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CRYOPRESERVATION OF A HUMAN BRAIN AND ITS EXPERIMENTAL CORRELATE IN RATS

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ABSTRACT

Several countries have established self-help cryonics groups whose mission is to cryopreserve human bodies or brains after legal death and ship them to cryonics organizations.

The objective here is to report the first case of human brain cryopreservation in Argentina as well as complementary experiments in rats. After legal death, the body of a 78 year old Caucasian woman was transported to a funeral home where her head was submitted to intracarotid perfusion with 5 l cold physiologic saline followed by the same volume of cold saline containing 13% DMSO and 13% glycerol. The brain was removed, temporarily frozen at -80ºC and shipped to a USA cryostasis facility. Three groups of rats were intracardially perfused with either fixative but not frozen (Reference group), vitrification solution VM1 (Control group) or the cryoprotection solution used in the patient (Experimental group). Control and Experimental brains were stored at -80ºC and subsequently assessed by immunohistochemistry for the adult neuron marker (NeuN), the immature neuron marker doublecortin (DCX), the dopaminergic neuron marker tyrosine hydroxylase (TH) and the presynaptic marker synaptophysin (SYN). The number of NeuN positive neurons remained unchanged in the experimental brain cortex whereas the number of immature DCX neurons in the hippocampus fell markedly in the cryoprotected brains. The results were highly variable for hypothalamic dopaminergic neurons. Confocal microscopy for SYN revealed that cryopreservation did not affect the synaptic network in the hippocampus. To our knowledge, this is the first report correlating a human cryoprotection procedure with results in complementary experiments in laboratory animals.

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INTRODUCTION

The rapid progress of cryobiology and freezing technology is giving rise to a growing certainty about the feasibility of reversibly cryopreserving organs at subzero temperatures, mainly for the purpose of creating organ banks **(1).** Initial evidence suggests that successful cryopreservation can be achieved in rabbit kidneys **(2)** as well as in whole sheep ovaries **(3).** Within this context, studies in lower animals have shown that the nervous system can be successfully cryopreserved and brought back to a functional condition by super fast freezing and thawing procedures, preserving the memory of trained specimens **(4).**These results increase the likelihood that mammalian brains, including human ones, can be reactivated to a highly functional state after vitrification.

In cryonics, brain cryopreservation is progressively becoming the method of choice over full body preservation mainly due to the growing consensus that in a future, when reanimation becomes possible, the rest of the body will be replaced by artificial components or newly grown organs. A practical reason for choosing brain over full body cryopreservation in countries where there are no cryonics facilities is that shipping a head or a brain is simpler and much less costly than doing so with a whole body.

Experimental nervous system cryopreservation is a severely underdeveloped field of research worldwide, with the number of research laboratories devoted to the topic being well below five. This situation prompted our neuroscience group to partly enter the field in an attempt to bridge an interventive procedure that has been employed in humans for decades, with animal experimentation aimed at validating and improving patient treatment. This constitutes an initial effort to bring standard biomedical research procedures to the field of cryonics.

In the present study, we attempted to compare the differences between the results in rat brains cooled using the vitrification protocol that the Cryonics Institute of Michigan employs in its patients, with those in rat brains preserved by means of a protocol similar to the one used in the first human brain cryopreservation procedure carried out in Argentina in September, 2018. In order to mimic the procedure used with the patient's brain we were forced not to store the rat brains in liquid nitrogen (LN2). Therefore, although we

used the vitrification procedure employed by the Cryonics Institute, in the present study we did not actually vitrify the rat brains as they were stored at -80ºC not -196ºC which means that the final storage temperature was above the glass transition temperature below which vitrification occurs. In this context the term vitrification is nominal without reflecting actual vitrification.We focused our analysis of rat brains on several neuronal populations as well as on the impact of deep freezing on the preservation of synapses in the hippocampus, a key brain region for memory function.

MATERIALS AND METHODS

Patient's brain cryopreservation

Patient clinical condition upon hospital admission- Patient Beatriz Lidia Billone (the full name of the patient is displayed with the consent of author MEA, her daughter), a Caucasian female, aged 78, with a clinical record of obesity, sedentary life, insulin-resistant diabetes as well as cardiovascular and renal deficiencies, was admitted on August 10th, 2018 to the Buenos Aires Cardiovascular Institute (ICBA), presenting a septic shock caused by an abdominal infection focus consequential to an intestinal perforation related to diverticulitis. She had legally donated her brain authorizing her daughter (MEA) to take the necessary steps for having her brain cryopreserved.

Perfusion procedure-On September 9th, 2018, at 9:30 a.m. the patient was declared legally dead. The hospital did not authorize anticoagulation of the patient before death. Immediately, her head was covered with ice bags. At 11:30 an ambulance arrived to the hospital and the body was placed in an ice-cooled box for transportation to a thanatology/funeral parlor where it arrived at 12:30. At 12:45, a surgeon and a funeral director began perfusing the brain by inserting a catheter into the right carotid artery and using a peristaltic pump to perfuse 5 l saline solution through the head. A cut was made on the left jugular vein to allow drainage of blood. The tubing connecting the saline reservoir, passing through the pump and conducting the pumped solution to the body was covered with water ice. Saline perfusion ended at 14:15 (perfusion period, 1.5 h) and was immediately followed by perfusion with 5 l cold saline containing 13% DMSO (v/v) and 13% glycerol (v/v). The pumping pressure was kept below 3 pounds.

