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# Cryopreservation of rat hippocampal slices by vitrification ☆

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## Abstract

Although much interest has attended the cryopreservation of immature neurons for subsequent therapeutic intracerebral transplantation, there are no reports on the cryopreservation of organized adult cerebral tissue slices of potential interest for pharmaceutical drug development. We report here the first experiments on cryopreservation of mature rat transverse hippocampal slices. Freezing at 1.2 °C/min to −20 °C or below using 10 or 30% v/v glycerol or 20% v/v dimethyl sulfoxide yielded extremely poor results. Hippocampal slices were also rapidly inactivated by simple exposure to a temperature of 0 °C in artificial cerebrospinal fluid (aCSF). This effect was mitigated somewhat by 0.8 mM vitamin C, the use of a more "intracellular" version of aCSF having reduced sodium and calcium levels and higher potassium levels, and the presence of a 25% w/v mixture of dimethyl sulfoxide, formamide, and ethylene glycol ("$V_{EG}$ solutes"; Cryobiology 48, pp. 22–35, 2004). It was not mitigated by glycerol, aspirin, indomethacin, or mannitol addition to aCSF. When RPS-2 (Cryobiology 21, pp. 260–273, 1984) was used as a carrier solution for up to 50% w/v $V_{EG}$ solutes, 0 °C was more protective than 10 °C. Raising $V_{EG}$ concentration to 53% w/v allowed slice vitrification without injury from vitrification and rewarming per se, but was much more damaging than exposure to 50% w/v $V_{EG}$. This problem was overcome by using the analogous 61% w/v VM3 vitrification solution (Cryobiology 48, pp. 157–178, 2004) containing polyvinylpyrrolidone and two extracellular "ice blockers." With VM3, it was possible to attain a tissue $K^+/Na^+$ ratio after vitrification ranging from 91 to 108% of that obtained with untreated control slices. Microscopic examination showed severe damage in frozen–thawed slices, but generally good to excellent ultrastructural and histological preservation after vitrification. Our results provide the first demonstration that both the viability and the structure of mature organized, complex neural networks can be well preserved by vitrification. These results may assist neuropsychiatric drug evaluation and development and the transplantation of integrated brain regions to correct brain disease or injury.

The cryopreservation of neural systems of varying complexity has been extensively reported over the past several decades [19,40,41,43,45,51,52,61]. These studies have been motivated by a variety of important therapeutic [8,25,26,43,45], investigational [24,26,51,52], and practical [2,22,24] goals.

However, surprisingly, the goal of using cryopreserved neural tissue systems for neuropharmacological drug screening does not seem to have been pursued. The feasibility of preserving pharmaceutically relevant electrical and synaptic functions in cryopreserved neural circuits was suggested by Pascoe's classic report of the complete recovery of the rat superior cervical ganglion, including the demonstration of unaltered synaptic transmission, following relatively rapid cooling to −76 °C [40], but no comparable demonstrations are available for defined cerebral tissues. Complex neural functions have been recovered after freezing cerebral tissue to relatively high subzero temperatures [48,51,52], but not after cooling to temperatures that would permit long term storage.

The hippocampal slice is a well-studied, well-characterized, and easily-testable model trisynaptic pathway that has been used for the screening of a variety of pharmacological agents [36,44,54]. Jensen and colleagues [27,49] reported the successful cryopreservation of immature rat hippocampi, as judged particularly from their survival and integration with host tissues following intracerebral transplantation [49]. Although their studies are encouraging for the use of brain tissue transplantation techniques, they are not aimed at providing adult tissues for neuropharmacological drug screening, and the use of immature tissue, which may differ from adult tissue both in ease of cryopreservation and in pharmacological responsiveness, would be generally inappropriate for this end. The cryopreservation of isolated fetal hippocampal cells has been reported [31,53], but, in addition to the drawbacks of immaturity for drug discovery, these cells cannot recapitulate all of the relationships of intact hippocampal tissue and may be altered by isolation procedures and lack of appropriate contacts with other cells.

Brain slice models are extensively used by the pharmaceutical industry (e.g., [42,46,50,54]). The ability to bank fully viable and functionally normal tissue slices for pharmacological evaluation of drug metabolism [9,10,58], toxicity [9], and responsiveness of chosen molecular and physiological targets to drugs in development [42,46] would allow pharmaceutical companies to more conveniently schedule experiments, more easily perform simultaneous evaluations across species [10,50], and more easily perform simultaneous screening of drug metabolism in multiple organ systems from the same species [9] while simultaneously reducing dependence on in-house tissue processing and

vivarium use. It should also allow sporadically procured viable human tissues to be more widely available for testing when and where needed [3,20–22,33]. To address the lack of demonstrated success in preserving functional integrated adult neural systems for pharmaceutical drug discovery, we sought to determine whether the rat transverse hippocampal slice could remain viable following cryopreservation by either freezing or vitrification. Our results show that although freezing was highly damaging to rat hippocampal slices, vitrification permitted the viability and structural integrity of this preparation to be very well preserved.

## Materials and methods

### Brain slice preparation

All procedures performed on rats were conducted with the approval of the Harbor-UCLA Animal Care and Use Committee and were in compliance with USDA guidelines. Male Wistar rats weighing 220–320 g were deprived of food and water for 18–20 hours prior to the experiments. They were anesthetized with isoflurane in a closed chamber, weighed, and transferred into shallow ice water in a second closed chamber also containing isoflurane. After 7–9 min of pre-cooling, the rats were removed from the cooling chamber and immediately decapitated with a guillotine. Brains were removed under ice-cold saline within 1–1.5 min and transferred to a beaker of artificial cerebrospinal fluid (aCSF) at 0–2 °C for 1 min. (The composition of aCSF was: 124 mM NaCl, 5 mM KCl, 22 mM $NaHCO_3$, 1.25 mM $NaH_2PO_4$, 1.25 mM $MgSO_4$, 2 mM $CaCl_2$, and 10 mM glucose.) The pH was set to 7.4 and maintained with 95% $O_2$/5% $CO_2$. The right hippocampus was removed and stored in the aCSF beaker at 0–2 °C while the left hippocampus was removed. Each hippocampal preparation was cut into eight to nine 475 μm thick transverse slices using a McIlwain tissue chopper. The slices were removed from the blade of the chopper using a soft sable brush and transferred to a dish containing aCSF at 4–6 °C. Finally, all slices were transferred to the two wells of an Oslo-type recording chamber (Fine Science Tools, Foster City, CA, USA) and incubated with aCSF at 34–37 °C for at least 1 h before being used in experiments. The total time required for slice preparation was 9.5–11 min. The volume of each well of the Oslo chamber was 2 ml. The slices were superfused with aCSF at

1.2–6 ml/min for full submersion conditions (all experiments except those shown in Table 1) or 1.2 or 4 ml/min for interface conditions (the experiments reported in Table 1). Modified versions of aCSF are described in the text. The RPS-2 [17] and LM5 [18] carrier solutions are described elsewhere.

