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Doublecortin cells and stroke

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Contents:

Abstract

Introduction

Doublecortin (DCX) is a microtubule associated protein expressed by migrating neural precursors. DCX is expressed in approximately 4% of all cortical cells in adult normal primate brain. DCX cells are is thought to play a role in plasticity or neural repair because their expression is enhanced locally in response to an acute insult made to the brain. That being said, it would be interesting to know how the expression of DCX is modified in stroke. The aim of my work is to study the expression of DCX cells in the cortex of patients having a stroke, compared to control patient.

Methods

DCX cells were quantified on DCX stained 5 µm thick formalin fixed paraffin embedded brain sections in 5 stroke patients and 3 control patients. . By using a computerized image analysis system (Explora Nova, La Rochelle, France), cortical areas were selected on injured and perilesional areas. Total number of cells was counted, whereall nuclei were colored in blue. Then the DCX cells were counted on the corresponding
DCX sections. The ratio of DCX cells over total cells was then calculated.

Results

We observed a similar ratio of DCX cells/total cells in the patients who died 1 day, 25 days and 1460 days after their stroke and in the control patients. However we found an important increase of the ratio DCX/total cells in the patients who died 2 and 13 days after stroke. In these two patients, the maximal amount of DCX cells could be observed in the perilesional areas.

Discussion and conclusion

The increase of DCX cells ration in patients died 2 and 13 days after stroke could be due to DCX cells being more resistant to degeneration after ischemia compared to surrounding cells. or to a recrutment of DCX cells, either from the cortical source of DCX cells or from the stem cell sub ventricular zone.
In order to be able to answer this question, the total number should be counted, which is methodologically challenging.

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

Introduction

Stem cells are undifferentiated cells that have the capacity to generate specialized cells and can divide to produce more stem cells.

In mammals, there are three types of this cells; embryonic stem cells, foetal stem cells and adult stem cells.

Embryonic stem cells are isolated from the blastocyte (4 days post fertilization). They create the three primary germ layers; ectoderm mesoderm and endoderm. Thus embryonic stem cells can produce all cell types of the adult body. We call this capacity totipotency.

Foetal stem cells are the primitive stem cells in the organs of foetuses or umbilical cord and are less numerous than the embryonic ones. Unlike embryonic stem cells, these stem cells are not totipotent and are specific of the foetal tissue where they are found. They have a high level of division and important potential of differentiation. We say that they are multipotent[1].

Adult stem cells represent a few cells present in every tissue of the body. These cells have the capacity to repair and maintain the tissue in which they are found (multipotency). For example, neural stem cells can create all neural cells (neurons, astrocytes, oligodentrocytes). The formation of new neurons in the nervous system is called neurogenesis. Neurogenesis is more active during pre-natal development but it remains present in adulthood.

There are in the human brain two distinct structures where neurogenesis remains during adulthood: the Sub-Ventricular Zone (SVZ) and the Sub-granular zone of the Dentate Gyrus[2]. Indeed, we observe in these two areas the generation of neurons from neural progenitor cells during adulthood.

More recently the neurosurgical research group of the CHUV (Centre hospitalier universitaire vaudois) in Lausanne has been able to harvest cells originating from adult primates cortex of humans and monkeys[3]. These cultured cells are neural ecosystems with characteristics of precursor cells. However unlike stem cells, their rate of replication is more indolent and after a few weeks of culture, a senescence can be observed. Cortical Doublecortine (DCX) cells have been identified to be at the origin of these neuronal ecosystems.

Doublecortin is a microtubule-associated protein that is transiently expressed in migrating neuroblasts in both developing and adult mammals, thus coordinating neuronal migration through the organization and stability of microtubules[4].

DCX was described for the first time [5] as a gene X-linked Lissencephaly and Doublecortex Syndrome, which are two human disorders with a disruption of cortical neuronal migration during development. Neuronal precursors express DCX over a period of about two or three

weeks until they matured into neurons. A high concentration of DCX cells has been found in the neurogenesis areas, Sub-Ventricular Zone and in the Hippocampus, so DCX in those areas reflect neurogenesis[6]. However, not only DCX cells are expressed in those specific locations in the adult, but they are also spread in the whole neo-cortex of adult human and non-human primates. Approximately 4.5% of all cortical cells express Doublecortin[3].

