PROGRESS REPORT –YEAR 1

HISTOLOGICAL AND ANATOMICAL PRESERVATION OF RAT BRAINS AFTER VITRIFICATION AND REWARMING

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PROJECT ORIGINAL ABSTRACT

The aim of the project is to assess the degree of histologic and anatomical preservation of rat brains after pre-mortem perfusion with a vitrification protocol used in human patients, scaled down for young rats, followed by a 3-stage cooling protocol that ends in liquid nitrogen. This protocol will be followed, between 2 and 7 days later, by rewarming and standard fixation, sectioning, and Nissl and immunohistochemical staining of relevant brain regions. Young adult Sprague–Dawley female rats will be placed under deep anesthesia and intracardially perfused with three cryopreservation solutions progressively enriched in ethylene glycol (EG), which will range from 10 to 70% EG. The third solution (termed VM1) includes 15% dimethyl sulfoxide (DMSO) as a permeabilizing agent. Different experimental groups of 4-5 rats each will be used. A control group which will be perfused with fixative (paraformaldehyde 4% in buffered saline) and not frozen (standard procedure) and the vitrified groups, which will be perfused with the vitrification solutions and frozen as indicated. Comparisons will be made for the different parameters under study in the different experimental groups, which will be perfused immediately or 90 minutes after death. This should clarify the impact of the length of time between death and vitrification on the preservation of brain structural integrity.

INTRODUCTION

Our initial objective was to set up the rat brain vitrification procedures in order to ensure reproducibility. We also wanted to compare the performance of our vitrification solutions (VM1) with the original VM1 solutions. We performed 6 pilot experiments. This was necessary as immunohistochemical analysis of a number of brain cell markers showed variability from experiment to experiment. Next, we performed full experiments 0 min after death (euthanasic perfusion) and 90 min after euthanasic perfusion with carrier solution alone, keeping the heads of rats covered with water ice until full vitrification. In these full experiments we used 2 and 6 months old rats in order to assess the effect of age on cryopreservation effectiveness. Below we describe our experimental design, methods and results.

MATERIALS AND METHODS

Animals

Young (2 months) and adult (6 months) female Sprague-Dawley rats were used. Animals were housed in a temperature-controlled room (22 ± 2ºC) on a 12:12 h light/dark cycle. Food and water were available *ad libitum*. All experiments with animals were performed in accordance to the Animal Welfare Guidelines of NIH (INIBIOLP's Animal Welfare Assurance No A5647-01).

Vitrification solutions

a) **Carrier solution** consists of 0.028 M KCl, 0.23 M glucose, 0.01 M Tris base (pH 6.8). This solution is kept at 4°C.

b) **10% w/w EG solution** consists of 10% ethylene glycol (EG) in 0.028 M KCl, 0.23 M glucose, 0.01 M Tris base (pH 6.8). This solution is kept at 4°C.

c) **30% w/w EG solution** consists of 30% EG in 0.028 M KCl, 0.23 M glucose, 0.01 M Tris base (pH 7.5). This solution is kept at 4°C.

d) **70% w/w EG solution VM1** consists of 70% EG, 31.8% (v/v) dimethyl Sulfoxide (DMSO), in 0.028 M KCl, 0.23 M glucose, 0.01 M Tris base (pH 7.85). This solution is kept at -20°C.

Brain perfusion

Animals were placed under deep anesthesia by injection of ketamine hydrochloride (40 mg/kg, i.p.) and xylazine (8 mg/kg, i.m.), and intracardially perfused. To this end the thoracic cavity of the rats was opened and the beating heart exposed; a 21G needle was inserted into the left ventricle. The needle was fitted to a 3.16 mm ID PVC tubing which was connected to a peristaltic pump (Minipuls 3, Gilson Instruments, France) and whose free end was immersed in a graduated cylinder containing the appropriate perfusion solution kept at water ice temperature. The flow rate of the pump was set to 5.2 ml/min. A small cut was made in the right atrium in order to allow drainage of the perfusate. A 21 G needle was inserted in the right ventricle for eluate fraction collection. The abdominal aorta was clamped in order to restrict perfusion to the head and the thoracic and brachial regions **(Fig. 1).**

