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Research report

Intraischemic but not postischemic hypothermia prevents non-selective hippocampal downregulation of AMPA and NMDA receptor gene expression after global ischemia

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Abstract

Hypothermia may afford histological neuroprotection induced by ischemia by preventing aberrant Ca^{2+} influx through NMDA (*N*-methyl-D-aspartic acid) or Ca^{2+}-permeable AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid) receptors. Expression of hippocampal GluR1(A), GluR2(B), GluR3(C) and NMDAR1 (NR1) subunits was investigated by in situ hybridization at 1 and 7 days after 10-min transient global ischemia in the presence and absence of intraischemic or postischemic brain hypothermia (30°C). At 1 day, normothermic ischemia markedly suppressed the expression of GluR1(A), GluR2(B), and GluR3(C) receptor mRNAs to a similar degree in the vulnerable CA1. Less vulnerable CA3a–c subregions were also acutely downregulated. NR1 mRNA expression was reduced in CA1 but to a lesser extent than AMPA mRNAs. At 7 days after normothermic ischemia, a time of marked CA1 cell loss, all three AMPA transcripts were nearly absent in CA1 while a percentage (33.9±7.2%) of NR1 mRNA remained. Intraischemic hypothermia fully blocked the damage and non-selective mRNA downregulations at 1 and 7 days. By contrast, postischemic hypothermia postponed neurodegeneration but only partially rescued the expression of AMPA and NR1 mRNAs at 7 days and not at 1 day after the insult. Therefore, hippocampal AMPA receptor mRNAs decline at a relatively similar rate after normothermic global ischemia and cellular neuroprotection by intraischemic hypothermia occurred independently of altered subunit composition of AMPA receptors. Since decreases persist within resistant neurons under the postischemic condition, AMPA receptor-mediated Ca^{2+} currents probably do not contribute to selective vulnerability.

Theme: Disorders of the nervous system

Topic: Ischemia

Keywords: Intraischemic hypothermia; Postischemic hypothermia; AMPA receptor; NR1 receptor; Delayed neurodegeneration; Selective vulnerability

1. Introduction

Toxic concentrations of intracellular Ca^{2+} due to overactivation of ionotropic NMDA-type glutamate receptors was proposed to contribute to delayed cell death following a neurological injury, such as loss of CA1 neurons after normothermic global ischemia [3,4,16,41,64]. More recently AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)-type glutamate receptors were implicated in the damage (the GluR2(B) hypothesis) [32–35,43,60–63], since they were discovered to be highly permeable to Ca^{2+} provided the GluR2(B) subunit is lacking [11,39,44,73].

The functional role of Ca^{2+} influx through NMDA and non-NMDA receptors in postischemic neurodegeneration is

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controversial [40]. Previous pharmacological studies appear to support the excitotoxic hypothesis in that both non-competitive and competitive NMDA and non-NMDA antagonists (e.g. MK-801, CGS 19755, and NBQX) reduce ischemic infarction even when drug administration is delayed [2,51,66,67,69,76]. By contrast, other studies have shown, that pharmacological neuroprotection may be the result of drug-induced hypothermia, induced by injection of either NMDA or non-NMDA antagonists, rather than by direct blockade of glutamate receptors [9,10,56,75]. In in vitro hypoxia–aglycemia studies, a neurotoxic role of glutamate receptors is supported whereby antagonism of the NMDA-receptor is efficacious against cell death under constant temperature [23,74]. For the AMPA receptor subtypes, there is opposing evidence as to whether downregulation of the GluR1(A) subunit is simultaneous or delayed with the other subtypes (GluR2(B), GluR3(C), or GluR4(D)) [17,28,42,49,60]. If all AMPA subunit subtypes decrease after ischemia then increases in Ca^{2+} permeability through AMPA receptors would not be achieved. Direct evidence against the GluR2(B) hypothesis comes from posttranscriptional editing studies of the GluR2(B) subunit whereby editing is unimpaired in CA1 after normothermic ischemia, which is also required for a postischemic rise in AMPA receptor-mediated Ca^{2+} permeability to occur and be toxic [46,63].

Intraischemic hypothermia is probably the most effective treatment for enduringly preventing postischemic neuronal injury [15]. An important mediator of hypothermic neuroprotection is the reduction of ischemia-induced glutamate release [7,14,41,53]. Postischemic hypothermia is also neuroprotective, particularly when prolonged postinsult treatment paradigms are used [12,18,19]. However, when cooling is delayed, critical factors such as the exact temperature monitored, hypothermia duration, or combined treatment with other agents such as antipyretics [22] or anti-inflammatory cytokines [25] determine the extent of CA1 hippocampal injury [20,21]. Therefore, hypothermia was used, herein, to manipulate any potential putative switch in AMPA receptor subunit composition to determine whether increased formation of Ca^{2+}-permeable AMPA receptors may be a common toxic mechanism of selective hippocampal postischemic cell death. In situ hybridization studies were carried out to compare GluR1(A), GluR2(B), GluR3(C), and NR1 mRNA levels throughout the hippocampus after transient global ischemia in the presence and absence of intraischemic or postischemic hypothermia.

