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Doublecortin cells and neurodegenerative disease

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Contents:

Abstract

Introduction : Doublecortin (DCX) is a microtubule associated protein expressed by migrating neural precursors. DCX is also expressed in approximately 4% of all cortical cells in adult normal primate brain. DCX expression is also enhanced locally in response to an acute insult made to the brain. This is thought to play a role in plasticity or neural repair. That being said, it would be interesting to know how the expression of DCX is modified in a more chronic insult, like in neurodegeneration such as in Parkinson's Disease (PD) and Alzheimer's Disease (AD). **The aim of my study is to study the expression of DCX cells in the cortex of patients having a neurodegenerative disease, compared to control patients.**

Method: DCX cells quantification on 9 DCX-stained 5 µm thick formalin fixed paraffin embedded brain sections: 3 Alzheimer's disease patients, 3 Parkinson's disease patients and 3 control patients. Each patient had several sections that we could stain with different stainings (GALLYA, TAU, DCX). By using a computerized image analysis system (Explora Nova, La Rochelle, France), cortical columns were selected on areas on the cortex with a lot of degeneration subjectively observed on GALLYA stained sections and on TAU stained sections. Then total number of cells was counted on TAU sections, where all nuclei were colored in blue. Then the DCX cells were counted on the corresponding DCX sections. These values were standardized to a reference surface area. The ratio of DCX cells over total cells was then calculated.

Results : There is a difference of DCX cell expression between Alzheimer's Disease patients and control patients. The percentage of dcx cells in the cortex of an Alzheimer's patient is around 12.54% ± 2.17%, where as in the cortex of control patients, it is around 5.47% ± 0.83%.

On the other hand, there is no significant difference in the ratio of DCX cells over total cells between parkinson's patients and control patients, both having around 5% of DCX cells.

Discussion: There is a dramatic increase of DCX expression in AD (12.5%) compared to PD and controls (5.5%). The increase in DCX ratio in AD may have two potential causes:

1.The increased ratio is due to DCX cells being more resistant to degeneration compared to surrounding cells which are degenerating due to AD, leading to the cortical atrophy observed in AD patients. So the decrease of total cells without any change in the number of DCX cells makes the ratio bigger in AD compared to the controls.

2.The increased ratio is due to an actual increase in DCX cells. This means that there is some neural repair to compensate the degenerative process, just like the repair process observed in acute lesions to the brain.

This second idea can be integrated in the broader point of view of neuroinflammation. The progression of the disease would trigger neuroinflammation and the process following the primary inflammatory response which is neural repair. So our study can show that the increase in DCX cells is an attempt to repair the degenerated neurons, in the context of neuroinflammation triggered by the physiopathological progression of the disease.

Key words : Doublecortin, Neuroplasticity, Neurodegenerative, Neuroregeneration, Immunohistology

Introduction :

In our increasingly ageing population, neurodegenerative diseases represent a great challenge to deal with. Diseases such as Alzheimer's Disease (AD) or Parkinson Disease (PD) have a grossly common mechanism : the slow degeneration of neurons followed by their death, thus creating long term symptoms progressively worsening and debilitating the patient. At late stages of both diseases, histological findings show a major cortical atrophy[1],[2]. In the present report, we will study a cortical cell population called doublecortin positive cells, or DCX cells. We will evaluate whether DCX cells are affected in both diseases the same way by neurodegeneration.

DCX is a microtubule associated protein which stabilizes the filaments of microtubule, allowing the growth cone to proceed correctly[3]. Therefore, it is expressed by migrating neural precursors[4][5].

A high concentration of DCX cells has been found in the Subventricular zone and in the hippocampus which are well known locations of neurogenesis throughout development[4][6]. So Doublecortin expression in those zones reflects neurogenesis. But a recent study[7] showed that not only DCX cells are expressed on those two locations in the adult, but they are also spread in the whole neocortex of adult human and non human primates, unlike the rodents. Approximately 4.5% of all cortical cells express Doublecortin[7]. These cells are thought to play a role in plasticity or neural repair. Therefore, DCX is considered as a marker for neuroplasticity.

Furthermore, some studies[7][8] show that the expression of DCX is enhanced locally in response to an acute insult made to the brain, which could mean that there is an attempt for neuroregeneration. Indeed, this could mean that DCX cells are associated with migrating cells involved in repair mechanisms and local plasticity.

That being said, we want to know how the expression of DCX is modified in a more chronic insult, like in neurodegeneration such as in Parkinson's Disease and Alzheimer's Disease.

So the aim of my study is to characterize the distribution of DCX cells in PD and AD patients' postmortem brain slices and compare with control patients.

The hypothesis being that there is a modification of dcx expression in the cortex of patients having a neurodegenerative disease.

Method :

This report is based on 9 human brain tissues, obtained from the Department of Pathology of the Lausanne University Hospital (CHUV): 3 Parkinson patients (Number A0200269, A0300080 and A0300084), 3 Alzheimer patients (A0800047, A0500097 and A0700131) and 3 control patients (A0900090, A0900100 and A0900079). All human material is acquired in accordance with the CHUV local ethical committee.

Three different steps were necessary to complete this study : immunohistochemistry optimization, data collection and result analysis.