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At about 16:00 brain removal began and ended at 17:15. The optic nerves and other connections were carefully severed. The brain was immediately immersed in a 5 l plastic beaker containing 9.3% DMSO and 9.3% glycerol in saline **(Fig. 1)**. The beaker was placed in a Styrofoam box that was covered with water ice. It was sealed and stored, first in a refrigerator at +2 ºC and later the same day, at -16ºC in a standard freezer. The brain appeared to be essentially blood-free and did not freeze at -16°C, implying good uptake of cryoprotectants into the brain. The temperature of the container was monitored with a digital thermometer (range +70 to -50ºC). Two days later, dry ice was added to the freezer in order to lower the temperature further and next day the beaker (wrapped in towels to isolate it from the dry ice), was placed into a box with dry ice. Finally, the box was transported to a research laboratory where it was stored in a deep freezer, initially at -70ºC and later at -80ºC. The beaker remained there for about 7 weeks until it was shipped by air to the United States for long-term storage at Alcor in Scottsdale, AZ. Transportaion indry ice to the US took between 2 and 3 days.

Animal experiments

Rats

Adult (5 months) male 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).

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. The above solutions are water-based. 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) whose free end was immersed in a graduate cylinder containing the appropriate perfusion solution kept at water ice temperature. The flow rate of the pump was set to 13 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 effluent fraction collection. In these effluent fractions the refractive index was measured in order to verify that at the end of each perfusion step the collected effluent (described below) had the refractive index of the corresponding pure perfusion solution, thus ensuring complete washout of the previous fluids. It should be pointed out that confirming a complete washout of the vascular system does not imply that a complete washout occurred in brain tissue. The abdominal aorta was clamped in order to restrict perfusion to the head and the thoracic and brachial regions. In order to insert a thermal probe into the brain of rats, a small hole (using a dental bur head 2.5 mm in diameter) was drilled 5 mm anterior to bregma in order to insert a sensing probe of a thermometer so that temperature could be monitored at a depth of 1 mm, at the level of the deeper cortical neuron layer **(5)**. At the end of perfusion, brains were rapidly removed, stored at -20°C for 2 h, then transferred to a -80°C deep freezer until ready for processing. Graded warming was attained by transferring the brains from -80ºC to -20ºC for 2 h and finally to room temperature (21-.23 ºC) After thawing, experimental brains were immersed in fixative solution overnight (-20 ºC). Finally, they were serially cut into 40 µm thick coronal sections on a vibratome.

Experimental Design

In pilot studies we had determined that in each experimental day we were able to properly handle 4-5 animals. Since the experiments involved 8 animals, we split each experiment into two consecutive days.

Three groups of animals were formed, namely,

REFERENCE (group with fixative)**,** rats were perfused with 100 ml phosphate buffer (PB) 0.1M followed by 250 ml fixative solution in a conventional way and not cooled.

CONTROL, (group with vitrification solution) rats were perfused with 100 ml carrier solution, followed by 100 ml 10% EG solution. Then, 100 ml 30% EG solution and finally 150 ml VM1 solution. The protocol used for this group is based on CI procedure in patients and was adapted to rats.

EXPERIMENTAL (group with solution A + Solution B), rats were perfused with 100 ml Solution A, in order to wash out blood. The peristaltic pump was turned off and rats were left connected to the pump for 5 h (in an attempt to mimic the time elapsed during patient transportation from hospital to the funeral home) with their heads fully but selectively covered with water ice. At the end of the waiting period the pump was turned on and 50 ml Solution A were additionally perfused, followed by 150 ml Solution B. It is worth mentioning that our aim was to compare the standard, preferred procedure of attempted vitrification, which we designate as the "control" protocol since it is the one that should normally be used, to a protocol that approximates the one we found necessary to employ under adverse conditions, which has not previously been evaluated and therefore is designated as the "experimental" protocol.

Perfusion solutions for control and experimental rats

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 in storage 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. This solution is kept in storage at 4°C.

d)**70% w/w EG solution (VM1)** consists of 70% EG and 31.8% (v/v) dimethyl Sulfoxide (DMSO), containing 0.028 M KCl, 0.23 M glucose, 0.01 M Tris base **(Suppl. Table 1).** This solution is kept in storage at -20°C.

Freezing solutions (used for the experimental rats)

a) **Solution A** consists of sterile 0.9% (w/v) NaCl (saline) kept in storage at 4°C.

b) **Solution B** consists of 13% (v/v) DMSO and 13% (v/v) glycerol in saline solution kept in storage at -20°C

Fixative solution

a) Perfusion fixative: Phosphate buffered paraformaldehyde 4% (pH 7.4) for perfusion of the Reference group.

b) Cryopreservation fixative: 300 g/l sucrose, 10 g/l polyvinylpyrrolidone (40K, MW), 30% (v/v) EG in phosphate buffered paraformaldehyde 4% (pH 7.4), for fixing and storing brains overnight at -20ºC.