The fact that the brain has the capacity to generate new neurons raises the question of how neurogenesis is affected after a brain injury. For example, stroke is an acute brain damage caused by a stop of blood supply, representing a leading cause of neurological disability in humans.[7] Recovery after stroke is long and often incomplete.

The role of neural stem cells and precursor cells is not completely elucidated but is certainly crucial to help the patient recovering after stroke. Indeed, we observe in rodent with ischemic stroke an increase of cells proliferation in the SVZ. [11] Thus, we can say that endogenous neural stem cells supply the injured adult brain with new neurons but this supply is not sufficient for all the patients to recover their entire motor capacities. [7]

Unlike neural stem cells, little is known about the destiny of DCX cells after a stroke.

The team from Lausanne has already [3] demonstrated in monkeys that the neural ecosystems originating from the DCX could improve motor function recovery after having been reimplanted in the same individual close to a motor cortical lesion. Now the question is whether the cortical DCX cells naturally found in the cortex also participate to brain repair after a stroke.

The aim of my study is to analyze the histological distribution, morphology and characterization of DCX-positive cells in the cortex of patients who died from an acute stroke, and to compare it with control healthy subjects.

Material and Methods

1. Material

This report is based on 8 human brain tissues obtained from the Department of Pathology of the Lausanne University Hospital (CHUV) cells. All human material was acquired in accordance with the CHUV local ethical committee. This table summarized the clinical characteristics of each patient.

5 stroke patients

Patients	Time between stroke and death	Cause of stroke	Injured territory
A100046	1 day	Intern Carotid stenosis (M1 segment)	Sylvian artery territory
A900227	2 days	Proximal and distal vasospasm of anterior cerebral circulation	Anterior céeréebral artery territory
A080090	13 days	Embolism of cerebral anterior artery and middle céeréebral artery	Sylvian artery territory
A800154	25 days	Right sylvian artery thrombus	Sylvian artery territory
A900190	4 years	Preoperative distributive shock (lung transplant)	Junctional infarcts

Table. 1: Clinical characteristics of stroke patients

3 control patients (death related to another cause)

Patient	Cause of death
A100154	Cardiac transplant
A080172	Three vessels coronary disease
A090018	Pancreatic adenocarcinoma with liver metastasis

Table. 2: Clinical characteristics of control patients

2. Methods

Immunohistological characterization was done for DCX with AB 2253 guinea pig anti-DCX, (Milipore, Temecula, California, USA). The immunoreactions were revealed for immunohistochemistry with biotinylated secondary antibody (goat anti-guinea pig, Vector Burlingame, CA, USA) followed by the immunoperoxydase Vectastain Elite system (PK 6100, Vector Laboratories) and DAB substrate kit (SK-4100, Vector Laboratories). The counterstaining was obtained with Mayer's hematoxylin solution (MHS32, Sigma-Aldrich, Buchs, Switzerland), Cresyl violet acetate and hematoxylin solution Gill no. 2 (GH23, Sigma-Aldrich, Buchs, Switzerland). The DAB immunostainings were observed under light microscope (BX40 Olympus).

The protocol used for immunohistochemistry of DCX is as following:

-the sections were dried 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%, 95% and 70%
-wash 1 time with tap water
-wash 1 time quickly with Potassium phosphate buffer salt 10 mM, PH6.9 (KPBS) 1x
-wash in KPBS 2x5 min