### Viability assessment

The parameter optimized in the current studies as a presumed index of viability is the $K^+/Na^+$ ratio. A high $K^+/Na^+$ ratio is clearly a prerequisite for neuronal viability and electrical activity. The $K^+/Na^+$ ratio has been used for evaluation of the functional viability of brain tissue [29], cultured astrocytes [5], heart tissue [56], wounded muscle [4], kidney tissue [16], and whole kidneys [47]. Following removal of the cryoprotectant and reincubation of slices in aCSF at 35 °C for $\geqslant 1$ h, slices were placed in groups of two or three into 2 ml polyethylene microcentrifuge tubes. To each tube, 0.7 ml of isotonic (0.3 M) mannitol in water was added to wash ions from the extracellular space of the slices. The tube was gently agitated by hand for 1.5 min, then the mannitol was discarded and the procedure was repeated. After removing residual solution, 50 µl of 3% w/v aqueous trichloroacetic acid (TCA) was added. $K^+$ and $Na^+$ concentrations were read in the TCA supernatant at least 24 h later using a flame photometer (Instrumentation Laboratories), and the concentration ratio is reported as $K^+/Na^+$. The percent recovery is reported as $100\% \times (K^+/Na^+)_{treated}/(K^+/Na^+)_{controls}$.

### Cryoprotectants, cryoprotectant treatments, and vitrification procedure

Following initial incubation for 60 min or more at 35 °C in aCSF to allow recovery from the shock of slice preparation, eight hippocampal slices were placed into a small polyethylene cylinder with a nylon mesh floor. Slices were then exposed to cryoprotectant by submersion of the base of the cylinder into a series of solutions of increasing and then decreasing cryoprotectant concentration. Due to the delicacy of the slices, no stirring or agitation was employed to speed diffusion. A variety of cryoprotectant addition and washout protocols were evaluated. Protocols for $V_{EG}$ and VM3 were empirically optimized based on the response of the $K^+/Na^+$ ratio to changes in step size, duration, and temperature, given the difficulty of theoretically modeling this highly unusual and poorly-understood system. Because of the large number of studies needed to arrive at apparently optimum addition and washout protocols, we report only the final protocols used for each cryoprotectant system studied. For clarity of presentation, these final protocols are given in the Results. Mannitol was included in the washout media to avoid osmotic shock as described in the Results.

The primary cryoprotectant formulas studied were $V_{EG}$ and VM3. $V_{EG}$ [14,16] consists of 16.84% w/v ethylene glycol, 13.96% w/v formamide, and 24.2% w/v dimethyl sulfoxide (total concentration: 55% w/v of the cryoprotective solutes of $V_{EG}$). Dilutions of $V_{EG}$ are reported as absolute total concentrations of $V_{EG}$ solutes, e.g., "25% w/v $V_{EG}$" is a dilution of $V_{EG}$ that contains a total concentration of 25% w/v cryoprotectant. VM3 [16] is a more stable and more advanced vitrification solution containing 22.3% w/v dimethyl sulfoxide, 12.86% w/v formamide, 16.84% w/v ethylene glycol, 7% w/v polyvinylpyrrolidone K12, 1% w/v (final concentration) "Supercool X-1000" ice blocker [60], and 1% w/v "Supercool Z-1000" ice blocker [59]. The physical properties of VM3 have been described elsewhere [13,18]. $V_{EG}$ and VM3 are commercially available from 21st Century Medicine, Rancho Cucamonga, CA (www.21cm.com).

After cryoprotectant introduction, slices to be vitrified were abruptly transferred, on their mesh support, to an aluminum block partly immersed in liquid nitrogen so as to cool the upper block surface to −130 °C. A piece of hydrophobic weighing paper placed on the block prevented adhesion of the sample cylinder to the block. Slice placement on the block raised the local block temperature (as measured by a thermocouple placed into a hole drilled into the metal block) to −125 °C over 4 min. Slice temperature, as measured by thermocouple, also reached −125 °C in this time. The block and slice temperature returned to −130 °C, which is about 3 °C below the glass transition temperature of $V_{EG}$ and 3–4 °C below the glass transition temperature of VM3, in 4–5 min. An additional 3–7 min were allowed at −130 °C before rewarming. Slices were warmed by being transferred to excess vitrification solution at −10 °C. Thermocouples placed directly into slices indicated average warming rates of about 2370 °C/min between $T_G$ and $T_M$.

*Freezing procedure*

Cryoprotectants were introduced using the slice transfer technique described above and at temperatures described below. Slices were then frozen in 1 ml polyethylene containers containing two slices and 0.3 ml of cryoprotectant solution per container. Four containers with closed lids were placed into a foam box that floated on a non-toxic organic fluid such as hexane that in turn was cooled by dry ice. Each container self-nucleated by means of a copper wire that penetrated through the floor of each container and through the floor of the foam box, allowing rapid heat conduction from a point within the cryoprotectant solution to the external heat sink sufficient to induce nucleation at the site of the copper wire. Cooling profiles were measured during two experiments involving freezing with 30% v/v glycerol, both of which gave closely similar results. In these experiments, the measured temperature within the samples fell at about 2–3 °C/min until the crystallization front reached the thermocouples near −10 °C, which is essentially identical to the melting point of 30% glycerol. After the temperature reached −10 °C, the cooling rate became constant at 1.2 °C/min until the samples reached −40 °C, slowing down somewhat thereafter. When cooling to below −20 °C was needed, sample containers were allowed to reach −50 °C before being transferred to dry ice for further cooling. Warming was accomplished by transferring the container to a water bath at 20 °C; warming rates were not measured.