Immunohistochemistry and immunofluorescence

Immunohistochemical procedures were performed on free floating 40 µm thick sections. For each animal, separate sets of sections were immunohistochemically processed using anti-doublecortin (DCX) goat polyclonal antibody (1:1000; c-18, Santa Cruz Biotech., Dallas, Texas), a nuclear marker of immature neurons,anti-neuronal nuclear antigen (NeuN) mouse monoclonal antibody (1:500;MAB377, Chemicon Inc.,Temecula, CA), a nuclear marker of mature neurons and anti-tyrosine hydroxylase (TH) rabbit polyclonal antibody (1:500;CalBiochem, New York).

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-goat serum(1:300, BA-9500, Vector Labs), horse anti-mouse serum (1:300, BA-2000,Vector Labs.) or horse anti-rabbit serum (1:300,BA-1100 Vector Labs), as appropriate, for 120 min, rinsed and incubated with avidin-biotin-peroxidase complex (ABC Kit) for 90 min. Finally, they were incubated with the DAB chromogenic reagent 0.08% $H_2O_2$for 2 minutes. In the case of DCX staining, sections were counterstained with the Nissl method (0.5% cresyl violet solution at 37ºC for 2 minutes) to visualize anatomical landmarks. All sections were mounted with Vectamount (Vector Labs) to use for image analysis.

For immunofluorescence staining, rabbit primary antibody against the presynaptic vesicle marker synaptophysin (SYN, Proteintech, Rosemont, IL; cat# 17785-1-AP) 1:500 was used. Goat anti-rabbit IgG conjugated with Alexa 488 (1:300; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were used as secondary antibody. Anti-SYN was used to assess the integrity of the synaptic network at the hippocampus after the experimental/control processes. Finally, sections were incubated for 10 min with the fluorescent DNA stain 4',6-diamidino-2-phenylindole (DAPI) to stain the nuclei and

mounted with Fluoromount. Then, sections were observed, and images captured using an Olympus confocal microscope (FV1000, Olympus, Japan)

Morphometry

Image analysis

In each brain block, one out of six serial sections was selected to obtain a set of non-contiguous serial sections spanning the dorsal hippocampus, cortex and hypothalamus. For this task, we used an Olympus BX-51 microscope attached to an Olympus DP70 CCD video camera (Tokyo, Japan).

We focused our analysis on mature neurons, immature neurons, dopaminergic neurons and synapsis. For synapsis determination, we used a confocal microscope (Olympus FV1000).

Thickness of the cortex and the hippocampus

To assess the thickness of the cortex and the hippocampus, three noncontiguous NeuN-stained sections were sampled every 240 µm along the anterior–posterior axis corresponding to Bregma -3.12 to 3.84 mm **(5).** The cortex region thickness was measured in three selected zones (retroesplenial, motor and somatosensorial primary cortex) using the Measurement tool of a digital image analyzer (Image Pro Plus v5.1, Media Cybernetics). The hippocampal region thickness was measured from inflexion point of CA1 region to ventral part of dentate gyrus (DG) granular cells using the same tool.

Mature neuron analysis

Mature neurons were detected as NeuN immunoreactive (NeuNir) cells. With the aim to determine number and diameter of cells, three sections per animal and eighteen fields per section in the primary and secondary motor cortex corresponding to Bregma -3.12 to -3.84 mm **(4)** were digitally assessed using the Manual Tag tool of the same image analyzer software. Cell diameter was measured as described for thickness in a minimum of 30 nuclei per section (540 per animal).

Immature neuron analysis

Immature neurons were identified as double cortin immunoreactive (DCXir) cells in the hippocampal DG. Three sections per animal in the whole DG were sampled. The DCXir cell

number was estimated by a modified version of the optical dissector method **(6).** DCXir cell diameter was measured as described for mature neurons.

Dopaminergic neuron analysis

Dopaminergic neurons were identified as tyrosine hydroxylase immunoreactive cells (THir) in the paraventricular nucleus of hypothalamus (PaVH). One section per animal corresponding to the Bregma -1.32 **(5)** was selected to sample the whole PaVH. THir cell diameter was measured as described for mature neurons.

Pre-synaptic analysis

Z-stack confocal images were captured using a 20x magnification objective (NA 0.75) and zoom 3X, with a Z depth of 1.01 µm/slice. Both fluorescent channels were sequentially acquired. The corresponding Maximum Intensity Z-projection was recorded**.**

Statistics

Analysis of the variance (ANOVA) was used to evaluate group differences. Tukey's method was chosen as a post hoc test. P values <0.05 were considered to indicate significant differences.

RESULTS

Patient's brain

After dissection the organ appeared free of residual blood, suggesting that perfusion had been effective despite the fact that the patient had not been anticoagulated before death (**Fig. 1)**. There were a few small superficial scratches and the tissue appeared significantly softened.