Figure 1. Panel A provides an overlook of the design of a typical vitrification experiment. Rats are perfused with a peristaltic pump (background). They are perfused via the left ventricle. The proximal rat is a control animal being perfused with fixative only. The other two animals are being perfused with one of the vitrification solutions. Notice that the heads of the experimental animals are covered with water ice. **Panel B** shows, on the left, a box with ice where graduated cylinders with the different perfusion solutions are kept during perfusion. A digital thermometer used to measure inner brain temperature can be seen against the wall. **Panel C** displays an experimental brain after vitrification perfusion. The organ appears like a fresh one.

Experimental Design

Pilot Experiments

Three groups of animals were formed (3 rats per group) namely, CONTROL, VITRIFIED-CI and VITRIFIED-OWN, and were perfused as follows:

The Control Group was perfused with 100 ml physiologic saline (0.9% NaCl) followed by 150 ml phosphate buffered para-formaldehyde 4% (pH 7.4) which acts as a fixative.

Vitrified Groups were perfused with 100 ml of 10% EG (CI or OWN, as appropriate) solution followed by 100 ml 30% EG solution (CI or OWN, as appropriate) and then by 150 ml of VM1 solution (CI or OWN, as appropriate).

At the end of perfusion, brains were rapidly removed. The control brains were stored in para-formaldehyde 4% (pH 7.4) overnight (4ºC).

The vitrified brains were placed at -20ºC for approximately 2 h, then transferred to a -80ºC deep freezer and after approximately 24 h, were placed in liquid nitrogen. Subsequently, the brains were removed from the liquid nitrogen and left at room temperature for about 15 min. They were then stored in para-formaldehyde 4% (pH 7.4) overnight (4 ºC).

Finally, all brains were maintained in cryopreservative solution at -20 ºC until use. For histological assessment, brains were cut coronally in 40 µm-thick sections with a vibratome.

RESULTS

In the first three pilot experiments a comparative study was carried out in order to compare the optical properties and performance of the VM1 solutions prepared at the CI and the same solutions prepared in our laboratory with our own reagents. As it can be seen in **Table 1**, both versions of VM1 displayed the same refractive indices at the three concentration levels of EG used (10, 30 and 70%). After perfusion, the brains were weighed and measured. We could verify that the results with both VM1 solutions were quite comparable. Immunohistochemical results were also comparable within the high variability limits we faced in those initial studies. We therefore considered that our VM1 solutions were essentially equivalent to the CI's preparations and for the rest of the experiments adopted our VM1 solutions.

Table 1. Comparative performance of VM1 solutions prepared at the CI and in our own laboratory.

Solution Origin	Own	CI	Own	CI	Own	CI
% EG	10		30		70	
ºB	10.9	11.3	22.3	22.1	50.4	51.9
Δ (g/ml)	1.04	1.05	1.09	1.09	1.24	1.24
Brain weight (N=3) After perfusion (g)	-	-	-	-	1.22∀ 0.36 X∀SEM	1.33∀0.16
Brain length (mm)	-	-	-	-	22.6∀0.5	21.7∀0.6
Brain width (mm)					12.2∀0.8	14.0∀1.2

20% w/w: sucrose solution, 20.0°Bx (1.0830 g/ml)
ºB are degrees Brix (adimensional) which are used to measure sucrose concentration. Δ= volumetric density in g/ml. Brain variables were determined after perfusion with 70% EG solution.

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Full Experiments

Two full experiments were performed, one using young rats and the other one using adult animals. Other than that, the experimental design was identical for both **(Fig. 1).**

Experimental Design

Full Experiments

In the pilot studies we had determined that in one day we were able to properly handle 5 animals. Since each experiment involved 4 animals per group, two experimental groups and a control group, we split each experiment into two consecutive days. Each half of the experiment was fully symmetrical with the other half.

