Cannabinoid receptor type 1 in the human foetal brain and its changes in Down's syndrome

Ph.D. thesis

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1. Introduction

The proper development of the central nervous system (CNS) depends on the temporally and spatially coordinated interaction of multiple signaling systems. The endocannabinoid system (ECS), – which consists of the cannabinoid receptors, endogenous cannabinoids (eCBs) and their metabolic apparatus – represents one of these signaling units. During the formation of the cerebral cortex, eCBs acting on cannabinoid receptor type 1 (CB_1R) control neuronal proliferation, migration and particularly the establishment of appropriate neural connectivity. Recently, the microtubule-binding protein Superior Cervical Ganglion-10 (SCG10) has been identified as a key downstream element of CB_1R signal transduction participating in axonal wiring.

Our knowledge about whether errant eCB signaling contributes to the pathogenesis of developmental brain disorders is rather limited. The leading genetic cause of intellectual disability, Down's syndrome (DS) is a complex neurodevelopmental disorder characterized by impaired neurogenesis, astrogliosis, inhibitory interneuron predominance, dysfunctional axon growth and synaptogenesis. In the hippocampus of adult $Ts65Dn^{+/+}$ mice (the most widely used mouse model of DS),

CB_1R expression and function are increased, and its pharmacological inhibition restores neurogenesis and memory processes. Moreover, in neurospheres derived from foetuses with DS, SCG10 mRNA expression is markedly reduced.

2. Objectives

As multiple CB_1R-regulated neurodevelopmental events are pathological in DS, we hypothesized that the neuroarchitectural impairments in DS are associated with the alterations of the ECS during brain morphogenesis.

Thus, we first systematically analysed the expression of CB_1Rs in human foetal brains with DS and in age-matched controls, and wished to answer the following questions:

I. What is the morphological appearance of CB_1R^+ profiles and does it differ between DS and control foetal brains?

II. Is there a difference in the temporal and spatial appearance and disappearance of CB_1R^+ profiles between DS and control foetal brains?

III. Is there a quantitative difference in the expression of CB_1R^+ profiles in distinct brain areas between DS and age-matched control foetal brains?

Next, we examined the response of cortical neurons derived from neonatal $Ts65Dn^{+/+}$ and wild-type littermate mice upon

CB_1R stimulation, with a particular interest in SCG10 protein availability. We aimed to answer the following questions:

IV. Is there a difference in the subcellular distribution of the SCG10 protein between $Ts65Dn^{+/+}$ and wild-type cortical neurons?

V. How does the stimulation of CB_1Rs affect SCG10 protein availability in $Ts65Dn^{+/+}$ cortical neurons compared to wild-type cortical neurons?

VI. Does CB_1R stimulation leads to a different neurite growth response in $Ts65Dn^{+/+}$ cortical neurons?

3. Methods

3.1. Human samples, preparation of brain tissues, immunohistochemistry

We have used human foetal brain samples collected by the Brain Bank of the Institute of Neurology at the Medical University of Vienna, Austria. Samples were obtained from spontaneous or medically induced abortions. To map CB_1R distribution, a total of 13 male, 14 female and 3 foetal brains with unknown sex were selected. These samples had no genetic disorders, head injury or neurological disease and we acknowledged them as controls. Another 10 male, 8 female and 5 foetal brains with unknown sex but all with DS were selected for comparative purposes. In both groups, the age of the samples varied between gestational week 14 and 40.

For light microscopy, 3 μm thick sections were prepared then immunolabelled with a polyclonal anti-CB_1R antibody made in rabbit (gift from Ken Mackie). Antibody binding was visualized by using 3-3'- diaminobenzidine chromogen and sections were counterstained with haematoxylin to optimize orientation. For confocal laser scanning microscopy, pre-treated sections were exposed to a mixture of mouse anti-NeuN and rabbit anti-CB_1R antibodies. Immunoreactivities were revealed by carbocyanine

3- or 5- tagged secondary antibodies raised in donkey and Hoechst 33,421 was used as nuclear counterstain.

3.2. In vitro neuropharmacology, protein analysis, immunocytochemistry

Neocortical tissue from neonatal wild-type and littermate Ts65Dn$^{+/+}$ mice was dissociated enzymatically and plated for Western blotting or for immunocytochemistry. Neurons were stimulated by the full CB_1R agonist WIN55,212-2 (500 nM) for 30 minutes.

For Western blotting, neurons were lyzed immediately after stimulation. After centrifugation, protein samples were separated by SDS-PAGE, transferred onto PVDF membranes and subsequently exposed to primary antibodies (acetylated tubulin, GAPDH). For signal detection, HRP-conjugated secondary antibodies were used.

For immunocytochemistry, stimulated neurons were kept alive for another day. After immersion-fixation and pre-treatment, cells on coverslips were exposed to rabbit anti-SCG10 and mouse anti-β-III-tubulin primary antibodies. Carbocyanine 2- or 3-tagged secondary antibodies were used for visualization and nuclei were counterstained by Hoechst 33,421.

3.3. Imaging

Human samples were analysed by combining high-resolution digitalised light and confocal laser-scanning microscopy. For the morphometry of mice cortical neurons, confocal laser-scanning microscopy was used.