*Light and electron microscopy*

Control slices were incubated at 35 °C and then transferred to glutaraldehyde fixative for standard embedding, sectioning, and staining for transmission electron microscopy (TEM). Other slices were frozen and thawed or vitrified and restored to normal conditions at 35 °C as described in detail below, then fixed and processed for TEM as above. Parallel samples from the same groups in the same experiments were evaluated by $K^+/Na^+$ ratio to allow the morphological results to be related to the physiological results. Thick sections were stained with toluidine blue for light microscopy. All TEM work was conducted at the Harbor-UCLA Department of Pathology. Light photomicrography was done at 21st Century Medicine using a Spot RT Imaging video camera and

Spot image capture and processing software (Diagnostic Instruments, Sterling Heights, MI) to obtain digital images.

*Statistical testing*

Differences between means were tested using Student's *t* test.

## Results

*Freezing injury*

Untreated fully immersed control hippocampal slices were found to have $K^+/Na^+$ ratios of about 1.1–1.5 (Fig. 1, results plotted at 37 °C; mean ratio ∼1.35 for the first experiment and ∼1.2 for the second experiment), and the $K^+/Na^+$ ratio was found to be responsive to both glycerol and to freezing and thawing. Adding 30% v/v glycerol by the
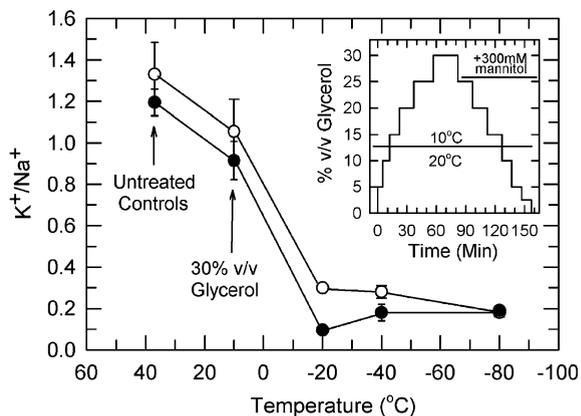


Fig. 1. Effects on rat hippocampal slices of exposure to 30% v/v glycerol and freezing in 30% v/v glycerol to three subzero temperatures. Two separate experiments are reported (white points, one experiment; black points, second experiment; all points for a given experiment are connected to indicate the progression of injury with each step of the protocol from glycerol exposure only (at 10 °C) to glycerol exposure plus freezing (points below 0 °C) for each individual experiment). The white points below 0 °C show the effect of freezing to the indicated temperatures and thawing immediately. The black points below 0 °C show the effect of freezing to the same temperatures and holding at those temperatures for 12 h before thawing. Inset: the protocol used for adding and removing glycerol. The horizontal line in the inset indicates that glycerol exposure was at 20 °C for concentrations of 10% v/v and below, and at 10 °C for concentrations above 10% v/v. Mannitol was present at 0.3 M throughout all washout steps, including the final step of transfer into glycerol-free aCSF. Slices were fully immersed in aCSF during 35 °C post-treatment incubation prior to testing for $K^+/Na^+$ ratio. Means ± 95% confidence limits.

protocol given in the inset resulted in a moderate drop in $K^+/Na^+$ ratio to about 0.8−1.3 (results plotted at 10 °C). Slices frozen to −20, −40, or −79 °C in 30% glycerol and immediately thawed (white points) were severely injured, regaining a $K^+/Na^+$ ratio of only about 0.3 (∼27% of the pre-freezing $K^+/Na^+$ ratio) at the first two temperatures and 0.2 (∼18% of the pre-freezing ratio) at the lowest temperature. Storing slices frozen with the same protocol at the three studied subzero temperatures for 12 h (black points plotted below 0 °C) did not further increase injury at −79 °C and possibly at −40 °C, but caused additional severe injury at −20 °C. These results were not improved by lowering the glycerol concentration to 10% w/v (freeze–thaw recoveries from −79 °C were 13% of pre-freeze $K^+/Na^+$ ratios) or by replacing glycerol with 20% v/v dimethyl sulfoxide (freeze–thaw recoveries from −79 °C were 21% of pre-freeze $K^+/Na^+$ ratios) (detailed data not shown).

Microscopic examination of brain slices fixed after freezing and thawing in 10% glycerol, cryoprotectant washout, and incubation at 35 °C for ⩾60 min showed two predominant kinds of cells. The majority were essentially obliterated (Fig. 2A), and the neural processes in these areas generally had the appearance of spherical vesicles instead of visibly two-dimensional fibers. A minority of cells retained some structural integrity but were considerably shrunken and distorted, and most likely moribund (Fig. 2B). The overall pattern is consistent with the possibility of intracellular freezing in a large proportion of neurons using the present freezing procedure. Although recovery might well be better after slower freezing, we did not investigate this possibility further as our main interest was to determine the possibility of preservation by vitrification.