Three groups of animals were formed, namely **CONTROL**, **0h** post mortem and **1.5h** post mortem and were perfused as follows:

The Control Group was perfused with 100 ml physiologic saline (0.9% NaCl) followed by 150 ml phosphate buffered para-formaldehyde 4% (pH 7.4) which acts as a fixative.

The 0h group was perfused euthanasically with 100 atml 0ºC carrier solution, followed by 100 ml 10% EG solution, 100 ml 30% solution at 0ºC and 150 ml 70% EG solution at 0-20ºC. In all experimental animals 0.5 ml eluate fractions were collected every 10 min.

The 1.5h group was initially perfused with 100 ml carrier solution, then the pump was stopped and the animals left on standby for 90 min before restarting the perfusion process with 100 ml 10% EG solution and so on.

At the end of perfusion, the brains were rapidly removed. The control brains were stored in para-formaldehyde 4% (pH 7.4) overnight (4ºC).

The 0h and 1.5h vitrified brains were placed at -20ºC for approximately 2 h, then transferred to a -80ºC deep freezer and after approximately 24 h, were placed in liquid nitrogen. Subsequently, the brains were removed from the liquid nitrogen and left at room temperature for about 15 min. They were then stored in para-formaldehyde 4% (pH 7.4) overnight (4 ºC). For histological assessment, brains were cut coronally in 40 µm-thick sections with a vibratome.

RESULTS

For illustration purposes **Table 2** shows the raw data of typical one-day full experiment for one rat from each experimental group. Notice that in the 1.5h group, animal´s brain temperature keeps dropping after initial perfusion reaching around 4ºC (90 min post carrier perfusion). Inner brain temperature (temperature probe is inserted 15-20 mm inside the brain from above) remains lower in the 1.5h rats during vitrification perfusion.

Table 3 shows the variations in the refractive indexes in the different perfusion fractions in rats euthanasically perfused (0h rats). After 100 ml perfusion the refractive indices of the eluate is essentially the same than that of the corresponding pure solution, indicating that the previous solution has been completely displaced by the current one. For the 70% perfusion solution we chose to perfuse 150 ml in order to make sure that the brain tissue was thoroughly embedded in it. Again, eluate refractive index at the end of perfusion corresponded to that of the pure 70% EG solution. The results were quite comparable for the 1.5h animals.

Table 2. Inner brain temperature variation during perfusion of representative 0h and 1.5h rats.

R1-0h	
Time (min)	Temperature (°C)

-5	33.2
0	30.4
5	25.4
10	17.0
15	15.6
20	14.3
25	11.9
30	10.4
35	9.0
40	8.7
45	8.8
50	8.5
55	8.8
60	9.4
65	8.9
70	8.7
75	8.7
80	8.7
85	8.5
90	8.3

R1-1.5h	
Time (min)	Temperature (°C)
-5 pre	33.4
0 pre	30.5
5 pre	25.0
10 pre	20.5
15 pre	16.8
20 pre	13.8
0 Vitrification	**3.9**
5 Vitrification	4.4
10 Vitrification	4.6
15 Vitrification	4.9
20 Vitrification	5.0
25 Vitrification	4.9
30 Vitrification	4.6
35 Vitrification	4.8
40 Vitrification	4.9
45 Vitrification	5.0
50 Vitrification	5.3
55 Vitrification	5.2
60 Vitrification	5.1
65 Vitrification	5.5

Table 3. Evolution of eluate refractive indices during perfusion.

Rat	R1-0h		R1-1.5h	
Perfusion time (min)	°Bx	g/ml	°Bx	g/ml
0	37.1	1.1642	36.3	1.1601
10	37.5	1.1662	37.1	1.1642
20	21.0	1.0875	21.4	1.0892
30	18.7	1.0772	18.8	1.0777
40	21.9	1.0915	22.1	1.0924
50	22.7	1.0951	22.5	1.0942
60	50.8	1.2375	43.3	1.1964
70	51.5	1.2414	50.5	1.2358
80	51.5	1.2414	50.4	1.2352
90	51.2	1.2397	-	-

ºB are degrees Brix (adimensional) which are used to measure sucrose concentration. Δ= volumetric density in g/ml. Brain variables were determined after perfusion with 70% EG solution.