Rat brain results

Anatomical findings

Brain cortex temperatures during saline perfusion reached similar levels than in the control group but reached lower levels after the 5-h standby period in the Experimental group, indicating that during the standby period brain core temperature steadily dropped **(Table 1).** Brain weight immediately after perfusion was (0∀SEM in g; n=3) Ref, 1.73 ∀ 0.07; Cont, 1.31 ∀ 0.13; Exper, 1.54 ∀ 0.16; no significant differences were detected

among groups. Concerning brain dimensions (fronto-caudal and lateral) no significant differences were detected either (data not shown). In all rats the refractive index of effluents after each perfusion step was the same as in the pre-perfusion solutions indicating that the washout for that step had been complete **(Suppl. Table 2)**.

After rewarming from -80ºC through -20ºC and final room temperature, the brains of the two cryoprotected groups displayed a similar appearance and size. The thickness of the cortex and hippocampus did not differ significantly among the three brain groups nor did cell diameter of NeuNir neurons from those regions **(Fig. 2 and Fig 3).**

Immunohistochemical findings

NeuN neurons- Immunostaining was somewhat fainter in the cryoprotected and control brain groups as compared with the nonfrozen Reference brains **(Fig. 3).** In quantitative terms, mature neuron numbers were comparable among the three groups whereas neuron diameter revealed a significant shrinkage of the experimental neurons. The two perfusion cryoprotective procedures did not appear to have a differential effect on NeuNir neuronal shape.

Hippocampal SYN immunostaining-Confocal microscopy of the presynaptic marker synaptophysin in the hippocampus revealed a well-preserved density of synaptic connections, a finding consistent with integrity of the hippocampal synaptic network **(Fig. 4).** The immunoreactive area of the synaptic buttons of this region did not show significant differences among the three experimental groups **(Fig. 4-plot).**

Hippocampal DCX neurons- The labelling of DCX neurons in the DG of the hippocampus fell markedly after freezing (experimental group) **(Fig 5)**. The loss of labelling was higher in the experimental than in the control brains (Fig 5-plot). DCX neurons in the Control and Experimental groups showed a significant shrinkage.

Dopaminergic neurons- In the hypothalamus, THir (dopaminergic) neurons and processes did not show, when visually assessed, a clear change in number **(Fig. 6)**. Morphometric analysis of TH cell diameter did not reveal significant changes among groups whereas it was difficult to draw statistical conclusions regarding immunoreactive TH cell numbers due

to high data scattering associated with high non-specific color background **(Fig. 6-images and plot)**.

DISCUSSION

The long-held doctrine that deep freezing is a physical process that irreversibly terminates life in animals or isolated organs has been increasingly challenged by experimental achievements and observations in nature. *In vitro* fertilization is a good example of the first case **(7, 8)** whereas the cryobiosis of tardigrades illustrates the latter statement **(9,10).** By the same token, the perception that cryopreserving a human body or brain after natural death in the hope that it may be reanimated by future biomedical technology, is an unrealistic or even utopic endeavor, has been progressively changing in recent years. Even agencies like NASA sponsor suspended animation research projects as this technology is considered essential for interstellar or even interplanetary travel **(11).** Consequently, a growing number of countries have established self-help cryonics groups whose mission is to cryopreserve human bodies or brains after legal death and ship them to a number of cryonics organizations established in the US and other countries. Such a self-help group was founded in Argentina a few years ago, and in September, 2018, the first human brain was frozen in the country and shipped to a cryonics facility in the US. Before shipping we kept the brain stored at -80 ºC instead of liquid nitrogen (LN2) temperature (-196 ºC). We reasoned that if the brain was temporarily stored in LN2 and then taken back to -80 ºC for shipping in dry ice, the sudden rise of temperature might damage the tissue.

Profiting from the fact that the group is associated with a neuroscience team carrying out experimental brain cryopreservation studies in rats, it was decided to perform a complementary experiment in rats comparing the results of a brain perfusion protocol used for this patient with a protocol based on the standard vitrification protocol used in patients by the Cryonics Institute of Michigan. A technical comment is in order here. Since the CONTROL rat brains were stored at -80ºC instead of -196ºC, they do not constitute rigorous controls but rather the best possible approximation. In this regard, the term “vitrification solution” used here should be considered as an operational designation that does not necessarily imply that the perfused brains are vitrified. In any case, we found no

histological evidence of ice formation. When ice forms in a cryopreserved brain and subsequently melts during vibratome sectioning, the ice crystals leave a typical footprint, cavities in the tissue. As can be seen in the micrographs, no such cavities are evident. Also worth mentioning is the fact that our results in rat brains are only an approximation to what may be expected to be observed in human brains. Although, size and other species-related differences will influence results in the two species, the rat brain is widely used in neuroscience as a model to study human brain physiology and pathology. The present study follows this criterion.

The histology of the two groups of rat brains was compared with that of nonfrozen brains perfused with fixative following standard procedures in neuroscience. This third group of brains was taken as a reference standard.