Immunohistochemistry optimization :

The first step of the study was to optimize the protocol used for staining of the 5 µm thick formalin fixed paraffin embedded brain sections with DCX. After several trials to find the correct dilution and the right antibody, the protocol used in this study was established :

- Dry the sections at 52°C for 30 min to melt the paraffin
- Hydration of brain sections : sequentially histosolve by dipping back and forth 10 times each in alcohol 100%, 94% and 70%
- Wash 1 time with tap water
- Wash 1 time quickly with PBS 1x
- Wash in PBS 1x for 2x 5 min
- Pre-heat Tris-EDTA pH 9.0 for 3 min in microwave
- Put the sections in Tris-EDTA for 10 min in microwave for antigen retrieval
- Wash 1 time quickly with PBS 1x, then 2x 5 min in PBS 1x
- Put the sections in 3% H2O2 in H2O for 10 min for quenching
- Wash 1 time quickly with PBS 1x, then 2x 5 min in PBS 1x
- Blocking : 30 min in PBS-Casein 0.5% and NGS 5%
- Primary Antibody (AB2253 guinea pig anti-DCX) overnight in PBS-Casein 0.25% at 4°C at 1/200 µl

- Wash 2x quickly then for 2x 10 min in PBS 1x
- Secondary Antibody (goat anti-guinea pig biotinylated (Vector, Burlingame, CA, USA) at 1/400 µl for 40 min in PBS 1x at RT
- Wash 2x quickly then 2x 5 min in PBS 1x
- Apply the ABC solution of the ABC kit for 30 min
- Wash 1x quickly then 2x 10 min in PBS 1x
- Revelation with DAB kit for 20 min
- Wash 1x with tap water, then 1x quickly under PBS 1x, then 2x 5 min in PBS 1x
- Dehydration of stained brain sections: sequentially dip back and forth 10x each in alcohol 70%, 94%, 100% and histoclear.
- Embedding with Eukitt

Stainings used :

Each patient had several sections that could be stained with different stainings : GALLYA, TAU and DCX. Cortical columns were selected on areas on the cortex with a lot of degeneration subjectively observed on GALLYA stained sections and on TAU stained sections. The TAU and GALLYA stainings were done by the University Department of Pathology at CHUV, based on standard staining protocols used for diagnoses.

GALLYA silver staining is used to detect senile plaques and neurofibrillary tangles[9] specific to the pathophysiology of Alzheimer Disease.

TAU is an antibody based staining directed against the Tau protein, whose pathological accumulation is common to many pathologies classified under the general name of tauopathies. This staining is counterstained with hematoxilin which colours the nuclei in blue.

Total number of cells was counted on TAU sections, where all nuclei were colored in blue. Then the DCX cells were counted on the corresponding DCX sections.

Histological analysis :

The DCX marked cells were counted thanks to the Mercator Software from Explora Nova, under the Olympus BX40 microscope. In each section, a cortical column was drawn in a region where there was a lot of degeneration, confirmed by the corresponding section stained in GALLYA and in TAU (obtained thanks to the collaboration of the University department of Pathology at CHUV). An area with a lot of degeneration was a region where there was subjectively a lot of staining for GALLYA and/or TAU. The column was then roughly divided into three layers.

Once the segmentation for the nucleus on TAU sections (obtained thanks to the collaboration of the University department of Pathology at CHUV) was defined and the cell surface limits from 20-500 μm^2 were applied, the software automatically counted the total cells. Visual control was made in order to make sure that the software was correctly identifying the cells. Then DCX cells were counted manually on DCX stained sections, as the software was not specific enough for those cells. DCX cells which were eligible for counting were brown stained cells with a “ghostly” nucleus, as DCX is a cytoplasmic marker.

All sections were counted in a random order and without knowing the diagnosis of the patient