-put the sections in Tris buffer with Ethylenediaminetetraacetic acid, PH 9 (Tris-EDTA) for 5 min in microwave (500W) for antigen retrieval
-wash 1 time quickly with KPBS 1x, then 2x5 min in KPBS 1x
-put the sections in 3% H2O2 in oxygene peroxide (H2O) for 10 min for quenching
-wash 1 time quickly with KPBS 1x, then 2x5 min in KPBS 1x
-blocking: 30 min in KPBS-Casein 0.25% + triton and normal goat serum (NGS) 5%
-primary Antibody (guinea pig anti-DCX) overnight in KPBS-Casein
0.25% + triton 0.3% at 4°C
-wash half of monkey brain tissue quickly with KPBS
-preparation of the Secondary Antibody (goat anti-guinea pig) at 1/400 µl in KPBS + monkey brain tissue overnight at 4°C
-wash 2x quickly then for 2x10 min in KPBS 1x
-secondary Antibody (goat anti-guinea pig) at 1/400 µl for 40 min at RT
-wash 1x quickly then 2x5 min in KPBS 1x
-apply the ABC solution of the ABC kit for 30 min
-wash 1x quickly then 2x10 min in KPBS 1x
-revelation with DAB kit for 20 min
-wash 1x with tap water, then 1x quickly under KPBS 1x, then 2x5 min in KPBS 1x
-dehydration of stained brain sections: sequentially dip back and forth 10x each in alcohol 70%, 94%, 100% and histoclear
-embedding with Eukitt

Histological analysis

The DCX marked cells were counted with the Mercator Software from Explora Nova, under the Olympus BX40 microscope. In each section, we randomly chose two areas within the lesion and two perilesional aeras. An injured area was a region with a lot of gliosis, less nuclei and an important extravasation of hematies around blood vessels.

Once the segmentation for the nucleus was defined and the cell surface limits from 25-250 μm^2 were applied, total cell and DCX cells were manually counted. For these two results, a ration could be calculated.

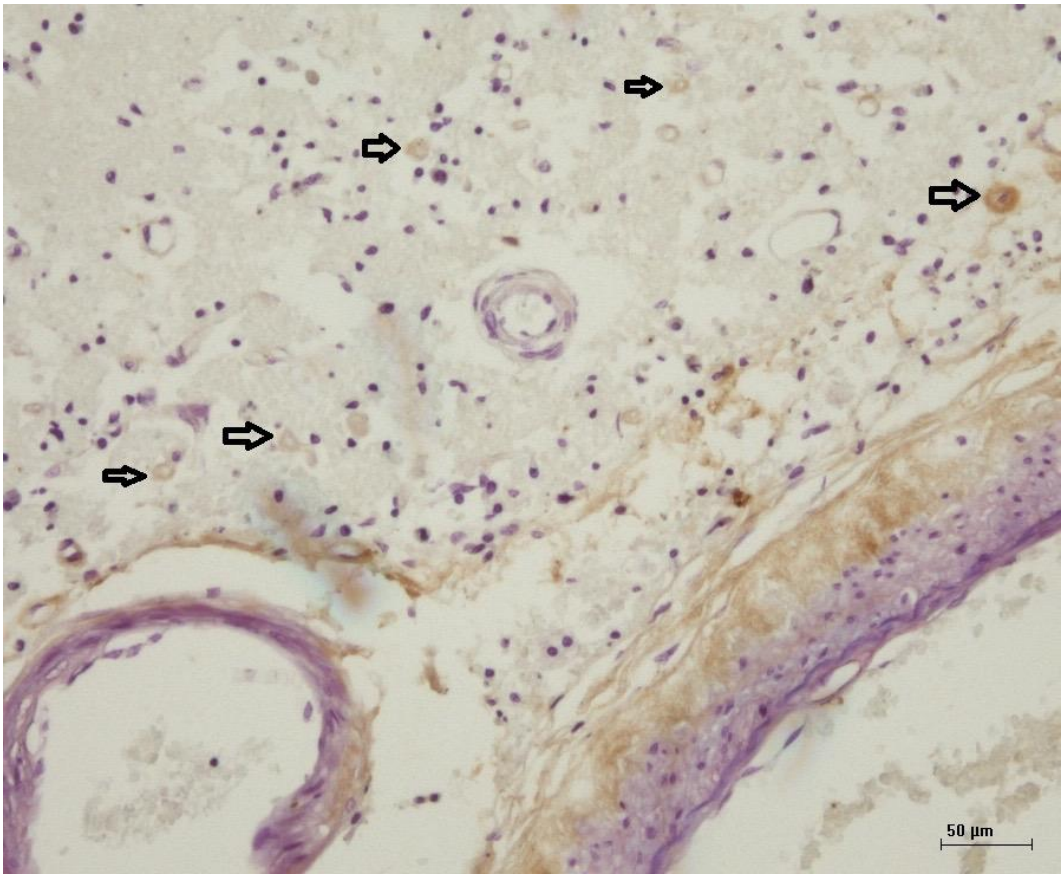

Fig2: The arrows are showing DCX cells in a perilesional area.