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Morphometric Results

Post thawing vitrified and fixwd brains and regularly fixed brains were serially cut into coronal sections 40 μm thick on a vibratome. For morphometric analysis brain sections were captured using an Olympus DP70 digital video camera attached to an Olympus BX51 microscope (Tokyo, Japan). Sections were analyzed using the ImagePro Plus (IPP™) v5.1 image analysis software (Media Cybernetics, Silver Spring, MA). For morphometric analysis of neurons from different neocortex regions, sections were immunostained for the mature neuronal marker NeuN. Neuronal diameter and roundness did not significantly change after rewarming but a 90-min cold ischemia in neocortex did change cell diameter in the hippocampus of adult rats **(Tables 4a and 4b)**. At regional level, the width of neither the neocortex nor the hippocampus was affected by either vitrification or 90 min cold ischemia **(Tables 4a and 4b)**.

Exptl. Group	Young Cortex	Cortex	Hippocampus

	Diam (μm)	Roundness	Width (μm)	
C	10.3∀0.5 (2)	1.6∀0.1 (2)	1,270∀40 (2)	1,126∀146 (2)
0h	8.3∀0.5 (2)	1.7∀0.3 (2)	1,214∀107 (3)	979∀81 (2)
1.5h	8.0∀0.8 (3)	1.7∀0.8 (3)	1,190∀58 (4)	1,009∀127 (4)

Table 4a. Morphometric analysis of the neocortex and hippocampus of vitrified young brains. NeuN-labeled cells (mature neurons); Data are expressed as X ∀ SEM (N)

Exptl. Group	Adult Cortex		Cortex	Hippocampus
	Diam (μm)	Roundness	Width (μm)	
C	8.9∀0.8 (2)	1.6∀0.1 (2)	1250∀18 (2)	1155∀121(2)
0h	7.5∀0.3 (2)	1.9∀0.3 (2)	1110∀90 (3)	1017∀90 (3)
1.5h	5.6∀0.5* (3)	1.5∀0.3 (3)	1220∀37 (4)	1188∀57 (4)

Table 4b. Morphometric analysis of the neocortex and hippocampus of vitrified adult brains. *Significant (P<0.05) versus corresponding control (C). Data are expressed as X ∀ SEM (N)

When another type of neurons was studied, namely the dopaminergic (TH) neurons of the hypothalamus, which are easier to characterize than the nigral dopaminergic neurons (lost in Parkinsonian patients) we found that 90 min cold ischemia had a significant effect on their size (diameter) **(Table 5).**

Exptl. Group	Young	Adult
C	16.2∀1.1 (2)	16.2∀0.9 (2)
0h	15.3∀1.4 (3)	16.1∀1.2 (3)
1.5h	14.1∀0.3* (4)	14.6∀0.7* (4)

Table 5. Morphometric

analysis (diameter in μm) of Tyrosine Hydroxylase (TH) neurons (dopaminergic neurons) in the hypothalamus of young and adult rats after vitrification. * Significant (P<0.05) versus corresponding control (C). Data are expressed as X ∀ SEM (N)

Immunohistochemical Results

Methods. Immunohistochemical procedures were performed on free-floating sections. For each animal, separate sets of sections were immunohistochemically processed using anti-doublecortin (DCX) goat polyclonal antibody 1:250 (c-18, Santa Cruz Biotech., Dallas, Texas), an anti-neuronal nuclear antigen (NeuN) marker monoclonal antibody (MAB377, Chemicon Inc.,Temecula, CA) and a rabbit anti-tyrosine hydroxylase (TH) polyclonal antibody (CalBiochem, New York) 1: 500. For detection, the Vectastain® Universal ABC kit (1:500, PK-6100, Vector Labs., Inc., Burlingame, CA, USA) employing 3, 3-diaminobenzidine tretrahydrochloride (DAB) as chromogen, was used. Briefly, after overnight incubation at 4ºC with the primary antibody, sections were incubated with biotinylated horse anti-mouse antiserum (1:300, BA-2000,Vector Labs.), horse anti-rabbit antiserum (1:300) or horse anti-goat antiserum (1:300, BA-9500, Vector Labs), as appropriate, for 120 min, rinsed and incubated with avidin-biotin-peroxidase complex (ABC Kit) for 90 min and then incubated with DAB. Sections were counterstained with Nissl method (0.5% cresyl violet solution at 37ºC for 10 minutes) to visualize anatomical landmarks and mounted with Vectamount (Vector Labs) to use for image analysis.