When we submitted the original version of this MS we were unaware of similar studies documented in the literature and were at the time unable to compare our results with other published data. When we received the manuscript for revision, a publication had appeared reporting the ultrastructural effects of prolonged normothermic (37 ºC) and cold (0 ºC) ischemia on the cerebral cortex of adult rats. The authors found that the sequence of degradative changes that occur in brain ultrastructure, ranging from early signs of energy depletion to advanced necrosis, proceed much faster under normothermic than under cold ischemic conditions. Thus, ultrastructural signs of advanced necrosis are observed after 36 h at 37ºC and after 2 months at 0ºC. The focus of this study is different from ours but the approach shows a similar intent to move experimental cryonics work towards the use of whole rodents **(12).**

It should be pointed out that the patient's brain was not perfused with vitrification solutions but with a cryoprotective formula. The same is true for the experimental animals.

Our results revealed that in quantitative terms, none of the two cryoprotective procedures had an adverse impact on hippocampal or cortical thickness. However, both freezing procedures reduced the immunolabelling of the different neuronal populations assessed although to a different extent. Thus, while mature (NeuNir) neurons were lightly affected, dopaminergic (THir) neurons showed a variable attenuation of the immunolabeling. The

doublecortin (DCX)-expressing granular immature neurons of the hippocampal dentate gyrus (DG), are highly excitable cells that have been hypothesized to underpin ongoing learning and memory formation **(13).** DCX neurons are highly vulnerable to aging **(14).** In the experimental brains the immature (DCX positive) neurons of the hippocampus showed the most unfavorable impact, particularly in the experimental brains. Although the latter observation suggests that the DCX neurons of the hippocampus may have sustained irreversible damage, it is important to mention that these neurons are necessary for learning but not for remembering **(15)**. Perhaps the more encouraging finding of the present study is that the density of hippocampal synapses (SYN immunoreactive area) was affected by neither of the cryoprotective protocols. If future studies show that brain synaptic density is not affected by cryoprotection procedures, this would suggest that the connectome retains an acceptable integrity after vitrification, which would let us expect that cryopreserved brains keep a substantial proportion of the information present in the nervous system at the time of death.

CONCLUDING REMARKS

Considering the lack of previous experience of our group with perfusion procedures in human patients, we are satisfied with the results achieved. We are aware that even in the US a significant proportion of patients cannot be perfused within an acceptable time frame after death and that transportation of the bodies to their ultimate destination, a cryonics organization, is often performed covering the bodies with water ice. In this context, the quality of our approach can be considered good. We hope that the expertise gained with this first case may allow us to optimize our cryoprotective procedures.

Perhaps the most original feature of the present paper is the use of an animal model to mimic the cryoprotective approach performed in our patient and compare the results with those obtained by approximating the well-standardized procedure used by the Cryonics Institute. Although the results in rat brains cannot be directly extrapolated to human brains, our experimental approach constitutes, in our opinion, a reasonable first approximation. Laboratory animals are widely used in neuroscience (and biomedical

research in general) to model neurological pathologies and assess neuroprotective strategies. Our approach brings the same principle to cryonics.

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Conflict of interests

The authors declare that none of them has conflict of interests in this study.