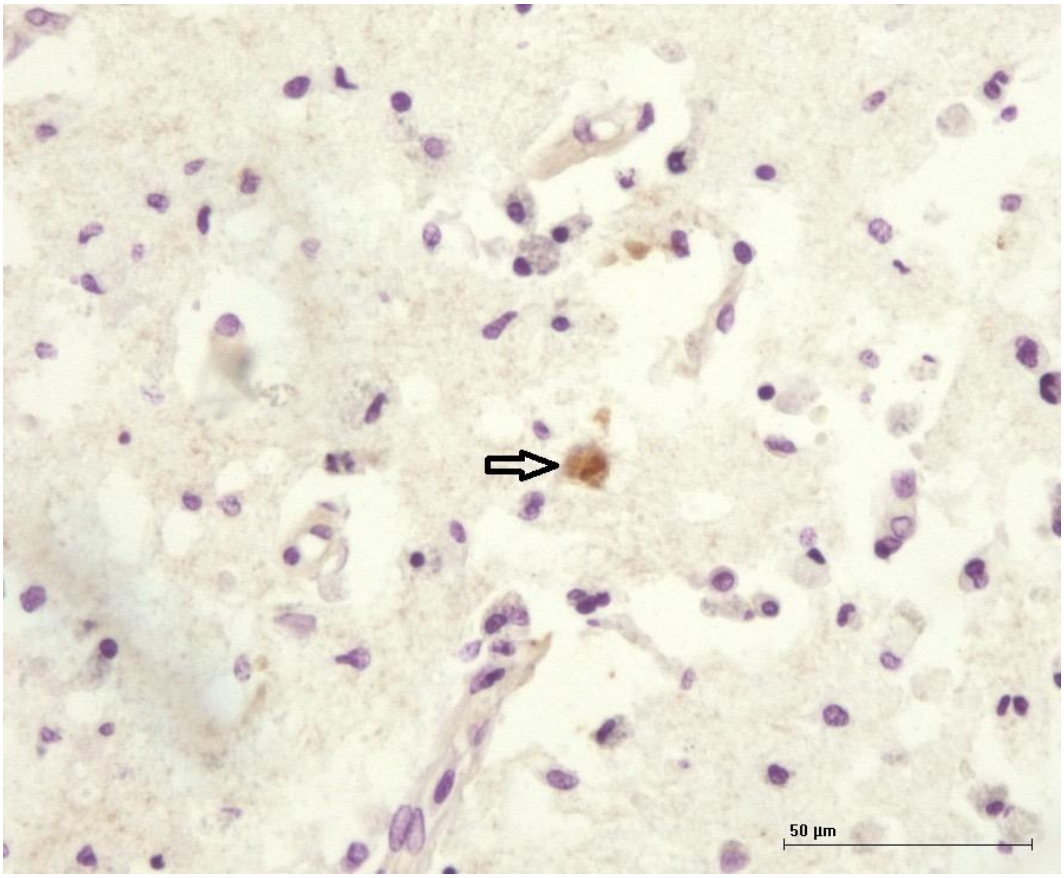

Fig3: The arrow is showing a DCX stained cells in an injured area.