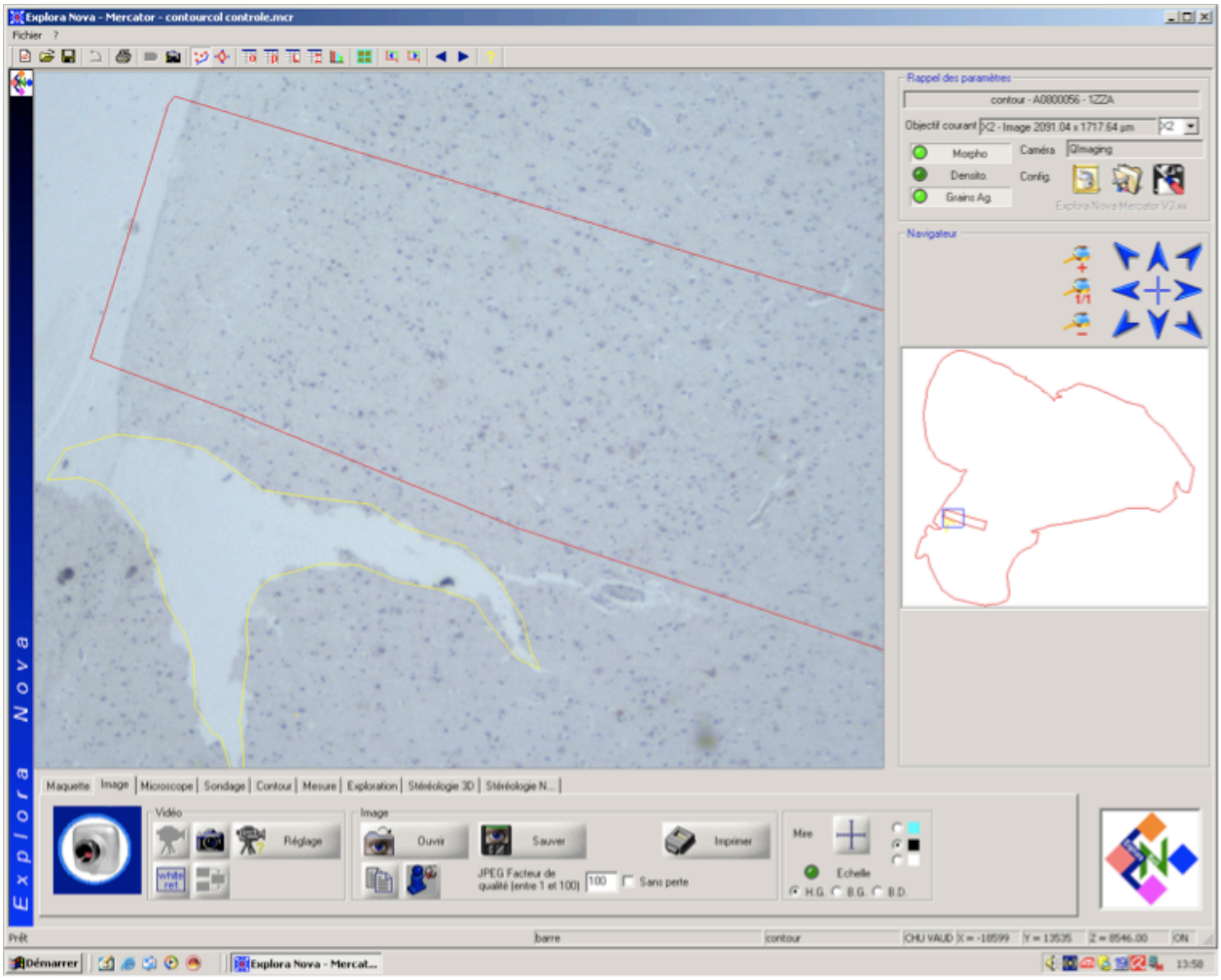


Fig1: Cortical column drawn on an area with a lot of degeneration (spotted previously on the corresponding section stained by GALLYA). Then the template with the column was used on TAU sections (shown in Fig.1) and divided roughly into three layers. The software counted the nucleus on TAU for total number of cells.

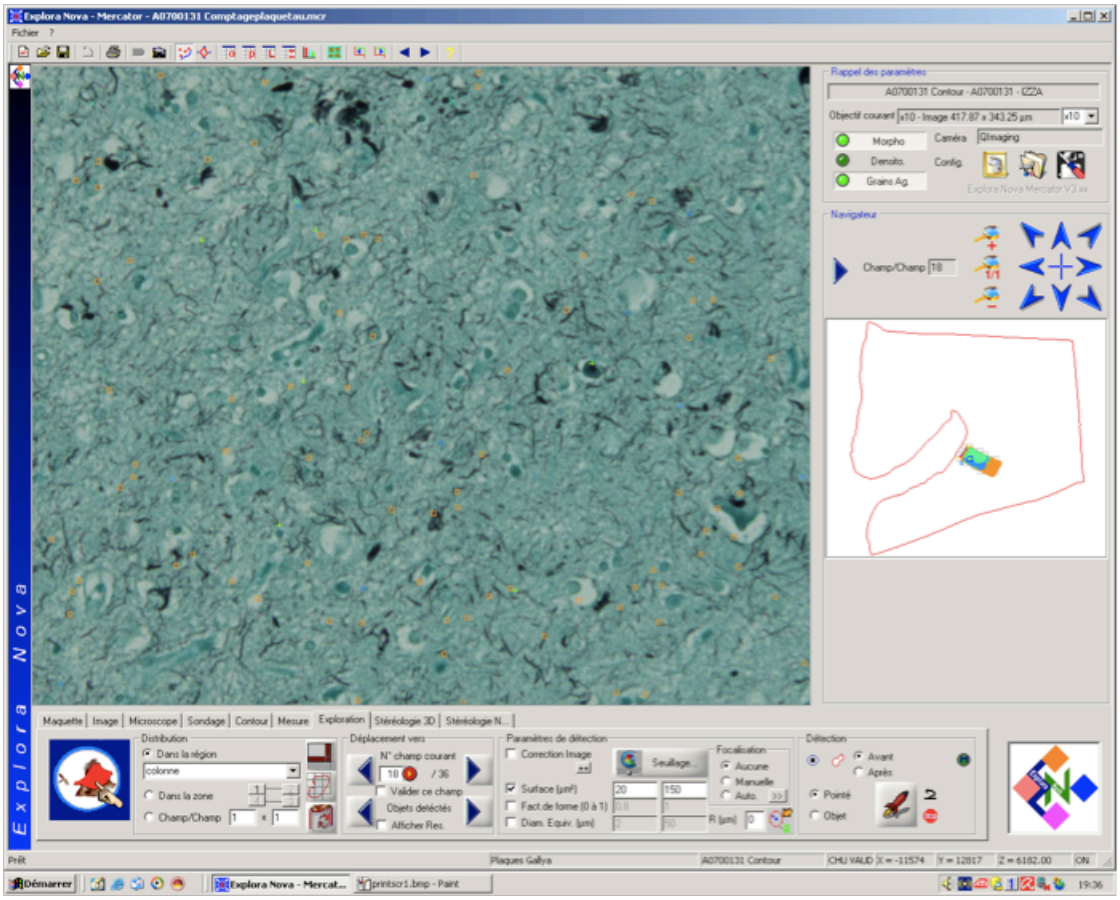


Fig.2: GALLY stained corresponding section used to select the area of the column where there is subjectively more degeneration seen. Degeneration is stained in black. To match the template of one patient between the different stained sections (GALLYA, TAU, DCX), some reference points were used near the column. When another staining was used of the same patient, the adjustment was made using those reference points (fig.3)