Nissl stain.- In order to assess the integrity of the brain histo-architecture, Nissl staining was performed on brain sections. Sections were thoroughly rinsed with phosphate buffered saline (PBS), put on gelatin-treated slides and air-dried overnight. Then, slides were briefly washed in distilled water and then immersed in a 0.5% cresyl violet solution at 37ºC for 10 minutes. Afterwards, they were washed rapidly in distiller water and dehydrated through a series of graded ethanol (95 % 2 to 30 min and 100% 2 x 5 min each) and cleared in xylene (2 x 5 min each). Slides were coverslipped using Vectamount (Vector Labs, Inc., Burlingame, CA, USA).

Results. As indicated above, **i**n general**,** the vitrification procedure did not affect morphometric parameters when rats were vitrified at the time of euthanasia.When they were left 90 min in standby after euthanasia, a certain degree of significant cell shrinkage was detetcted. It remains to be determined how the brain of old rats is affected by the same procedures. Immunoreactivity

appeared more sensitive to vitrification although different cell markers behaved differently. NeuN and DCX were highly sensitive whereas TH was not. The functional significance of these changes is difficult to predict. At anatomical level, neocortex and hippocampal thickness appeared well-preserved after vitrification in both young and adult brains. Here, the data for adult rats are not shown.

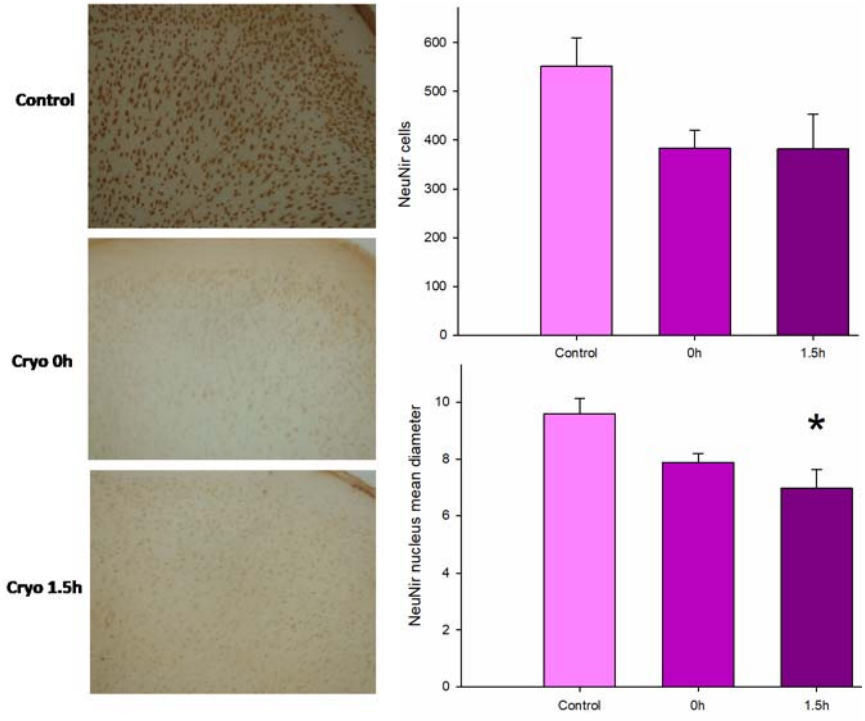

Figure 2.- NeuN imunohistochemistry of a young rat neocortex.As it can be seen nuclear NeuN immunohistochemical stainability is fainter in the two vitrified brains as compared with regularly fixed brains. As a consequence the cell count tends to fall without reaching significance. The mean nuclear diameter of neocortex neurons falls after 90 min cold ischemia. Bars over columns represent SEM values. *, P<0.05 versus control.