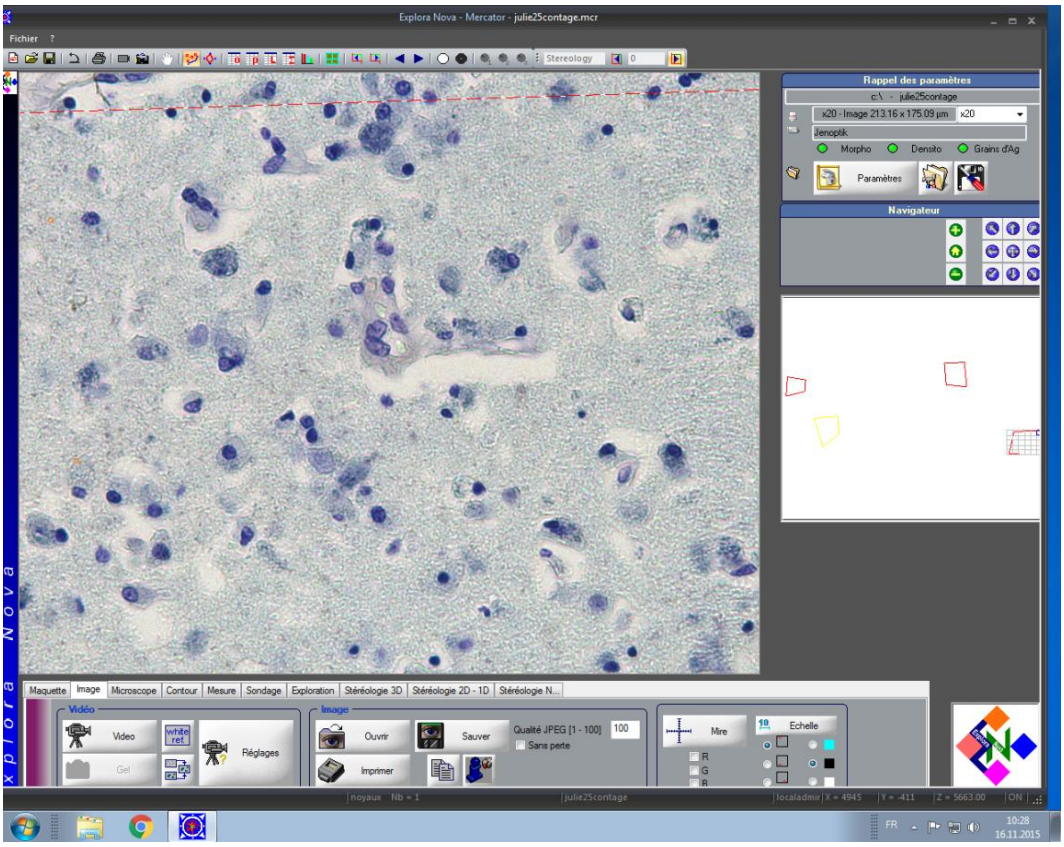

Fig4: On each section, 2 injured areas and two perilesional aeras were randomly selected. Then these areas were separated into several parts. Total cells and DCX cells were manually counted in order to optain a ratio.

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.

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

Results

1. Optimization of staining technique

- The first part of my study was to optimize the technique of DCX cells staining for an optimal counting. However, despite the fact that we applied the same protocol for each

section, we observe a heterogeneicity in staining. We decided to rate the quality of staining from 1 to 5 (1, being the weakest and the most diffuse staining, and 5, the strongest and cleanest staining). To simplify the description of the staining we decided to form 2 categories:

- **1-3**: weak and diffuse staining
- **4-5** : strong staining

The presence of diaminobenzidine crystals combined with iron ions was highlight because these elements could bias the counting.

Stroke sections	Quality of staining	Comments
A100046	1	Weak and diffuse staining
A900227	4	Strong staining
A080090	4	Strong staining
A800154	2	Weak staining
A900190	2	Presence Oydates Diaminobenzidine cristals combined with iron ions (presence of red blood corpuscules or hemolysis waste)

Table3. : Staining characteristics for stroke patients

Controls Sections	Quality of staining	Comments
A100154	5	Strong staining
A080172	5	Strong staining
A090018	5	Strong staining

Table 4: Staining characteristics for control patients

2. DCX/total cells ratio

We observed a similar ratio of DCX cells/total cells in the patients who died 1day, 25days and 1460 days after their stroke and in the control patients. However we found an important increase of the ratio DCX/total cells in patient who died 2 and 13 days after stroke. In these two patients, the maximal amount of DCX cells could be observed in the perilesional areas.

