on similar level.
4.5. CB₁R density in Alzheimer’s disease prefrontal cortex

AD Braak stage I-II samples showed the highest CB₁R density (control: 19.70 ± 3.48 DPM/mm², Braak I-II: 26.04 ± 5.28 DPM/mm², mean ± SEM). This is especially remarkable since in these early stages prefrontal cortex is neuropathologically intact. On the other hand our results suggest a continuous decrease of CB₁R population through AD progression, however, CB₁R density did not drop below control level not even in end-stages (Braak V-VI) of disease (22.25 ± 7.62 DPM/mm², mean ± SEM).

We also calculated average density for the combined control and AD Braak I-II groups, since neuropathologically intact specimens of AD Braak stage I-II brains had been used as controls in former studies. This resulted in masking of real differences between real controls and other AD sample groups (control and Braak I-II: 22.87 ± 4.96 DPM/mm², Braak III-VI: 23.51 ± 7.52 DPM/mm², mean ± SEM). Furthermore, alterations in AD Braak stage I-II could remain hidden. Our results suggest that all regions of AD Braak I-II stage brains have to be examined separately from controls in the future.

5. DISCUSSION

5.1. Dopamine D₂/D₃ receptor–G-protein signal transduction in Parkinson’s disease

Our results support previous findings that after chronic regular antiparkinsonian treatment in the caudate nucleus of PD patients the dopamine D₂/D₃ receptor density is decreased. This downregulation in dopamine receptors could be due to higher synaptical dopamine levels caused by chronic and maybe inadequate dopaminergic substitution.
Postsynaptic hypersensitivity of D₂ dopamine receptors has been reported, at least in early stages of Parkinson’s disease. This phenomenon can be considered as functional compensation against dopamine deficiency. According to our knowledge the relationship between dopamine D₂/D₃ receptor–G-protein coupling and dopamine D₂/D₃ receptor density has never been investigated in postmortem caudate nucleus, middle frontal gyrus and cingulate gyrus specimens of long-term L-DOPA treated PD patients.

On postmortem human PD caudate nucleus specimens we described for the first time that the decreased D₂/D₃ receptor density maintains D₂/D₃ receptor–G-protein signal transduction activity on the same level as that of the controls. This suggests the hypersensitivity of the remaining D₂/D₃ receptors, as well as the presence of a functional compensation of dopaminergic neurotransmission during the whole progression of treated PD. The early dopamine receptor upregulation and increased signal transduction may contribute to sustained therapeutic benefits of L-DOPA administration during initial phases of PD. On the other hand the unchanged receptor sensitivity could be involved in the development of dyskinesias, which are the most frequent side effects of long-term dopaminergic substitution therapy. The latter can be explained by the involvement of non-dopaminergic sources of endogenous dopamine synthesis and L-DOPA conversion into dopaminergic signal transduction, which results in dysregulation and oscillatory behavior of synaptic dopamine levels.

Similar to previous authors, regarding dopamine D₂/D₃ receptor density in middle frontal gyrus and cingulate gyrus we found no difference between control and PD groups. At the same time, PD samples showed increasing tendency of D₂/D₃ receptor sensitivity in both region. These data could be explained if we assume, that dopamine receptor density depends on active stimulation, which in PD associates with dysregulation of synaptic dopamine levels,
the latter caused by losses of dopaminergic terminals. Hence, the inappropriate dopaminergic stimulation could be enough to keep D$_2$/D$_3$ receptor density on control level, however, it does not assure normal signal transduction, which could lead to mild hypersensitivity.

5.2. Cannabinoid type-1 receptor density in Parkinson’s disease

Based on former researches it can be established that normal function of indirect and direct striatal pathways needs intact dopaminergic and cannabinoid systems. Striatal dopaminergic-cannabinoid imbalance, as well as cessation of normal synaptic plasticity (e.g. eCB-LTD) could be essential in the pathophysiology of PD. Our results refer to decreased dopamine D$_2$/D$_3$ receptor density in caudate nucleus of PD patients, whereas CB$_1$R density was unchanged. Neither of examined receptor densities showed alteration in postmortem middle frontal gyrus sections of PD patients. To our knowledge, this is the first report about unchanged CB$_1$R densities in caudate nucleus and middle frontal gyrus of PD patients following long-term L-DOPA treatment.

On the one hand in the background of this apparently intact CB$_1$R population could be reactive changes of inactive (‘reserve’) CB$_1$R population caused by neurodegeneration, which could hide a receptor loss. On the other hand, it is more likely that in PD the striatal medium spiny neurons and the corticostriatal terminals (structures containing the most of CB$_1$Rs) are not damaged, thus CB$_1$R population expressed by them remains intact. Furthermore, it is feasible that exogenous dopaminergic therapy cannot assure optimal D$_2$ receptor stimulation or normal modulation of signal transduction, regulated by dopamine-cannabinoid ‘synergism’. Subsequently, as a summary of our results, we could presume that in PD the stimulation of downregulated but hypersensitive dopamine D$_2$/D$_3$ receptor population is insufficient or inadequate to evoke eCB-LTD on corticostriatal terminals. At the same time, dopaminergic substitution could be suitable to some extent to ensure retrograde modulation, and in this
manner it could keep CB₁R density on control levels. To clear these assumptions further studies are required.