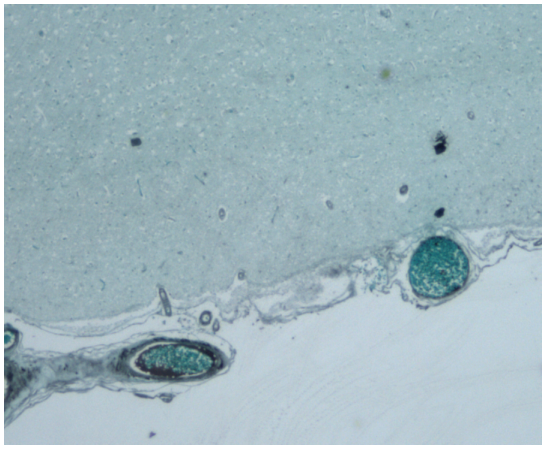


Fig.3: Example of fixed reference used: blood vessels are good fixed references because they are unchanged through the different sections of one patient used for different staining. By matching those vessels on another staining, we would be sure to be on the same area of interest

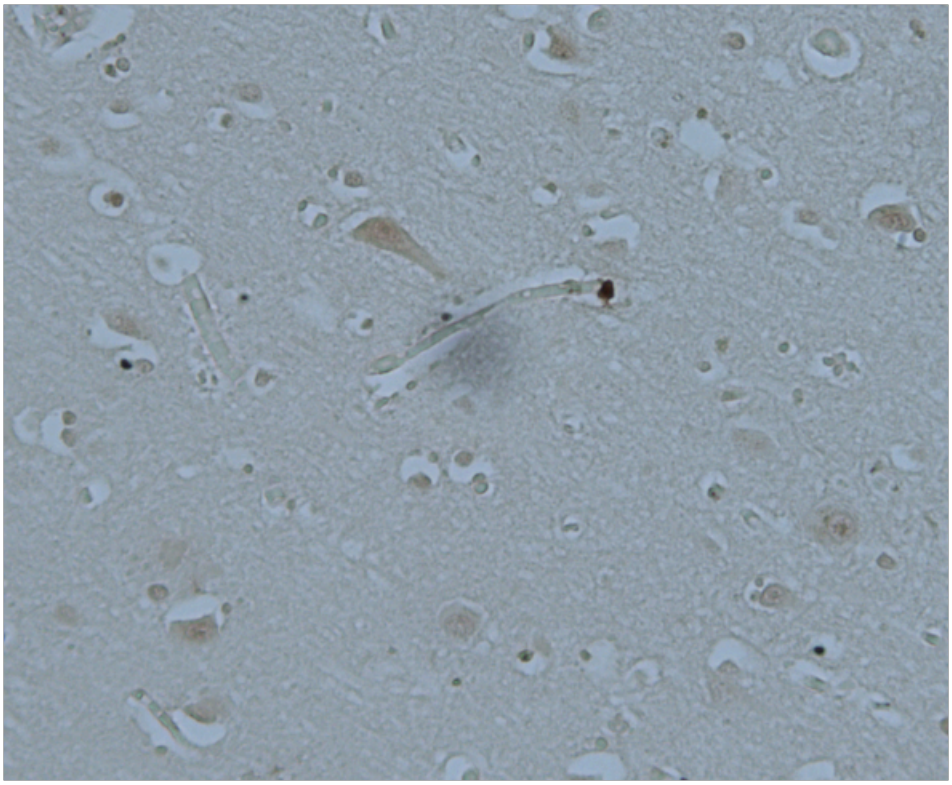


Fig.4: DCX stained cells.