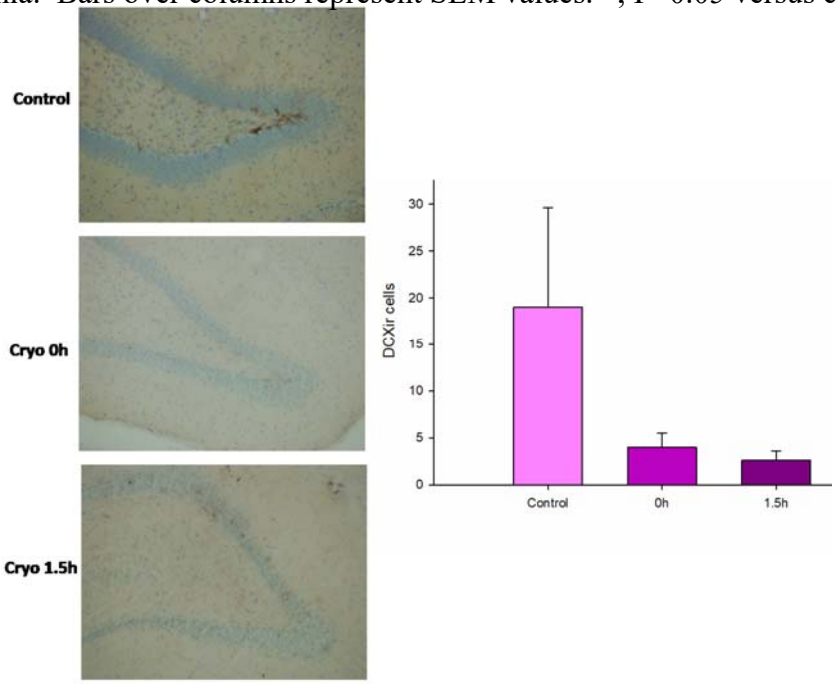

Figure 3.- Doublecortin (DCX) neurons in the hippocampus. These neurons play a crucial role in memory formation and retention. There are a limited number of them in the dentate gyrus of the hippocampus and DCX neurons are generated as new memories are formed. This is an area o adult neurogenesis. As the graph shows, the attenuation of immunohistochemical stainability makes some of the neurons nondetectable by the software so that apparent cell count falls.