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LEGENDS TO FIGURES

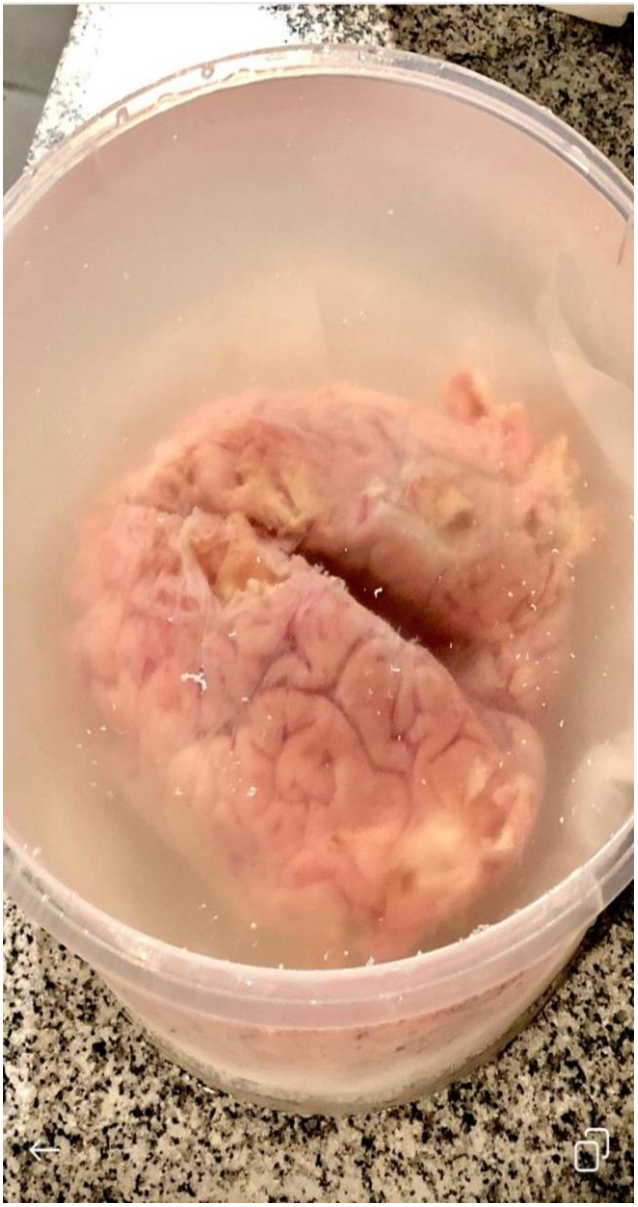

Figure 1- Patient's brain immediately after removal from the skull. The organ was immersed in cryoprotective solution as described in M&M, and kept there during the freezing process. No blood remnants are observed. The organ has a friable appearance. A slight scratch is observed at both parietal lobes, behind the central sulcus, in close proximity to the longitudinal fissure.

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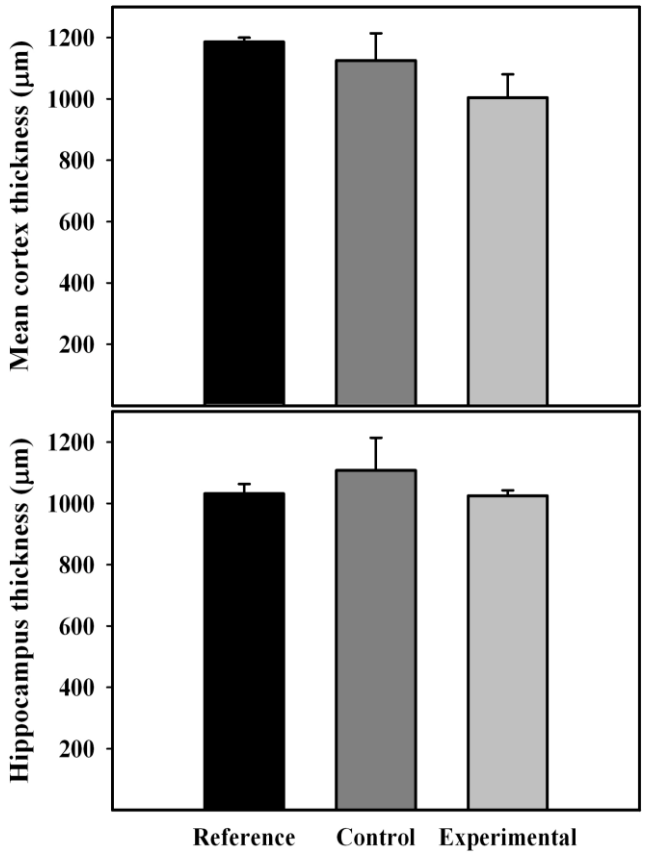

Figure 2-Thickness of the cortex and hippocampus nonfrozen (Reference), Control and cryoprotected (Experimental) brains. In both cases the thickness was determined as described in M&M**.** Reference, Control (high concentration), and Experimental (low concentration) brains.

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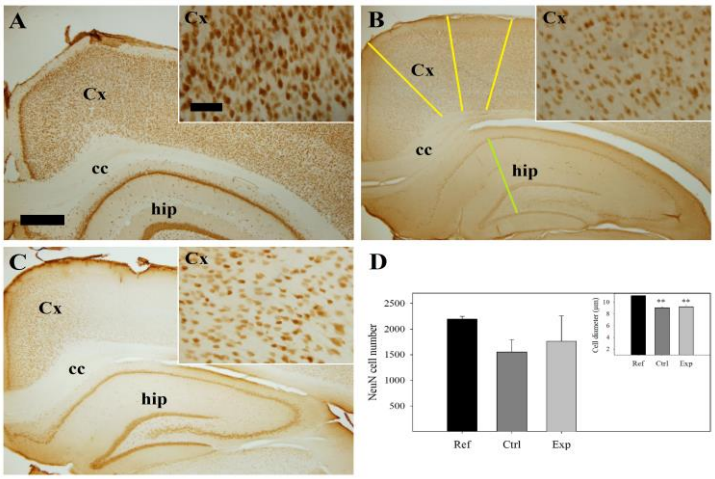

Figure 3-Mature (NeuNir) neurons in the cortex of the Reference (A), Control (B) and Experimental (C) brains. Immunolabeling is fainter in the frozen brains although in quantitative terms cortical NeuN neuron number is comparable in the three groups. In the frozen brains NeuN neuron diameter is significantly smaller than in the nonfrozen brains. Green lines and yellow line show the reference points where cortical and hippocampal thickness were measured, respectively. Cx, cortex; cc, corpus callosum; hip, hippocampus. Scale bar in main panels = 200 µm; scale bar in insets= 50 µm.Ref, reference; Ctrl, Control; Exp, Experimental.