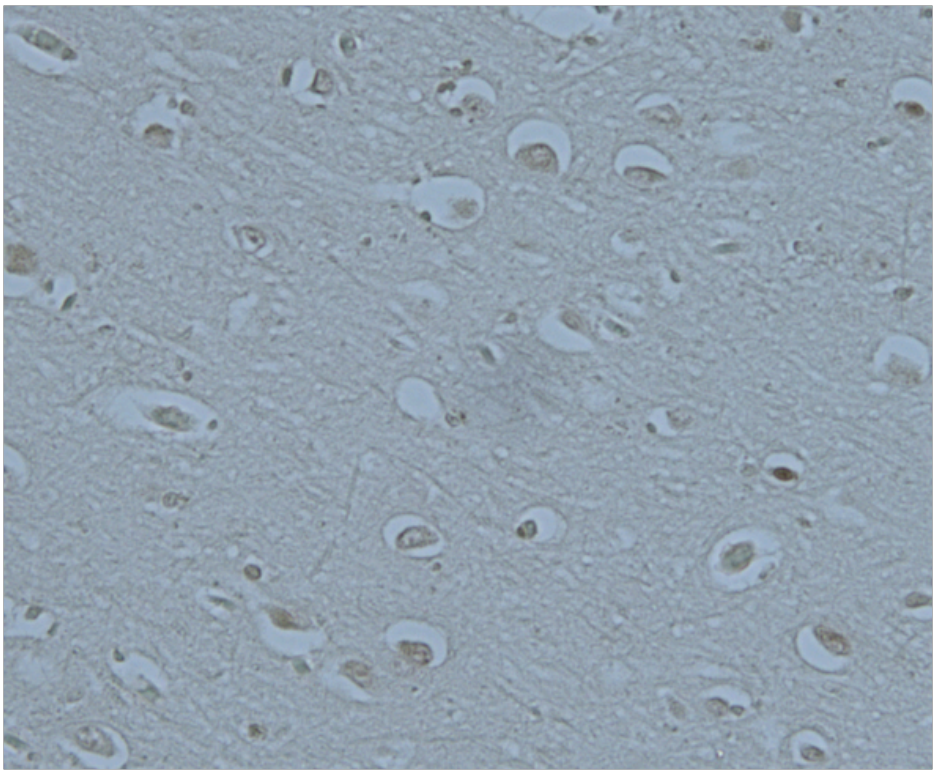


Fig.5: In the centre of DCX cells, we can see a lighter staining corresponding to the nucleus. DCX antibody is a cytoplasmic marker.

Data analysis:

Results analysis was done on Microsoft Excel using basic statistics tool.

Raw data was collected directly from the Mercator Software which automatically converted the values into a Microsoft Excel chart. Values of interest collected are: number of total cells, number of DCX cells, Surface area of a column.

Because the surface area of a column was different from one section to another, standardization was needed. For this purpose, the surface area of section A0900090 was randomly chosen as the surface reference for all the other sections and standardization was done with this surface area. The number of cells of each section was divided by the surface area of the section, then multiplied by the reference surface area. The new value was the standardized number of cells.

The percentage of DCX cells over total cells was then calculated.

Methodological results:

The first part of this study was optimization of the staining technique for an optimal cell counting by the software.

At the beginning, we didn't have satisfactory staining on the sections; indeed the sections were either inhomogeneously stained, or not stained at all. So we had to change this situation by modifying some parameters in the protocol.

First of all, two primary antibodies were available: AB18723 rabbit anti-DCX and AB2253 guinea pig anti-DCX. Some trials had to be done in order to determine which suits the study best. Those trials showed that there was better staining with guinea pig anti-DCX.

Then the intensity of the staining was not optimal, so we increased the time of DAB revelation to 20 minutes from 5 minutes.

The concentration was also adjusted to 1/200 µl for the primary antibody and 1/400 µl for the secondary antibody.

The new modified protocol was applied to the sections used in this report.

Results:

There is a difference of DCX cell expression between Alzheimer's Disease patients and control patients. As the chart shows, the percentage of dcx cells in the cortex of an Alzheimer's patient is 12.54% ± 2.17%, where as in the cortex of control patients, it is 5.47% ± 0.83%.

On the other hand, there is no significant difference in the ratio of DCX cells over total cells between parkinson's patients and control patients, both having around 5% of DCX cells (Parkinson: 5.52% ± 0.81%).

Parkinson	Total cells	DCX	DCX/tot cells [%]
A0200269	4695.23	227.26	4.84
A0300080	2200.41	141.12	6.41
A0300084	3684.23	195.02	5.29
Average			5.52
Stand. Dev			0.81

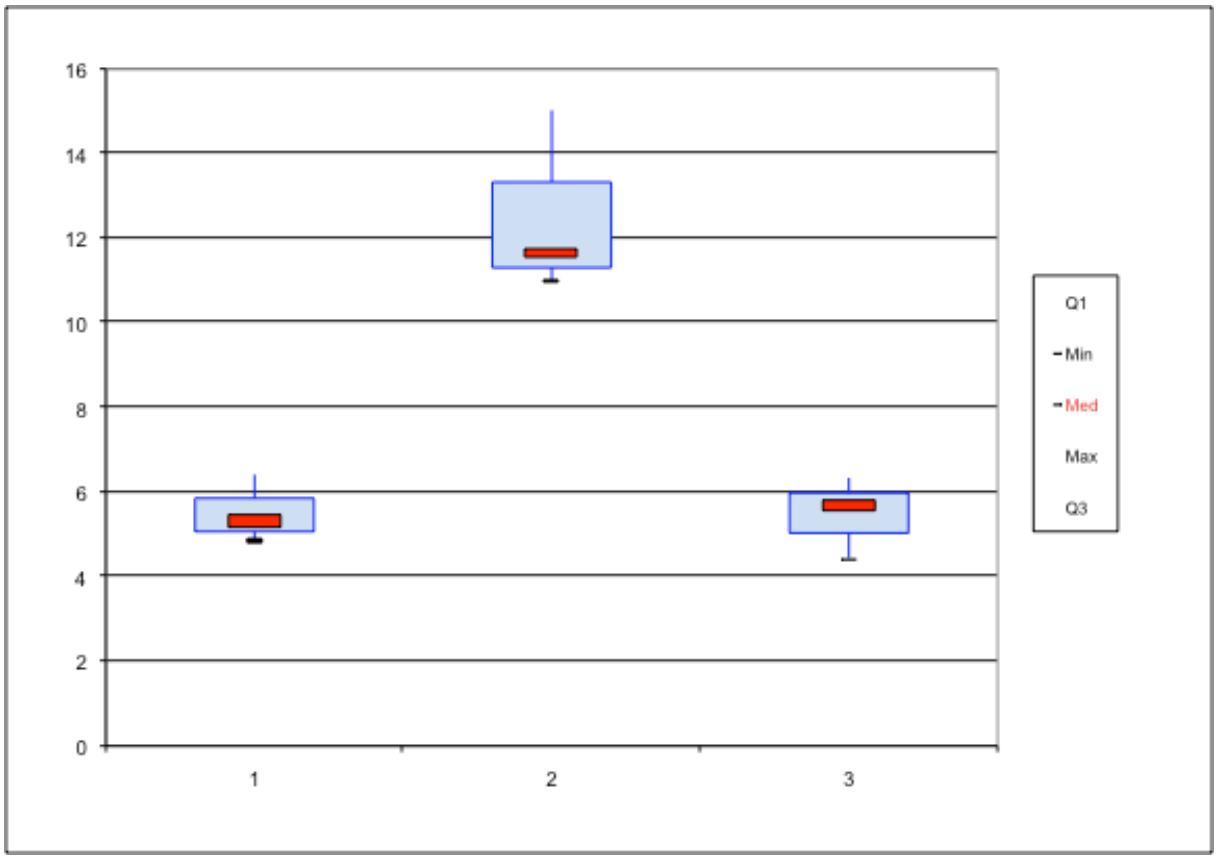
Chart1: Results for the three Parkinson's Disease sections