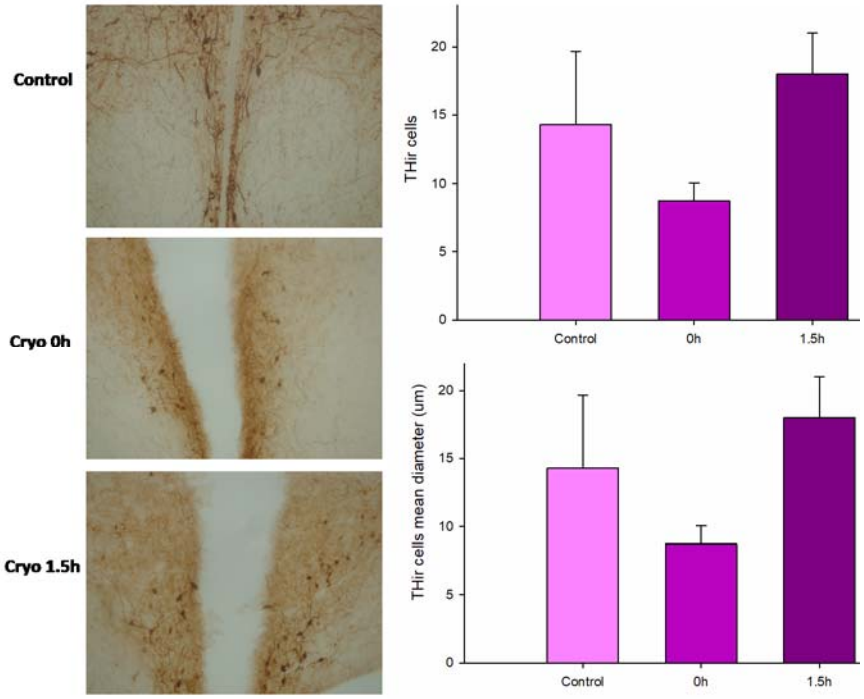

Figure 4.- Dopaminergic neurons (TH cells) in the hypothalamus of a young rat. Dopaminergic neurons are the neurons that are lost in the substantia nigra of Parkinsonian patients. In these neurons the stainability is preserved after vitrification and rewarming what means that the impact of vitrification is not the same in all neurons.

Mean cortex width

Mean hippocampal width

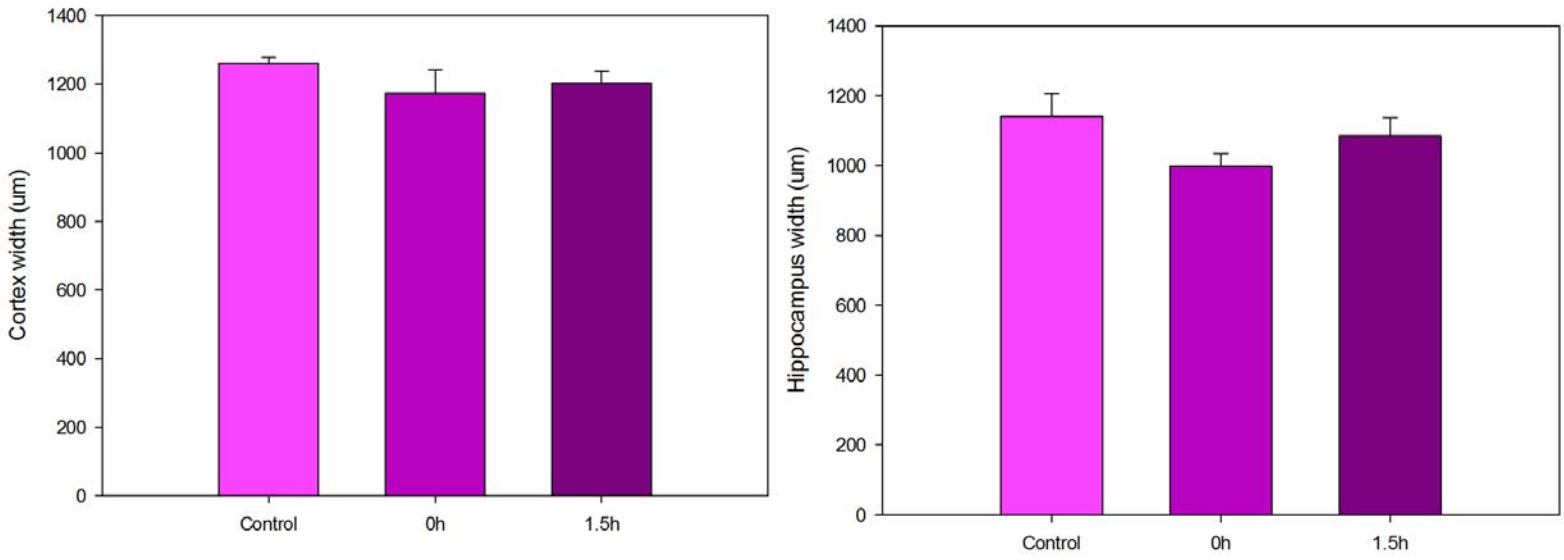

Figure 5 (previous page).- The neocortex and hippocampal width (or thickness) was morphometrically assessed in adult rats as illustrated in the upper left panel of Fig 6. No significant effect of either vitrification or 90 min cold ischemia was detected.