### Injury from hypothermia and 25% w/v cryoprotectant

To reduce the toxicity of the high concentrations of cryoprotectants that are needed for vitrification, hypothermic temperatures are needed for cryoprotectant addition and washout. For kidney slices, 0 °C is a suitable temperature for addition and washout in most cases. Fig. 3 shows the results of 16 independent control experiments on the effects of exposing rat hippocampal slices to 0 °C in the presence of different carrier solutions and candidate protective agents for varying times. There is an exponential decline with time in capacity to restore and maintain a normal $K^+/Na^+$ ratio after storage in aCSF (triangles) that is severe by 2 h of hypothermic exposure and reaches or approaches completion by 4.5 h. Based on the observations of Pakhotin and co-workers [37,39] indicating that aspirin and indomethacin can protect the guinea pig hippocampus during prolonged cold storage, many attempts were made to reduce the damaging effects of 0 °C exposure using these agents. However, Fig. 3 clearly demonstrates that neither aspirin (black symbols) nor indomethacin (dotted symbols) has any
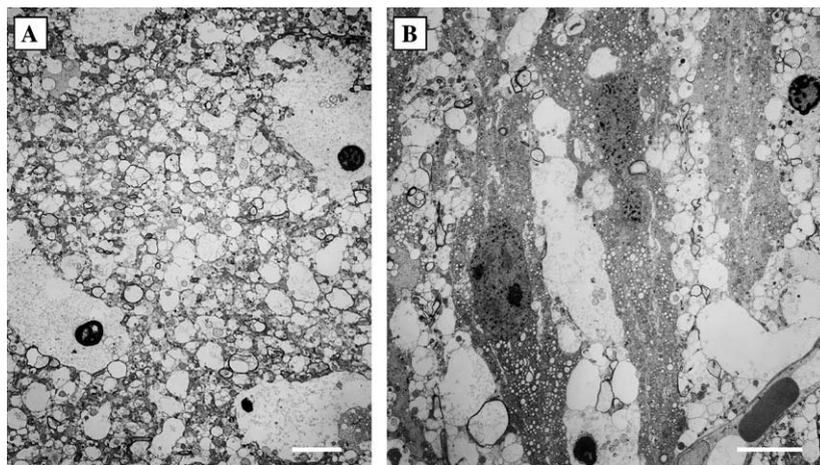


Fig. 2. Ultrastructural effects of freezing rat hippocampal slices with 10% w/v glycerol to −79 °C, thawing, removing cryoprotectant, and incubating the slices at 35 °C for 60–90 min. (A) Remnants of granular cells at the periphery of the dentate gyrus and adjacent neural processes. Cells appear devoid of cytoplasm and local neuropil appears disrupted. (B) CA1 cells with greatly distorted ultrastructure. (For examples of control ultrastructure and histology, see Figs. 7 and 8, respectively.) Scale bars = 10 μm.
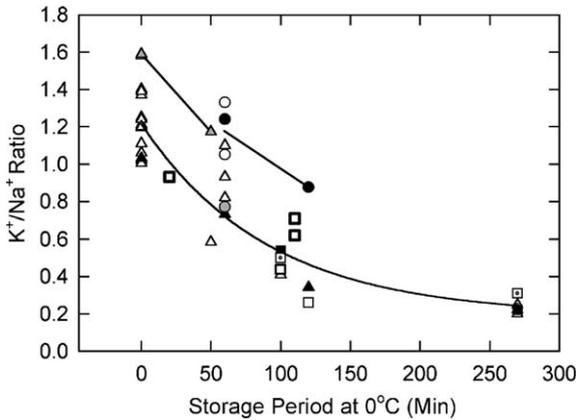
Fig. 3. Effect of hippocampal storage at 0 °C in the presence of different media and protective agents. Triangles, storage in aCSF. Circles, storage in aCSF modified to replace 2 mM $CaCl_2$ with 0.1 mM $CaCl_2$ and to replace 100 mM NaCl with 180 mM mannitol plus 10 mM KCl (solution A). Squares, storage in aCSF + 100 mM mannitol (maCSF). Black symbols, 0.5 mM aspirin. Grey symbols, 0.8 mM vitamin C. Dotted symbols, 45 μM indomethacin. Squares with medium-thickness sides, maCSF + 0.4 mM vitamin C plus 45 μM indomethacin. Squares with high-thickness sides, maCSF + 0.4 mM vitamin C plus 0.5 mM aspirin. Data from 16 independent experiments. Curved line, exponential curve fit to all data other than aCSF + 0.8 mM vitamin C (connected gray triangles) and solution A (black and white circles with line of best fit). All data obtained after rewarming slices to 35 °C for generally 60 min. Fully immersed slices. Each symbol represents the average of generally four replicate observations. Error bars are omitted for clarity, but the 95% confidence limits ranged from ±0.04 to ±0.28 $K^+/Na^+$ ratio units for stored slices and from about ±0.09 to ±0.4 for unstored slices, with most confidence limits being in the range of ±0.05–0.2 U.

protective effect for the rat hippocampus. Based on the well-known problem of colloid cell swelling under hypothermic conditions [28], many slices were stored in modified aCSF (maCSF, squares), consisting of aCSF plus 100 mM mannitol, with or without the above additives, but this intervention also appeared to have no influence on the course of hypothermic injury. Two other interventions, however, appeared to be protective. Addition of 0.8 mM (but not 0.4 mM) ascorbic acid to aCSF (joined gray triangles) seemed to raise both basal and post-storage $K^+/Na^+$ ratio. Modifying aCSF by replacing 100 mM NaCl with 10 mM KCl and 180 mM mannitol and reducing calcium levels from 2 to 0.1 mM seemed to protect the slices equally well (black and white circles). However, combining these two approaches negated the benefits of both in one experiment (gray circle), for reasons that are unclear. In addition, these two interventions raised

viability but did not appear to prevent the $K^+/Na^+$ ratio from declining at a rate similar to that observed in aCSF.

Fig. 4 shows that the injury incurred by 100 min of 0 °C storage in maCSF is absent at 10 °C. Further, exposure to 25% w/v $V_{EG}$ at 0 °C was not necessary to suppress toxicity, 10 °C exposure resulting in little or no damage. Interestingly, the same $V_{EG}$ treatment at 0 °C was clearly injurious, but it was less injurious than exposure to maCSF alone at the same temperature. These results indicate the existence of injury caused by hypothermic exposure that operates both in the presence and the absence of cryoprotectants, but that is partially mitigated by 25% w/v $V_{EG}$. Glycerol had no similar protective effect (data not shown). Exposure to 25% $V_{EG}$ at 15 °C was more injurious than exposure at 10 °C ($p = 0.0038$ by $t$ test; data not shown), so 10 °C was selected as the temperature of choice for the early stages of cryoprotectant loading in further protocols. We also varied the concentration of mannitol used as an osmotic buffer in aCSF during cryoprotectant washout at 10 °C. The mean $K^+/Na^+$ ratio was higher at 300 mM mannitol than at either 150 or 450 mM, and the difference between 300 and 450 mM was significant ($p = 0.012$; data not shown).