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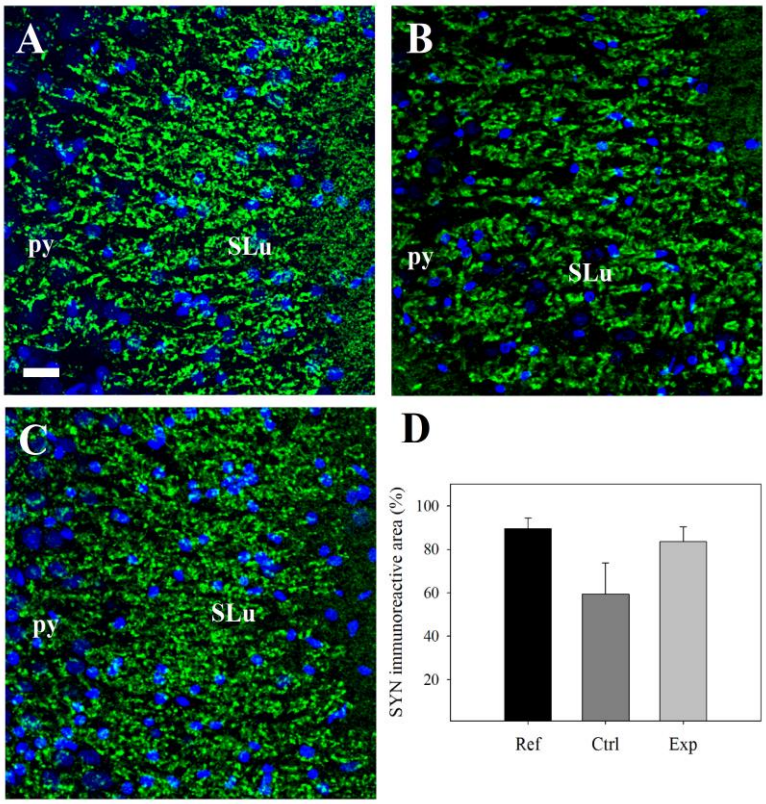

Figure 4-SYN immunolabeling in the CA3 hippocampus of the Reference (A), Control (B) and Experimental (C) brains. Confocal images represent a Maximum Intensity Z-projection display of synaptic buttons up to a depth of 15-20 SYN immunoreactive area does not differ significantly among groups. DAPI fluorescence corresponds to the cell nuclei in the same field ,.Py, pyramidal layer;Slu, Stratum lucidum. Scale bar= 20 µm.Ref, reference; Ctrl, Control; Exp, Experimental.

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This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

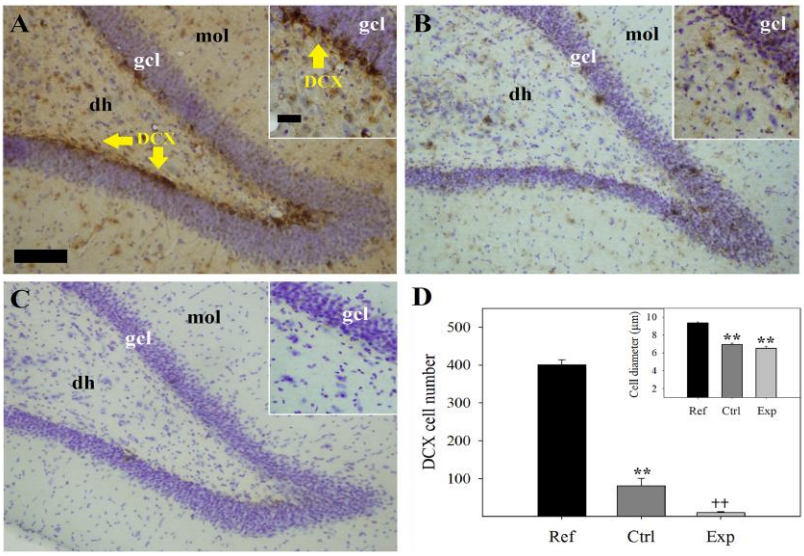

Figure 5-Immunohistochemistry of immature (DCXir) neurons in the hippocampal DG of Reference (A), Control (B) and Experimental (C) brains. The number of hippocampal DCX neurons (arrows) falls significantly in the Control brains and more so in the cryoprotected hippocampi. The DCX cell diameter also fell in the frozen hippocampi as compared with nonfrozen counterparts; dh, dentate hilus ; mol, molecular layer; gcl, granular cell layer; In the main panel of the plot asterisks indicate significant differences with the Ctrl group whereas crosses represent a significant difference of Exp versus Ctrl groups. ** or ⊥⊥, P<0.01 Scale bar in main panels = 100 µm**;** scale bar in insets= 25 µm**.** Ref, reference; Ctrl, Control; Exp, Experimental.