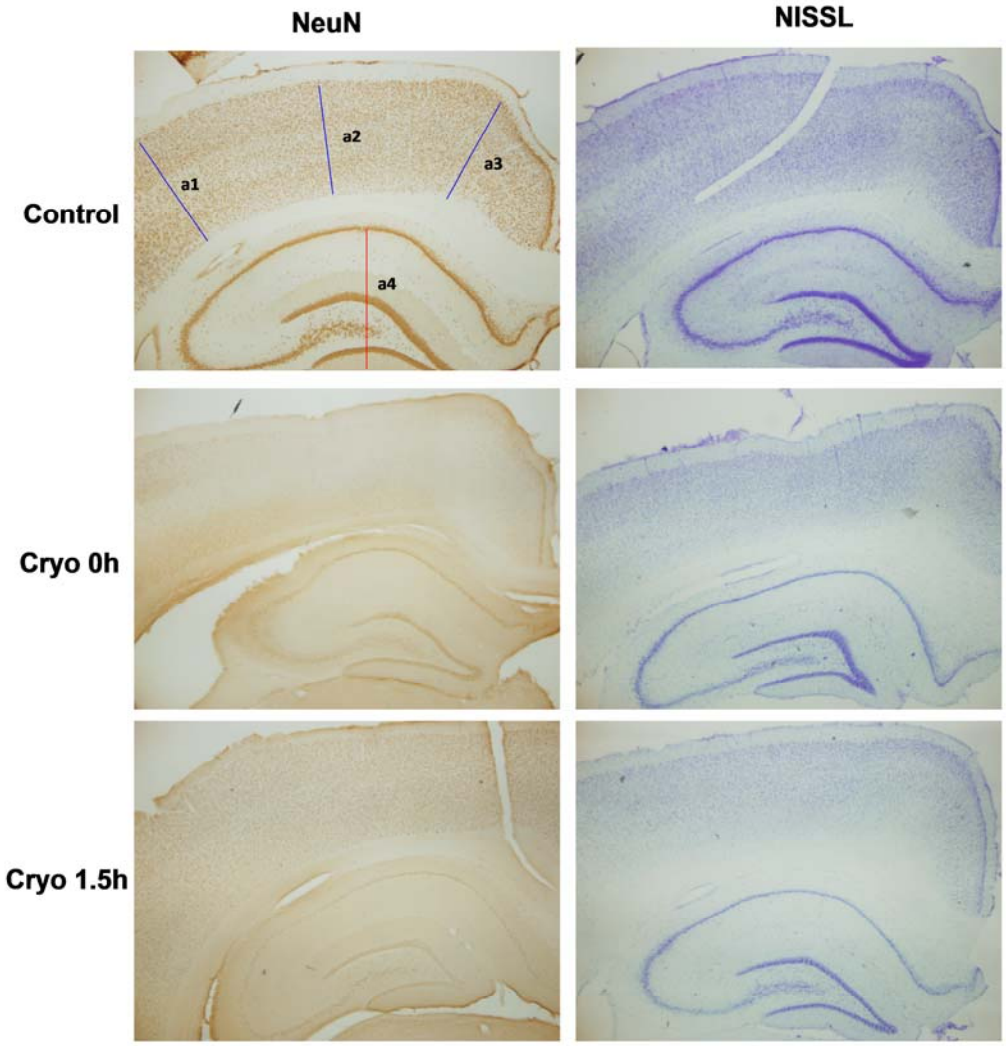

Figure 6.- This panoramic view of sagittal sections of the brain of representative adult animals of each group shows on the left NeuN immunohistochemical staining and on the left Nissl histochemical staining. Vitrification affects both types of staining. Nissl staining is less affected. Neocortex and hippocampal width (or thickness) was measured morphometrically as illustrated in the upper left panel. Blue lines correspond to neocortex. Red line to hippocampus.

CONCLUSIONS

The results so far obtained suggest that 90 min cold ischemia has a moderately unfavorable effect, at morphometric level, on brain preservation after vitrification. Vitrification itself affects the immunoreactivity of some neuronal markers but not of others.

OBJECIVES FOR YEAR 2

We plan to add a second longer standby time, possibly 5 or 6 h. We anticipate that such a standby time will have deeper effects on morphometric parameters.

We also plan to use a third age group consisting of old rats (around 20 months of age as available). This is an important age group as most patients are older individuals (>60).