Alzheimer	Total cells	DCX	DCX/tot cells [%]
A0800047	1452.63	168.88	11.63
A0500097	1910.22	286.94	15.02
A0700131	2809.41	308.44	10.98
Average			12.54
Stand. Dev			2.17

Chart2: Results for the three Alzheimer's Disease sections

Controls	Total cells	DCX	DCX/tot cells [%]
A0900090	2003	89	4.44
A0900100	804.44	45.23	5.62
A0900079	2928.67	185.53	6.33
Average			5.47
Stand. Dev			0.83

Chart3: Results for the three controls sections



Graph1: On horizontal axis: 1 is PD, 2 is AD and 3 is Controls. Max value is showed on the graph as the highest tip of the vertical line. Min is the lowest mark at the bottom of the box plot. Q1 is the lower limit of the box plot. Q3 is the upper limit of the blox plot. Med is the red line.

We can see a great increase in the DCX cell ratio between AD on one hand and PD, Controls on the other.

Between PD and Controls, we find a p of 0.4749 whereas AD and controls have a p of 0.0087 which well below p=0.05, showing that there is significant difference of expression between AD and controls, unlike PD and Controls which have a p of 0.47.

Discussion:

In this study, we show that control humans have approximately the same DCX expression than non human primates which is around 4.5% [7].

As an other study showed[10], we also confirm that there isn't any increase in the expression of DCX cells in the cortex of Parkinson's Disease patients compared to the Control Patients.

In this report, we show that the expression of DCX cells is modified in Alzheimer's disease.

There is a significant increase in the expression of DCX cells in the cortex compared to the cortex of control patients (12.54% for AD versus 5.47% for controls, with $p=0.008 <0.01$).

This is a dramatic increase which shows that even though Parkinson's Disease and Alzheimer's Disease have roughly the same pathophysiology (accumulation of protein followed by degeneration), there is something different happening that changes the expression of DCX cells in AD but not in PD.

First of all, the increase in the ratio of DCX cells over total cells can have two meanings: The first meaning is that there is the same absolute number of DCX cells between control and Alzheimer's cortex, but due to the cortical atrophy in AD, the absolute number of total cells is decreased in AD patients. So the decrease of total cells without any change in the number of DCX cells makes the ratio bigger in AD compared to the controls.

This could mean that DCX cells are "tougher" cells which resist neurodegeneration, while non DCX cells around are degenerating. The question then would be why those cells aren't degenerating and what makes those cells more resistant to neurodegeneration? This could be answered if we look at the role of doublecortin, which is to stabilize the microtubule for growth cone. So let us suggest that there is a process that makes the cell rearrange its cytoarchitecture using microtubules; the protein Tau would be implicated as it is a microtubule stabilizer just like doublecortin, but in mature neurons. Then if there is a neuropathological process that makes this tau protein to accumulate and form neurofibrillary tangles causing the cell to degenerate, then we could say that doublecortin cells have less probability to degenerate, because they will

have doublecortin itself to stabilize the microtubule instead or in addition of tau, avoiding a cell cytoarchitecture disturbance, thus avoiding the cell degeneration and making it more resistant.

Then another question could arise: what would be the role of those cells if they are not doing anything in the process of the progression of the disease, beside standing there doing nothing but resisting death?

The DCX cells being more resistant than the rest of the cells is one of the hypothesis. But this hypothesis is less likely to be true, because let's not forget that there is another neurodegenerative disease we are dealing with: Parkinson's Disease. Studies have showed that there is also some cortical atrophy in PD, meaning that there is also some cell loss [11][12][13]. But there is no difference in the DCX cell ratio between controls and PD, meaning that if there is cell loss in PD, there would be also Doublecortin cell loss in order to have the same ratio. Then why would DCX cells degenerate in Parkinson's Disease, while there would be no or little DCX cell loss in Alzheimer's Disease? Unless the degenerative process in PD is more toxic to DCX cells than the degenerative process in AD.

In order to confirm or reject this, we would have to find a method to count the absolute number of total cells in both AD and control patients in a given volume, then count the absolute number of DCX cells to highlight a loss of total cells in AD but no change in DCX cells number. This can't be done in this study because, the sections from controls and AD were different and possibly from different brain area, so the cortical layer can be different from one area to another, thickness wise. Furthermore, the angle of slicing the section can be different from one section to another and depending on the plane of slicing, there could be more or less cells, even in the same region.