RPS-2 is a solution developed for the simple cold storage of rabbit kidney slices and whole rabbit kidneys [7,15,30]. It is capable of maintaining the viability of rabbit renal cortical slices unchanged at 0 °C for 4 days, so we decided to explore its effectiveness for rat hippocampal slices. Our initial results with RPS-2 yielded recovery after 10 °C
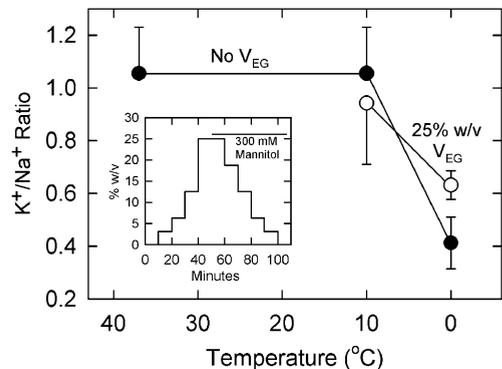


Fig. 4. Hypothermic injury in the absence (closed points) and presence (open points) of 25% w/v $V_{EG}$ solutes in maCSF. Inset: cryoprotection protocol (all steps at the temperatures indicated in the main panel). Pooled results from two independent experiments. Means ± 95% confidence limits. Fully immersed slices.

exposure (mean $K^+/Na^+$ ratio $\pm$ 1 SEM, 1.05 $\pm$ 0.06; no cryoprotectant present) equal to 109% of untreated maCSF control slices (0.96 $\pm$ 0.04, $p = 0.25$) and equal to 121% of maCSF controls at 10 °C (0.87 $\pm$ 0.04, $p = 0.05$), so we adopted RPS-2 for further experiments.

*Exposure to $\geqslant$50% w/v cryoprotectant and vitrification*

The general feasibility of cryopreserving slices by vitrification was first tested by raising the $V_{EG}$ concentration to 50% w/v (Fig. 5). When slices were treated with this concentration in aCSF at 0 °C (thick-walled circle), it was very damaging, but replacement of aCSF with RPS-2 eliminated most of this injury. In fact, when RPS-2 was used as the carrier, there was a trend for injury to be reduced rather than increased by temperature reduction below 10 °C (gray circles).

For complete vitrification, it was estimated that 53% w/v $V_{EG}$ is the minimum concentration required to vitrify at the cooling rates used in the present studies. Raising concentration to 53% in RPS-2 resulted in remarkably more injury than exposure to 50% $V_{EG}$, $K^+/Na^+$ falling even at −10 °C (open squares in Fig. 5). However, there was no further injury when slices so treated were then cooled to −130 °C, supporting the estimate that 53% is sufficient for vitrification.

Because toxicity appeared to be the limiting factor for the recovery of slices after vitrification with $V_{EG}$, we explored the use of VM3 [16,18] as an alternative vitrification solution. Because VM3 contains the X1000 and Z1000 ice blockers, we used LM5 as the carrier rather than RPS-2 since LM5 better maximizes the effectiveness of these ice blockers [18]. Successful use of VM3 required particularly gentle introduction and washout protocols, and results were erratic until the protocol employed in Experiment 151 of Table 1 (and highlighted in Fig. 6) was used. Nevertheless, several experiments showed that VM3 is intrinsically non-toxic (>90% recovery of $K^+/Na^+$ ratio; Table 1), and when slices treated successfully with VM3 were vitrified, there was no difference in recovery compared to VM3 exposure alone (Table 1).

Fig. 7 shows the ultrastructure of the stratum pyramidale and the dentate gyrus of control and vitrified/rewarmed rat hippocampal slices after recovery in aCSF at 35 °C. Figs. 7A and B show control CA1 cells and granular cells, respectively,
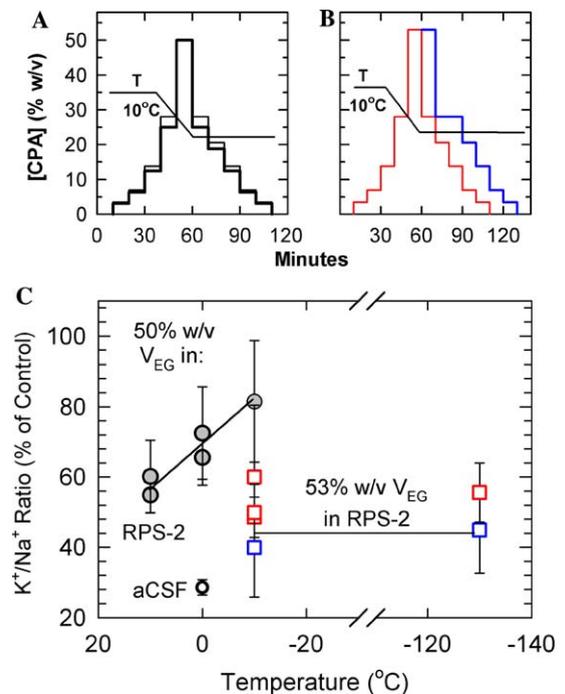


Fig. 5. Dependence of the toxicity of 50% w/v (gray and white circles in (C)) or 53% w/v (open colored boxes, (C)) $V_{EG}$ on temperature and carrier solution (aCSF or RPS-2), and comparison between the effects of vitrification and cryoprotectant exposure without vitrification. Line thickness and color for each symbol edge in (C) designates the protocol having the same line thickness and color in (A) and (B). (A) Addition/removal protocols for 50% w/v $V_{EG}$. (B) Addition/removal protocols for 53% $V_{EG}$. All steps were carried out at 10 °C except the peak concentration step and the first washout step, which were carried out at the temperatures (T) indicated in (C), and except for the case of the heavy line protocol in (A) (which corresponds to the thick-walled white circle in (C), for which all steps were carried out at 0 °C. The −10 °C 50% $V_{EG}$ protocol (medium line thickness) used 28% w/v $V_{EG}$ rather than 25% as the final step before and the first step after 50% $V_{EG}$, with proportionally higher intermediate concentrations below 28%. This same protocol was used in all experiments in (B) except for the use of 53% $V_{EG}$ instead of 50% $V_{EG}$ and except for longer times at 53% $V_{EG}$ and subsequent steps in one experiment (blue line protocol). Mannitol (300 mM) was present in all washout steps. Results plotted at −130 °C (open boxes) show the effects of vitrification and rewarming and are to be compared to the effects of incubation at −10 °C alone (open boxes). Results of 9 independent experiments. Means $\pm$ 95% confidence limits. Normalized to untreated controls (whose individual results were typical of untreated control results shown in Fig. 3) for clarity of presentation.