5.3. Cannabinoid type-1 receptor density in postmortem human Alzheimer’s disease prefrontal cortex

Our results refer to CB₁R upregulation in early stages of AD, which is followed by continuous decrease of receptor density during disease progression. This can be explained by different mechanisms. Previously other authors reported about intensification of synaptogenesis and the subsequent increase of glutamatergic and cholinergic signal transduction at the beginning of AD. Thus it is possible that CB₁R population increases due to newly formed synapses. On the other hand a long-lasting hyperexcitation could result in increased release of endocannabinoids, which in turn in heterosynaptic manner could completely block the GABAergic inhibition of pyramidal neurons. To prevent CB₁R saturation a reactive CB₁R upregulation could take place on presynaptic GABAergic terminals. The same heterosynaptic mechanism can happen on cholinergic terminals as well. Furthermore, heteromerization between CB₁R and acetylcholine and/or metabotropic glutamate receptors is also possible. In this way CB₁R would be able to improve or regulate altered signal transductions through a direct intracellular mechanism. Finally, in the background of possible CB₁R density increase reactive neuroprotective processes also emerge, which may involve this receptor type in a so far unknown manner. According to the explorative character of our research our aim was not to identify the precise mechanisms of a feasible CB₁R upregulation, but to emphasize the affection of CB₁R population in AD. The clarification of mechanisms is the task of future researches.
Our findings suggest a slow decrease of CB$_1$R density through the progression of AD. This can be explained by degenerative and inflammatory processes, resulting in an alteration of the neurons which mainly express this receptor type. In spite of this, CB$_1$R population remains at least on control levels even in the end-stages of AD. In the future these receptors could be target molecules in the treatment of AD.

Our data suggest that upregulation of CB$_1$R takes place in neuropathologically intact AD Braak stage I-II prefrontal cortex. This can occur as part of a functional reaction to alterations of – especially cholinergic and glutamatergic – signal transduction. Based on this we emphasize that, in contrast with former studies the neuropathologically intact specimens from AD Braak stage I-II brains should be investigated separately from controls in the future. This provides opportunity to reveal early reactive changes of endocannabinoid and other receptor systems, which could influence neurotransmission, as well as neuroprotection.

6. SUMMARY

We report for the first time, that in PD caudate nucleus signal transduction activity is not altered by long-term L-DOPA substitution therapy unlike dopamine D$_2$/D$_3$ receptor density. Our results show a possible mild increase of signal transduction of dopamine D$_2$/D$_3$ receptors in medial frontal and cingulate gyrus together with unaltered D$_2$/D$_3$ receptor densities in advanced PD. These support, that dopaminergic neurotransmission of distinct PD brain regions is affected in different extent. Finally, our results may provide us with additional details about the mechanisms of long-term complications (e.g. dyskinesia) caused by antiparkinsonian therapy.
To our knowledge, our study is the first to present on postmortem brain samples of long-term L-DOPA treated PD patients, that CB₁R densities in caudate nucleus and medial frontal gyrus remain unchanged. At the same time in PD patients caudate nucleus dopamine D₂/D₃ receptor density was clearly decreased. Our data suggest that in the caudate nucleus, during the progression of PD, the eCB-mediated synaptic plasticity, which is based on dopaminergic-eCB functional balance, could be altered by dopaminergic degeneration. It seems, however, that no reactive CB₁R density changes takes place.

This is the first study, to describe that the upregulation of CB₁R population precedes the appearance of neuropathological modifications in the prefrontal cortex of AD Braak stage I–II brains. The explanation could be the alteration of neurotransmission, as the earliest event in the pathophysiology of AD. Our results also support the idea that neuropathologically intact brain areas of AD Braak stage I–II brains should be studied separately from controls, in contrast to previous studies. Finally, CB₁R density even in end-stages of the disease was not below control level. In the future a preserved CB₁R population could serve as a target molecule for treatment during the whole progression of AD, due to CB₁R system’s wide connections in the central nervous system and immune system.
List of publications related to the dissertation

   Brain Res. 1453, 56-63, 2012.  
   DOI: http://dx.doi.org/10.1016/j.brainres.2012.03.014  
   IF: 2.879

   DOI: http://dx.doi.org/10.1016/j.brainresbull.2012.02.012  
   IF: 2.935

   DOI: http://dx.doi.org/10.1016/j.neuint.2011.11.004  
   IF: 2.659
List of other publications

DOI: http://dx.doi.org/10.1016/j.pemmed.2012.02.050

DOI: http://dx.doi.org/10.1016/j.jneumeth.2010.07.005
IF:2.1

DOI: http://dx.doi.org/10.1586/ERN.09.13

Total IF of journals (all publications): 10.573
Total IF of journals (publications related to the dissertation): 8.473

The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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