4. Results

4.1. Morphological appearance of CB_1R^+ profiles

We determined CB_1R distribution in cortical areas, hippocampal subfields and the cerebellum across the three trimesters of pregnancy in our control samples and then compared the findings with those in Down's syndrome foetal brains. In most of the investigated brain regions, CB_1R^+ profiles appeared as a meshwork of fine-calibre axonal fibres and varicosities. We observed CB_1R^+ varicosities amongst or around $NeuN^+$ perikarya, supporting their axonal identity.

4.2. Disrupted temporal dynamics of CB_1R expression in Down's syndrome during the second trimester

In control brains, during the early second trimester (between days 98-120) a dense bundle of CB_1R^+ fibres appeared at the border between the subventricular (SVZ) and intermediate zones (IZ) of the temporal and frontal cortices. In contrast, immunoreactive fibres were less and weakly visible in age-matched DS samples in the corresponding regions. We observed a similar phenomenon in the hippocampal formation: in control subjects, CB_1R^+ immunoreactive fibres were apparent in the Ammon's horn and in the fornix during the 4^{th} month of

gestation, which contrasted the poor immunolabeling in DS subjects.

Between gestational days 121-160, CB_1R distribution changed in both groups. In control brains, at the SVZ/IZ boundary of temporal and frontal cortices, CB_1R immunoreactivity weakened, whereas in DS cases the immunoreactivity of CB_1R^+ processes became more pronounced and exceeded that of control subjects. During this time period, in the allocortical hippocampi of both groups, CB_1R^+ varicose structures became more numerous but occured more often in DS subjects.

4.3. Development of CB_1R expression during the third trimester

We could not identify consistent differences between DS and age-matched control subjects during the last trimester of pregnancy. Temporal and frontal cortices lacked CB_1R^+ profiles in the previously investigated areas (SVZ/IZ boundary) in both groups. CB_1R^+ profiles populated all subfields of the hippocampal formation at approximately equivalent densities in DS and age-matched samples.

Of note, around day 240, in the molecular layer of the cerebellar cortex, we observed a meshwork of CB_1R^+ processes in DS but not in control brains.

4.4. CB_1R stimulation induces SCG10 degradation and tubulin ageing in $Ts65Dn^{+/+}$ cortical neurons

SCG10 accumulated in the perikarya, axonal varicosities and growth cones of both $Ts65Dn^{+/+}$ and wild-type neurons. In $Ts65Dn^{+/+}$ neurons $SCG10^+$ neurite segments were more proximal to the somata, as compared to wild-type neurons.

Stimulation by WIN55,212-2 resulted in excess SCG10 degradation in $Ts65Dn^{+/+}$ neurons, especially in their distal, motile neurite segments. In line with this observation, treatment with WIN55,212-2 also increased tubulin acetylation in neurons from $Ts65Dn^{+/+}$ but not wild-type littermate mice.

4.5. Neurons from $Ts65Dn^{+/+}$ mice exhibit slowed CB_1R-dependent neuritogenesis *in vitro*

Based on our SCG10 data, we challenged $Ts65Dn^{+/+}$ and wild-type cortical neurons with WIN55,212-2 for 30 minutes and allowed them to grow for another day. Under control conditions, $Ts65Dn^{+/+}$ neurons grew significantly slower than their wild-type counterparts. Remarkably, wild-type neurons had

moderately longer neurites on the third *in vitro* day (which can be interpreted as a relative resistance to low dose WIN55,212-2 exposure), whereas WIN55,212-2 occluded neurite outgrowth in $Ts65Dn^{+/+}$ neurons.

5. Conclusions

During our immunohistochemical analysis, we observed a transient CB_1R expression on developing white matter tracts in both healthy and DS-affected human foetal brains. However, we found that the temporal dynamics of CB_1R expression is distinct in DS: during the second trimester of pregnancy, the appearance of CB_1R^+ processes is delayed by at least a month, but stays high even at foetal periods when CB_1R expression in healthy developing brains becomes reduced.

Our *in vitro* experiments performed on cortical neurons derived from neonatal $Ts65Dn^{+/+}$ transgenic and wild-type littermate mice showed that CB_1R activation leads to excess SCG10 degradation and tubulin acetylation, and consequently slowed CB_1R-dependent neuritogenesis in $Ts65Dn^{+/+}$ neurons. These results indicate a neuronal hypersensitivity to CB_1R stimulation in this DS mouse model.

In summary, we propose that the neuroarchitectural impairments in DS are associated with the temporal and functional deterioration of the ECS, with a profound impact on proper axonal wiring.

6. Bibliography of the candidate's publications

Publication related to the thesis:

Patthy Á, Hanics J, Zachar G, Kovács GG, Harkany T, Alpár A. Regional redistribution of CB1 cannabinoid receptors in human foetal brains with Down's syndrome and their functional modifications in Ts65Dn$^{+/+}$ mice. Neuropathol Appl Neurobiol. 2023 Feb;49(1):e12887. doi: 10.1111/nan.12887. PMID: 36716771.

Publication not related to the thesis:

Patthy Á, Murai J, Hanics J, Pintér A, Zahola P, Hökfelt TGM, Harkany T, Alpár A. Neuropathology of the Brainstem to Mechanistically Understand and to Treat Alzheimer's Disease. J Clin Med. 2021 Apr 7;10(8):1555. doi: 10.3390/jcm10081555. PMID: 33917176; PMCID: PMC8067882.

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