and Figs. 7C and D show the ultrastructure of the CA4 region and dentate gyrus granular cells after vitrification with VM3, rewarming, and 35 °C incubation. The vitrification experiment shown is a worst case example in which the overall recovery

Table 1
Successful vitrification of rat hippocampal slices using VM3[a]

| Experiment number | Control $K^+/Na^+$ | $K^+/Na^+$ after | | % of Control $K^+/Na^+$ | |
|---|---|---|---|---|---|
| | | VM3 only | Vitrification | VM3 only | Vitrification |
| 151[b] | 2.71 ± 0.25 | | 2.92 ± 0.18 | | 107.9 |
| 152[b] | 2.71 ± 0.19 | | 2.53 ± 0.11 | | 93.3 |
| 135[c] | 3.03 ± 0.33 | | 2.75 ± 0.07 | | 90.7 |
| 140[c] | 3.41 ± 0.16 | 3.20 ± 0.14 | 3.13 ± 0.12 | 93.7 | 91.5 |
| 123[d] | 2.71 ± 0.23 | | 2.49 ± 0.08 | | 91.9 |
| 124 | 2.63 ± 0.15 | | 2.76 ± 0.08[e] | | 104.9 |
| 124 | 2.63 ± 0.15 | | 2.51 ± 0.43[f] | | 95.3 |

[a] Means ± 1 SEM. Absolute ratios are higher than in Figs. 1, 3, and 4 due to use of interface incubation conditions in the Oslo chamber at 35 °C rather than full immersion incubation prior to assessment of $K^+/Na^+$. The carrier solution was LM5 in all cases.
[b] The first 5 addition steps (2, 4, 8, 16, and 30% w/v) were at 10 °C and lasted 10 min each. VM3 was then added at −10 °C (for 25 min, with solution changes at 10 and 20 min). The first washout step was 31% + at −10 °C for 10 min ("+" signifies the presence of 300 mM mannitol). The last 7 washout steps were 16% +, 8% +, 4% +, 2% +, 1% +, 0% +, and 0% w/v at 10 °C for 10 min each, where 0% equals LM5.
[c] Same protocol as in experiments 151 and 152, but the first washout step is 30%+ and the 1%+ washout step is omitted.
[d] Same protocol as in experiments 135 and 140, but the slices were held in VM3 for 30 min rather than for 25 min before vitrification, and this solution was not changed during equilibration in VM3.
[e] Same protocol as experiment 123, but slices were held in VM3 for 20 min before vitrification.
[f] Same protocol as experiment 123, but slices were held in VM3 for 25 min before vitrification.
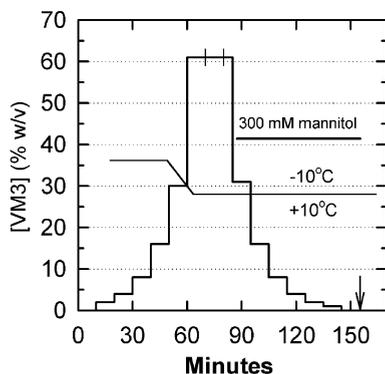


Fig. 6. Final protocol for the addition and washout of VM3. Tick marks at 61% (full-concentration VM3 step) indicate slice transfers to successive VM3 solutions. Arrow at $t = 155$ min indicates transfer from LM5 + 300 mM mannitol to LM5. Ten minutes were then allowed for the washout of mannitol prior to transfer to warm aCSF. Heavy horizontal line indicates the presence of mannitol during the washout steps. Light horizontal and diagonal lines indicate the thermal protocol (−10 °C above the lines, +10 °C below the lines).

of $K^+/Na^+$ was only 77% of control (Experiment 139). This slice showed a mixture of cells and neuropil that were indistinguishable from controls (Fig. 7C) and cells that displayed the obliterated or condensed morphologies seen in the frozen/thawed slices (Fig. 7D), suggesting inadequate cryoprotectant uptake in the latter cells (failure to vitrify or to remain ice-free on warming).

Fig. 8, on the other hand, compares control and fully successful vitrified/rewarmed rat hippocampal slices on the light microscopic level in order to convey the large scale preservation of structure and viability obtained with our final vitrification procedure. (A) Shows a control region of the CA1 portion of the stratum pyramidale. (B) Shows the overall structure of a slice preserved at −130 °C by vitrification in Experiment 151 of Table 1 and subsequently incubated in aCSF at 35 °C for >60 min. The CA1 area of this slice is shown at higher magnification in (C), and the CA3 region is shown in greater detail in (D). Both the CA1 and CA3 areas of the vitrified/rewarmed slice appear to be fully intact, confirming the overall preservation evident in (B), and all other regions examined also appeared to be perfectly preserved. These histological results were in agreement with ultrastructural observations for the same experiment indicating preservation after vitrification, rewarming, and warm incubation essentially equivalent to that observed in untreated control slices (data not shown).