The second meaning is that the modification of expression in the DCX cells ratio shows that there is an effective increase in DCX cells, meaning that there is some neuroregeneration taking place.

This increase in DCX cells in Alzheimer's disease can be compared to the increase in DCX highlighted by the study on primates with acute brain insult. Indeed, with this

comparison, we can argue that there is an attempt of repair which is initiated to compensate the chronic cell loss in Alzheimer's Disease. This hypothesis is more likely in a sense that there is loss of neurons by degeneration in AD and the brain is trying to compensate this loss by new neurons, or at least by creating some new connections with preexisting neurons. In normal state, the 4 to 5% of DCX cells observed in the cortex of controls can be neural repair elements standing by in the cortex like a sentry, ready to act if there is a cell loss. Of course the question, "why not in Parkinson's Disease?" is still here but this could be argued by saying that the progression of Parkinson's Disease is due to Lewy Body intracellular accumulation, whereas in Alzheimer's, the progression of the disease is due to intracellular neurofibrillary tangles and due to extracellular accumulation of beta-amyloid senile plaques. There is an extracellular component in Alzheimer's Disease which is not here in Parkinson's Disease. So the extracellular component can be a trigger to initiate an attempt for neuroregeneration.

This idea can be integrated in the broader point of view of neuroinflammation. Indeed, some studies[14][15] show that neuroinflammation is involved in the pathophysiology of Alzheimer's disease. The progression of the disease would trigger neuroinflammation and the process following the primary inflammatory response: neural repair.

Our study can show that the increase in DCX cells is an attempt to repair the degenerated neurons, in the context of neuroinflammation triggered by the physiopathological progression of the disease.

Everything that has been said above takes into consideration the fact that the increased expression of DCX cells is beneficial against the progression of Alzheimer's disease. But another hypothesis can be implied: the increased expression of DCX cells is deleterious for the development of the disease[15]. Maybe, in some stage of Alzheimer's disease, there is a deregulation of neuroplasticity and the increased DCX is a marker of high turnover in neuroplasticity which could be eventually toxic to the cells. Indeed we could imagine that in an early stage of the disease, there is a high activity of synaptic change that would need neuroplasticity and due to this increased neuroplasticity, there would be an increased accumulation of proteins, like tau, involved in structural change and whose accumulation would form the famous neurofibrillary tangles in the cells, causing them to degenerate eventually.

Basically the question would be: Is increased DCX expression slowing the progression of the disease by acting as a neuroprotection tool from the body to repair the cell loss, or is the increased DCX one of the causes of the development of the disease, or just a simple marker of the disease?

A lot of questions are arising from my results, but they can't be answered in this study which was limited due to time and several other factors.

Limits:

First of all, the cortical thickness and the cell density is different from one patient's section to another depending on the localization of the section in the brain and the slicing plane of the brain tissue. This means that we can't do comparative study of the absolute values of number of cells. That's why at this level we can't put forward a definite hypothesis on the increase of the DCX/total cells in Alzheimer's Disease. In order to answer this, we will have to costain the sections with DCX and NeuN, which is a specific marker of neurons, and see whether the ratio of DCX cells over NeuN cells is modified or not.

We also don't know the cause of death of the patients, whether they died due to the neurodegenerative disease or something unrelated for example. This underlines another limit of this study: we don't know the stage of the disease. This is important because if we knew the stage of the disease, we could look if DCX is more increased in early stages or in late stages. Ideally, we would need a DCX ratio evolution study throughout the disease, for example in order to know if DCX is increased in reaction of neurodegeneration or if it is increased before the cell loss. With the study on histological sections, we lack a certain timeline analysis.

Another limit is the quality of the sections, because the sections we were given by the Department of Pathology of the Lausanne University Hospital were old sections stored since a long time ago. Maybe this long storage time could have affected the quality of staining. But the fact that the results confirm the same ratio in control patients of DCX cells over total cells as the one found in a previous study on non human primates[7] – i.e 4-5% - shows that the results of this study can be taken seriously.

Perspectives:

Now that we confirmed that there is no significant change in DCX cells expression in Parkinson's Disease, we can concentrate on Alzheimer's Disease, where we showed a significant increase in DCX expression. The next step would be to confirm this increase on a broader series of Alzheimer's patient's brain sections.

We can also go further in our analysis of DCX cells overexpression by looking more in detail the DCX distribution throughout the cortical layers. This could be done by defining the different cortical layers within the defined column chosen for cell counting. We can see if DCX cell expression is overall increased throughout all cortical layers or only in a few layers.

We can also check if there is a correlation between the amount of degeneration seen by GALLYA or TAU staining and the increase of DCX cells. First globally by choosing different columns in the same section, for example one column where there is high degeneration taking place and one in an area where there is less degeneration; then locally by choosing within the same column, an area where there is high degeneration and see if locally there is an increase in DCX expression compared to another area in the same column where there would be less degeneration.

One study[7] showed that there is not only one kind of DCX cells but several subpopulation. During this study, we saw that there is indeed at least three morphological categories of DCX positive cells: large DCX cells, small DCX cells and twin DCX cells, which are a couple of DCX stained small cells found next to each other. We could compare the ratio of those subpopulation of DCX cells and see if there is a difference which could help us characterize the role of those cells.