	Total cells/1	Total cells/2	DCX/1	DCX/2	DCX/Total cells/1	DCX/Total cells/2	Average	Standard deviation
A100154	195	1042	9	36	4,6%	3.4%	4%	0,84%
A080172	289	457	11	18	3,8%	3,93%	3,86%	0,09%

A090018	276	378	27	42	6,8%	11,11%	8,95%	3,04%

Table 5: DCX/total cell ratio in control patients

Patient	Total cells/1	Total cells/2	DCX/1	DCX/2	DCX/Total cells/1	DCX/Total cells/2	Average	Standard deviation
A100046	263	0	276	0	0%	0%	0%	0%
A900227	183	136	17	35	9.23%	25.73%	17,48%	11,66%
A080090	415	269	90	30	21,68%	11,15%	16,5%	7,44%
A800154	606	705	12	8	1,98%	1,13%	1,55%	0,60%
A900190	641	1150	7	17	1,09%	1,47%	1,28%	0,27%

Table 6: DCX/total cell ratio in stroke patients (perilesional area)

Patient	Total cells/1	Total cells/2	DCX/1	DCX/2	DCX/Total cells/1(%)	DCX/Total cells/2(%)	Average	Standard deviation
A100046	493	1061	3	10	0,60%	0,94%	0,77%	0,24%
A900227	120	124	30	91	25%	73,38%	49,19%	34,20%
A080090	61	240	17	106	27,86%	44,16%	36,01%	5,76%
A800154	576	319	2	7	0,34%	2,19%	1,26%	1,30%
A900190	176	1180	11	18	6.25%	1,52%	3,88%	3,34%

Table 7: DCX/total cell ratio in stroke patients (lesional area)

Chart5Graph1: Time between death and stroke/DCX cells

Discussion

The first part of my study was to optimize the staining. Despite the fact that we applied in each section the same protocol, we saw a difference in the quality of staining between

sections. This heterogeneity could be explain by the fact that the sections given by the Department of Pathology of the Lausanne University Hospital were old sections stored since a long time ago with different preparation methods and different time between death and brain slicing. This long storage could explain the bad quality of RNA and that's the reason why we couldn't apply molecular techniques on these tissues.

All these factors could have affected the quality of the staining. Furthermore, even if immunological detection is a very used method, it seems important to mention the fact that many factors could influence the fixation reproducibility. So we must consider every tissue individually, comparing injured and perilesional areas in each section.

It is also important to consider the fact that, on a very damaged tissue, the antibody could not recognize the target anymore so that's the reason why a tissue without any staining was excluded.

However, 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[3], 4–5% shows that the results of this study could be reproductible.

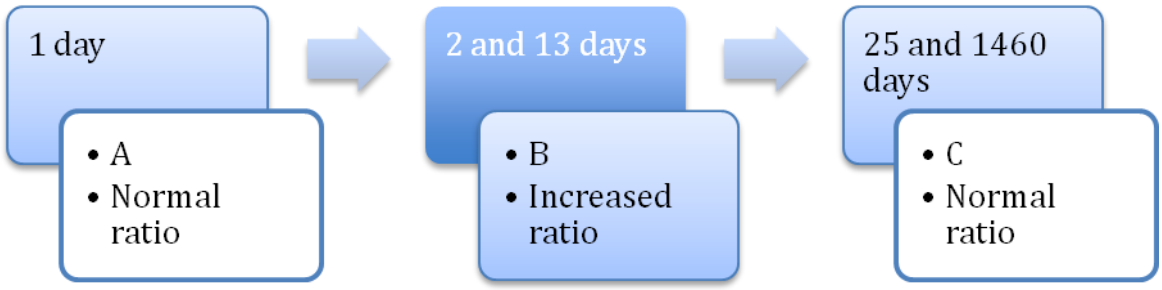

Fig 5: Evolution of DCX ratio in a stroke area

A: Patient died 1 day after stroke -> normal ratio

In the section of cortex from the patient who died one day after the ischemic lesion, we did not see DCX cell proliferation. Several hypotheses could explain this result.