## Discussion

The effects of freezing in these experiments were more severe than expected based on the results of Jensen et al. [26,27], who used cooling rates similar to ours to freeze whole immature hippocampi with the loss of only about 35% of the granular cells of the dentate gyrus. Our apparent nucleation temperature of −10 °C is close to the melting point of 30%
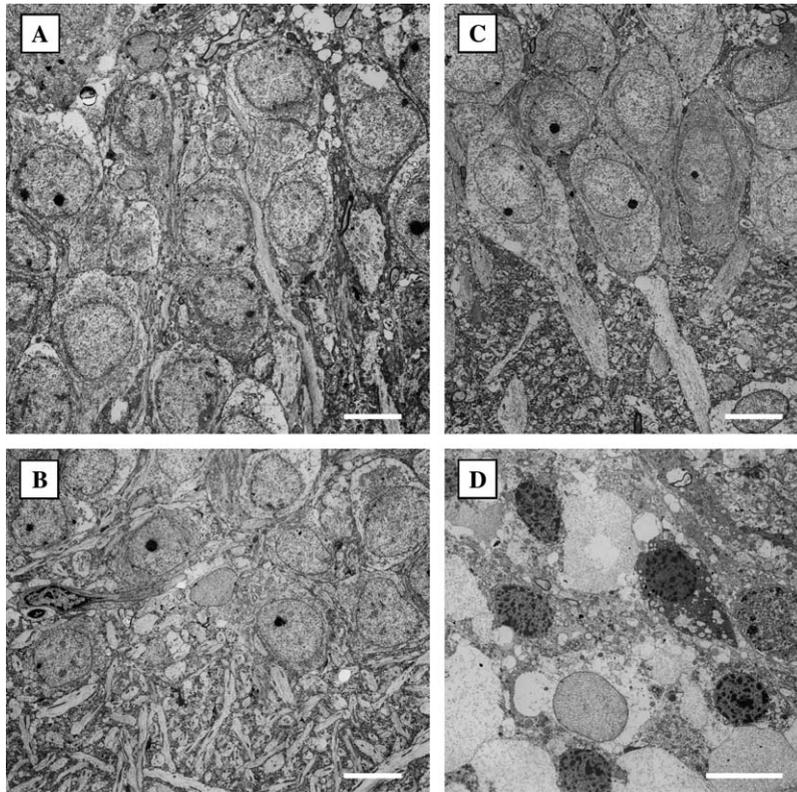
Fig. 7. Ultrastructure of control and incompletely recovered vitrified/rewarmed rat hippocampal slices. Vitrified/rewarmed slices were cooled to −130 °C, rewarmed, washed free of cryoprotectant and incubated in aCSF at 35 °C for >60 min before being fixed in low-osmolality Karnovsky's. (A) Control CA1 cells. (B) Control granular cells at the edge of the dentate gyrus, with adjacent dendrites. (C) Undamaged vitrified/rewarmed CA4 cells. (D) Damaged vitrified/rewarmed granular cells (dentate gyrus). Scale bars all represent 10 μm.

glycerol and not far below the melting point of 10% glycerol, so the extent of supercooling prior to nucleation in our experiments was apparently very small and would not normally be expected to produce a high likelihood of intracellular ice formation [34,35]. Nevertheless, the morphological results of Fig. 2 are suggestive of extensive intracellular ice formation, and this might be possible given the suppression of thermal rebound in our cooling method [12]. In addition, the optimal cooling rate of cerebral tissues has not been well-defined and could be slower than 1 °C/min due to, for example, the presence of myelinated nerve processes and minimal extracellular space, both of which might make water loss unusually slow. However, sufficient injury from any source could well result in the development of lytic degrees of cell swelling and the kinds of images seen in Figs. 2A and B during the period of metabolic challenge at 35 °C, so intracellular freezing is not a necessary explanation for the extensive damage seen.

It remains to be seen whether any freezing method can match the excellent results shown to be obtainable with vitrification in the present studies. According to the "80/20 rule" observed by some investigators [55], vitrification tends to yield much better results (∼80% recovery) than freezing (∼20% recovery) for contractile systems or cartilage, and one might expect this situation to apply to brain slices given their delicacy. Our present results, showing recoveries of >90%, are consistent with or even better than expectation based on the "80/20 rule." Our results are also strikingly superior to those obtained with isolated fetal hippocampal cells, of which only 12% survived freezing in 10% dimethyl sulfoxide and 24 h of subsequent culture [53].

Our experiments demonstrated a tendency for relatively rapid deterioration of rat hippocampal slices held at 0 °C in aCSF. This tendency is consistent with observations on profoundly hypothermic canine subjects indicating a very limited window
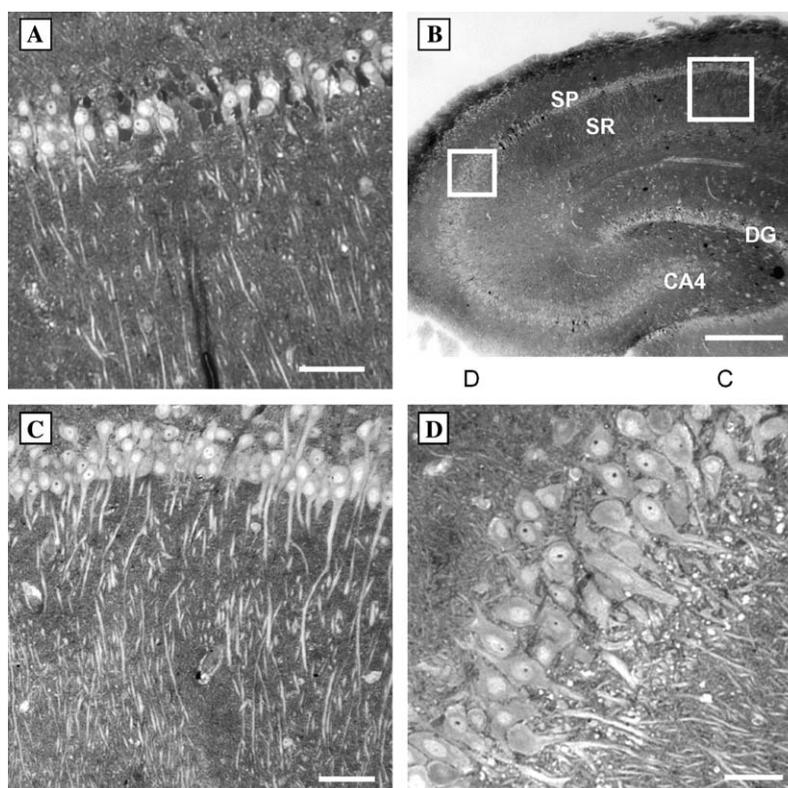
Fig. 8. Histological integrity of fully recovered vitrified/rewarmed rat hippocampal slices after cryoprotectant washout and 35 °C incubation for >60 min. (A) Control CA1 cells and their apical dendrite field in the stratum radiatum (from Experiment 140, Table 1). Scale bar, 50 μm. (B) Overall appearance of the greater part of the stratum pyramidale (SP) and dentate gyrus (DG) in a vitrified–rewarmed–reincubated rat hippocampal slice (from Experiment 151, Table 1), showing the full integrity of the entire structure after cooling to and rewarming from −130 °C. SR, stratum radiatum; CA4, CA4 region of the stratum pyramidale. Boxes indicate regions of the CA1 and CA3 fields shown in greater detail in (C) and (D) (the letter below each box refers to the panel that shows a magnified image of the zone within the box). Scale bar equals 500 μm. (C) Expanded view of the CA1 cells and their apical dendrites from (B), showing fully intact structures after vitrification and rewarming. Scale bar represents 50 μm. (D) Expanded view of the CA3 cells and their apical (right) and basilar (left, Schaffer collateral outputs) dendrite fields from (B), showing completely normal anatomy after cooling to and rewarming from −130 °C. Scale bar represents 40 μm.