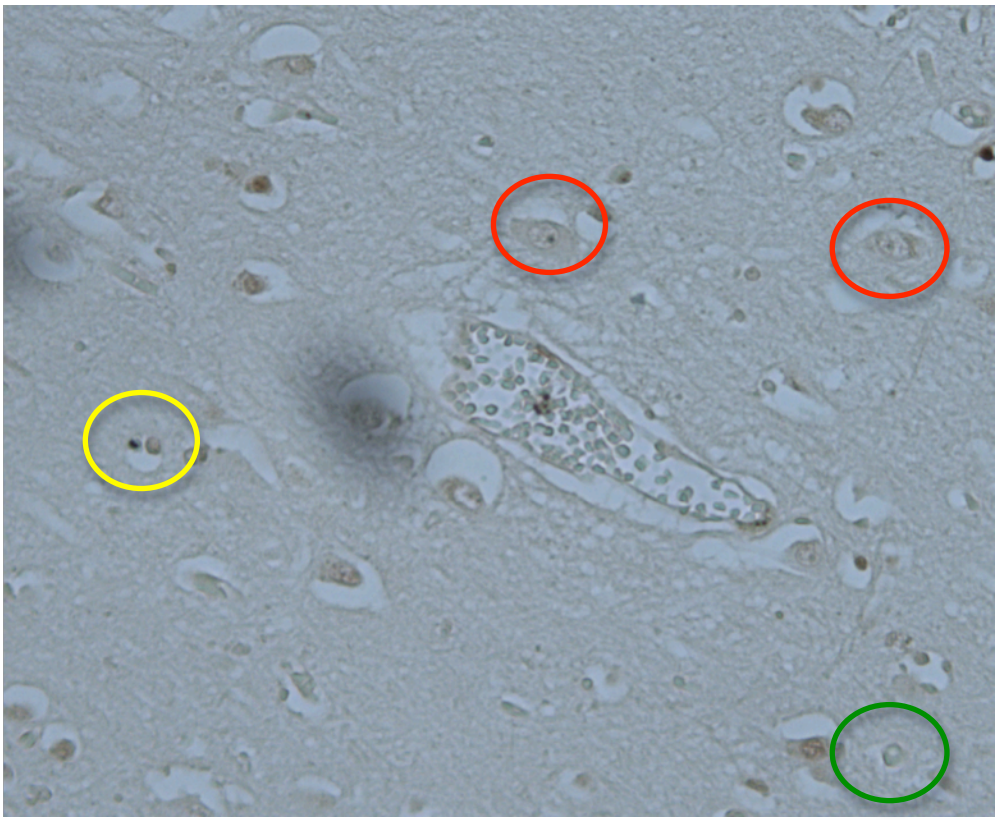


Fig.6: Red circles indicate large DCX cells, the yellow circle indicates the "twin" DCX cells and the green indicates the small cells.

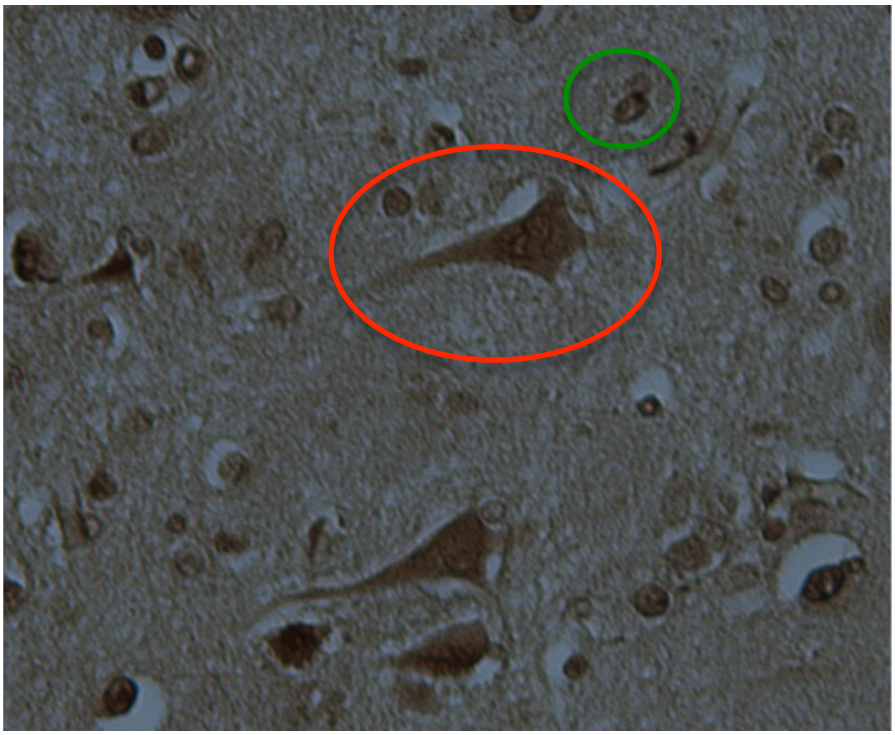


Fig.7: Another example of the difference between large DCX cells and small DCX cells

Conclusion:

At the beginning of this study, we had one fundamental question: was the expression of Doublecortin positive cells modified in neurodegenerative disease such as Parkinson's Disease and Alzheimer's disease? We showed that this study managed to answer this question by highlighting a modification in Alzheimer's disease of DCX cells expression implicated in neuroregeneration and neural repair. But because of this answer, many other questions are raised. The answer to those questions could lead to new research and therapeutical strategies targeting the cause of the disease itself. But the quest for those answers will need further studies which are way beyond the time frame and the level of a simple master's research project.

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Dr. Jocelyne Bloch, MD:

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