First of all, it could be a false negative result because of the bad quality of the staining.

In order to confirm or reject this hypothesis, we would have to find a section stained without the second antibody.

The second hypotheses could be that a latency is needed to see DCX cells close to a lesion. This can easily be explained by the fact that after a stroke, the DCX cells have to migrate a considerable distance to reach the lesion.

B: Patient died 2 and 13 days after stroke -> increased ratio

There are several hypotheses to explain the increase of DCX ration seen in patients who died 2 and 13 days.

First of all, it seems that DCX are much more numerous than nucleus. This observation could mean that DCX cells are "tougher" cells which resist neurodegeneration while non DCX cells around are degenerating. The question then would be why DCX cells aren't degenerating and what makes those cells more resistant to neurodegeneration? This special robustness could be explained by the role of DCX cells which stabilize the microtubule for growth cone.

To confirm this observation we would have to find a method to count the absolute number of total cells in both injured and control patients in a given volume, then count the absolute number of DCX cells to highlight a loss of total cells in stroke but no change in DCX cells number. This couldn't be done in this study because the cortical thickness and the cell density was different from one patient's section to the other depending on the localization of the section in the brain and the slicing plane of the brain tissue. This means that we couldn't establish the cells absolute value. That's why at this level we couldn't confirm this hypothesis.

The second interpretation to this DCX cell increase could be a recruitment of DCX cells towards the lesion. Here again, the only way to prove it would be to obtain the cell total count that should be increased compared to normal.

The last hypothesis is that the DCX cells migrate from the SVZ, as previous[15] studies seemed to demonstrate in rodent. However, since rodents don't have DCX cells in their cortex, it is difficult to compare these results to the one observed in human brain. Furthermore, the distance between SVZ and the lesion cortex being very large, 48 hours seems to be a very short period of time to allow migration of DCX cells from the SVZ to reach the lesion.

C: Patients died 25 and 1460 days after stroke -> normal ratio

First of all, the very low rate of DCX cells found in section of patient died 25 days and 4 years after stroke could mean either that these cells disappeared or that the DCX cells transformed to mature cells.

Here again the only way to prove it would be to obtain the total cell value. However considering our previous studies on autologous brain cell transplantation, we believe that these cells have the potential to differentiate to mature brain cells.
Indeed, previous study [3] in monkey shows that stained DCX cells were becoming neurons.

Here again the only way to prove it would be to obtain the total cell value. However considering our previous studies[3] on autologous brain cell transplantation, we believe that these cells have the potential to differentiate to mature brain cells.

This being said, it seems important to discuss about the role of DCX cells in stroke.
In adult rodents, DCX+ cells are only found in the stem cell niches located in the subventricular zone and in the hippocampus. However the DCX cells can migrate from this niches to lesions elsewhere in the brain. It has also been demonstrated that the number of DCX+ cells positively correlates with the recovery of functional deficits after stroke. Furthermore, transgenic knockout DCX rodents exhibit worse outcome after a stroke both in the short and long term on a consistent basis[4,5].

In non human primates, the conditions are different, since DCX cells are also found in the cortex. Nevertheless there is also a correlation between the DCX cells and stroke. Indeed a study[3] showed an increase of neurogenesis after stroke that could be related to motor capacities improvement. However, no study could confirm these facts in humans. Therefore if the neuron increase is confirmed, it is still difficult to establish a clear link between new neurons and motor capacities improvement.

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.

Perspectives

In our study DCX/total cell ratio changed depending on the time after stroke. However the number of sample we studied is small and to confirm the increase and decrease of DCX/total cell ration, we need to collect more data.

It would be also interesting to observe and count the DCX cells not only close to the lesion site but also in the rest of the brain.

Finally we could continue our observation and get more information about the distribution of the DCX cells within the different layers of the cortex. Moreover, knowing from our previous paper[15] that cortical DCX cells are categorized in different subpopulations, we could also determine if one subtype of DCX subpopulation is more affected by these post stroke changes.

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