of safety for the brain during hypothermic circulatory arrest, a model that may mimic slice static storage better than continuous hypothermic perfusion models [30]. The record for the longest period of circulatory arrest during profound hypothermia that is consistent with subsequent full neurological recovery (3 h) was set by Haneda et al. in 1986 [23] and has not been superceded to this day. According to a prominent 2004 review, "exsanguination cardiac arrest periods lasting 120 min can be reversed, but normality is difficult to achieve, and significant brain damage is found at necropsy" [57].

Unlike Pakhotin et al. [38,39], we were not, despite considerable effort, able to block this cold storage injury using either aspirin or indomethacin. This disagreement may be due to species differences between the guinea pigs of Pakhotin et al. and rats. Pakhotin et al. used aspirin only as a pretreatment, whereas we used it as a pretreatment, as an additive to hypothermic storage solutions, as a supplement present only after hypothermic storage, or in combinations of these ways. In no case was a consistent protective effect seen, and sometimes an apparently negative effect was seen. However, we did develop preliminary evidence for a protective effect of 0.8 mM vitamin C, although not for protection by 0.4 mM vitamin C. Changing the composition of the carrier solution also seemed to help, but whether the mechanism of protection is related to reduction of sodium level, elevation of potassium level, or reduction of calcium level remains to be determined.

Whatever the mechanism(s), the ability of carriers other than aCSF to ameliorate cold storage injury in brain slices appears to be critical for successful brain slice vitrification, because adding and removing all cryoprotectant at 10 °C and above does not seem to be feasible, and cold storage injury in aCSF or maCSF below 10 °C appears to proceed in both the presence and absence of cryoprotectants. Fortunately, cold storage injury was reduced or eliminated when RPS-2 (Fig. 5) or LM5 (Table 1) were used as carrier solutions. Interestingly, diluted $V_{EG}$ but not glycerol was able to somewhat reduce cold storage injury. This effect remains unexplained, but forms an interesting comparison to the results of Collins et al. [6] who found that 0.3 M glycerol and 0.3 M 1,2-propanediol were able to ameliorate hypothermic perfusion injury to rabbit kidneys.

Even when cold storage injury is obviated, ideal results appear to require careful choice of both the vitrification solution and the addition and washout protocol. Our best results were obtained with VM3, which also appears to be less toxic to kidney slices than $V_{EG}$. Our use of VM3 was motivated by the large increase in injury seen when $V_{EG}$ levels were raised from 50 to 53% w/v. However, we did not definitively rule out the possibility that this increased injury was the preventable result of crossing an osmotic threshold. Even so, the 61% w/v VM3 solution was able to give better results than 50% w/v $V_{EG}$ solutes, which provides strong support for the use of VM3 for hippocampal slices. Bone cell preparations, which may be hardier, were effectively preserved with 95% of full-strength $V_{EG}$ plus 1% Supercool X-1000 [32].

We find it noteworthy that a solution developed from experiments on rabbit renal cortical slices (VM3) has now been successfully applied to the vitrification of systems as diverse as mouse ova [16] and rat hippocampal slices. For brain slice banking to be maximally useful, it will be helpful if the same method can be used to bank slices from different species and from different regions of the brain, and evidence of applicability to widely different systems is therefore encouraging.

The $K^+/Na^+$ ratio assay used in these experiments has been supported elsewhere as a generally applicable viability test for organized tissues [4,5,11,16,29,47,56], and appears to be well suited for screening brain tissue viability. Sodium and potassium transport is a prerequisite for cerebral electrical activity and the avoidance of excitotoxic membrane depolarization and is a major energy-consuming process in brain tissue. It is also required for cell volume maintenance, which is particularly important in brain tissue, and for normal protein synthesis [1], and it is responsive to changes in membrane permeability, ATP production, and the sodium–potassium ATPase ion pump, all of which are critical for brain tissue viability and functionality. In the present studies the $K^+/Na^+$ ratio was sensitive to hypothermic injury, cryoprotectant toxicity, osmotic injury, and freezing injury, and appeared to be well correlated with the morphological integrity of the hippocampus. The virtually complete preservation of normal morphology even after 35 °C incubation in aCSF for prolonged periods confirms the results of the $K^+/Na^+$ ratio assay in indicating essentially full recovery of the viability of the rat hippocampal slice preparation after vitrification and rewarming.

In conclusion, our results provide the first demonstration that the viability of organized adult brain tissue neural networks can be well preserved by vitrification. Our results support the possibility of preserving hippocampal slices for pharmacological and physiological testing and provide new support for the possibility of neural system transplantation for medical applications. Now that viability has been demonstrated, it is feasible and appropriate to proceed to detailed neurophysiological examination of hippocampal slices after vitrification and rewarming to conclusively demonstrate their potential utility for the screening of psychoactive drugs, and experiments of this kind are currently in progress.

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