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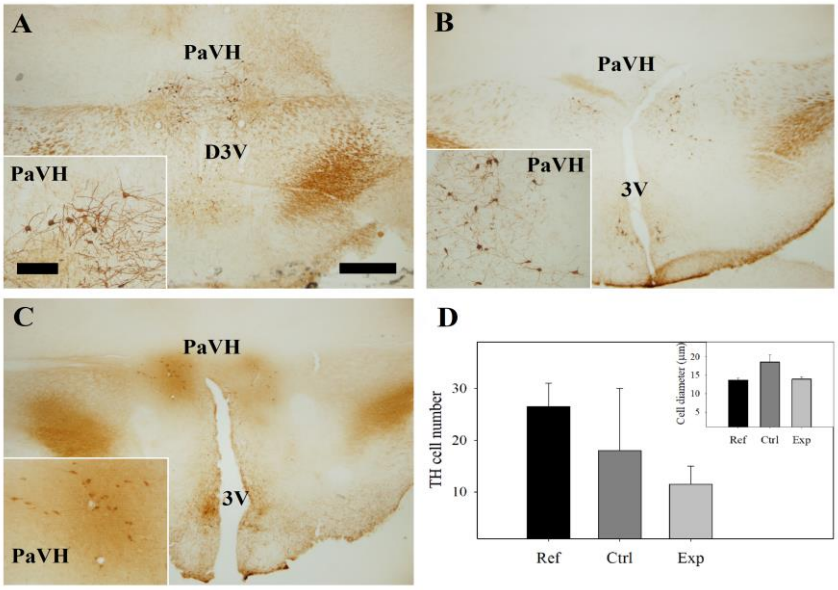

Figure 6-Immunohistochemistry of dopaminergic (THir) neurons in the hypothalamus of Reference (A), Control (B) and Experimental (C) brains. D3V, dorsal third ventricle; 3V, third ventricle; PaVH, paraventricular hypothalamic nucleus. Although the size of the TH neurons is preserved after freezing, there is a large data scattering in TH immunoreactive cell numbers. **.** Scale bar in main panels = 200 µm; scale bar in insets= 50 µm**.** Ref, reference; Ctrl, Control; Exp, Experimental

Table 1. Brain cortex temperature time-course during perfusion

	Experimental	**Controls**
Exp. time (min)	**Temperature (°C)**	**Temperature (°C)**
-5	36.8±0.3	-
0	35.1±0.6	-
5	27.6±1.1	-
10	23.9±0.9	-
15	20.6±0.6	-
5-h standby		
295		22.3±0.4
320	3.6±0.1	20.2± 0.5
340	5.6±0.2	18.8±0.6
245	7.1±0.2	17.8±0.6
350	7.1±0.2	17.2±0.5
355	7.0±0.2	16.7±0.5
360	7.3±0.3	16.5±0.6
365	7.4±0.3	15.6±0.4
370	7.7±0.3	15.2±0.6
380	8.5±0.3	14.1±0.4

385	8.6±0.3	13.6±0.3

Temperature data are expressed as 0± SEM. N=2. Temperature 22.3±0.4ºC and

Time, 295 min represents -5 min pre-perfusion in the control group

Supplementary Table 1. Composition of perfusion solution VM1

VM1 Component	Volume (ml)	Weight (g)	% (w/w)
DMSO solution	31.8	34.03	30
EG	70	77.7	70
Total	100*	111.73	100

* The reason why 31.8 ml DMSO solution plus 70 ml EG result in 100 ml instead of 101.8 ml is the volume reduction caused by mixing two liquids of different polarities.

Supplementary Table 2- Refractive indices and densities in effluents collected from the right ventricle during perfusion of vitrification solutions

A. Control Group

Perfusion time (min)	°B*	density (g/ml)**
0	36.6±1.1	1.16±0.1
10	38.3±1.3	1.17±0.0
20	29.0±1.9	1.12±0.0
30	13.0±1.0	1.05±0.0
40	18.0±1.3	1.07±0.0
50	34.4±2.3	1.15±0.1
60	52.4±0.3	1.24±0.0
70	55.3±0.1	1.26±0.0

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B. Experimental Group

Perfusion time (min)	°B	density (g/ml)
0	2.2±0.0	1.01±0.0
10	1.2±0.1	1.00±0.0
20	1.2±0.0	1.00±0.0
30	1.6±0.2	1.01±0.0
40	19.5±1.9	1.08±0.1
50	22.7±0.2	1.10±0.0
60	22.3±0.3	1.09±0.0
Perfusion time (min)	°B	density (g/ml)
0	2.2±0.0	1.01±0.0
10	1.2±0.1	1.00±0.0
20	1.2±0.0	1.00±0.0
30	1.6±0.2	1.01±0.0
40	19.5±1.9	1.08±0.1
50	22.7±0.2	1.10±0.0
60	22.3±0.3	1.09±0.0

C. Calibration Standards

Control Group	°Bx	g/ml	Exptl. Group	°Bx	density (g/ml)
20% w/w sucrose	19.8	1.09	20% w/w sucrose	20.1	1.08
Carrier	39.2	1.17	Saline	1.3	1.01
10% w/w EG	12.1	1.05	DMSO/glycerol	23.9	1.10
30% w/w EG	21.5	1.09			
70% w/w EG	55.9	1.27			

* °B stands for Brix index which is a unit of measurement of refractive index that some commercial refractometers use to readily measure sugar concentration (like sucrose) in solution

** ºB and densities are expressed as X±SEM (N= 3 in all cases).

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