2. Materials and methods

2.1. Surgical preparation

The study was carried out in male Wistar rats. Animals were fasted overnight but were allowed free access to water. Atropine sulfate (0.5 mg/kg i.p.) was injected 10 min before anesthesia to dry out tracheal secretions which otherwise might cause ventilatory obstruction in intubated animals. Anesthesia was induced with 3.5% halothane in a mixture of 70% nitrous oxide and a balance of oxygen. Rats were orally intubated and mechanically ventilated. During ventilation, the animals were paralyzed with pancuronium bromide (0.6 mg/kg i.v.). Temperature probes were inserted into the rectum and temporalis muscle, and separate heating lamps were used to maintain rectal and cranial temperatures at 36.5°C to 37.5°C (Mon-a-therm 7000; Mallinckrodt Inc.). Polyethylene catheters were introduced into the right femoral artery and vein for blood pressure recording and blood sampling. Mean arterial pressure (MABP) was measured via an indwelling femoral arterial catheter connected to a precalibrated Statham pressure transducer (Model P23XL, Viggo-Spectramed Inc.) and was recorded continuously (Model RS3400 polygraph; Gould Inc.). Serial measurements were made of arterial blood gases and pH (Model ABL 330, Radiometer America Inc.) and plasma glucose (Model 2300 Stat; Yellow Springs Instrument Co. Inc.).

2.2. Global cerebral ischemia

Rats underwent bilateral common carotid artery occlusions for 10 min with simultaneous reduction of MABP to 50 mmHg by withdrawal of arterial blood to produce high-grade forebrain ischemia, as previously described [68]. Full physiological monitoring was continued for 3 h into the postischemic period. Arterial and venous catheters were then removed, all incisions were closed and rats were returned to individual cages with free access to food and water for a survival period of 1 or 7 days. Sham-operated control rats underwent all experimental procedures except for the initiation of brain ischemia.

2.3. Experimental groups

Three groups were studied at each survival time (1 and 7 days). Group A: Normothermic ischemia: intraischemic and postischemic cranial temperatures were maintained at 36.5–37°C during the 3-h observation period. Group B: Intraischemic (cranial) hypothermia (30°C) was induced during the 10-min ischemic period followed by a 3-h normothermic recirculation period (cranial temperature 36.5–37°C). Group C: Postischemic (cranial) hypothermia: rats underwent normothermic ischemia (36.5–37°C) followed by a 3-h period of cranial hypothermia (30°C).

To achieve brain hypothermia, cranial temperature was maintained at the 30°C by means of a warming lamp and a cooling fan (delivering liquid nitrogen vapor) placed above the rat's head. Rectal temperature was separately regulated by heating lamps and controlled between 36.5 and 37°C in all groups throughout the experimental period.

2.4. Histological analysis

Animals were decapitated and brains rapidly removed and immediately frozen in *N*-methyl-butane at −35°C. Fresh frozen coronal sections (20 μm) were mounted and air-dried onto gelatinized slides. Neuronal cell loss was monitored in hippocampal structures by histological examination using the Nissl (thionin) stain. Prior to in situ hybridization experiments thionin staining was performed on adjacent air-dried sections from all animals. After in situ hybridization, emulsion-dipped sections were developed in D19 Kodak developer, then counterstained with hematoxylin/eosin for morphological resolution of neurotoxicity. Slides were dehydrated in graded ethanols, cleared, coverslipped, and photographed.

2.5. In situ hybridization

[^{35}S]UTP-labeled RNA probes were transcribed from GluR1(A), GluR2(B), and GluR3(C) (AMPA receptors), and NR1 (NMDA receptor), subunit cDNAs as described [32,34]. In brief, fresh frozen coronal sections (20 μm) were subjected to acetylation and incubated for 2 h at 50°C with 100 μl of prehybridization solution. For hybridization, adjacent sections from each brain were incubated with the ^{35}S-labeled RNA probes (10^6 cpm/section, 1 ng/ml) overnight at 50°C. After washing, sections were treated with RNase A (20 mg/ml) and dehydrated in ethanol. Slides were apposed (for 24–72 h) to Kodak XAR-5 film to generate autoradiograms. For light microscopy, high resolution studies, slides were dipped in photographic emulsion (Kodak NTB-2) and exposed for 2–3 weeks at 4°C. Since exposure times for each probe vary considerably as does percent cpm incorporation for each probe following transcription reactions, autoradiograms were scanned (Scion Image) and comparisons were made from brain images on the same film (NIH Image). The anatomy of brain images from autoradiograms and hematoxylin/eosin-stained sections was assessed from the atlas of Paxinos and Watson [59]. Microscopic examination was performed for every structure described. Photomicrographs of histology were obtained using a Nikon Eclipse 800 microscope under light optics and a Spot color digital camera (Micron Optics).

2.6. Quantification and statistical analyses

Serial sections were cut at the level of hippocampus and mounted onto 15–20 different slides. Thus, every slide contained three different levels labeled for each probe, and the experiment was repeated in duplicate. To achieve quantitative determinations of mRNA expression levels for the four probes examined, brain sections from animals with normothermic and hypothermic ischemia and from control sham-operated rats were cut in the same experimental session, incubated with the same solutions of RNA probes on the same day, and apposed to the same sheet of film. Autoradiograms were scanned with a Sony CCD camera and images were quantified with NIH Image software (Macintosh 940). Mean optical densities of pixels overlying hippocampal subfields for each riboprobe from a minimum of four sections per rat were averaged, and film background densities were subtracted. Pixel size was small compared to structures examined.

Densitometric measurements are reported as grand means±S.E.M. of individual means from a minimum of 3–5 rats at 1 or 7 days, in the presence or absence of intraischemic or postischemic hypothermia. Optical density measurements were calculated for each brain region and were normalized to corresponding regions from control sham-operated animals. Optical density means of each probe were compared by two-way analysis of variance with post hoc Fisher Protected Least Significant Difference measures (ANOVA). Percentages calculated from each area were also compared with Student's unpaired *t*-test. Significance was set at $P<0.05$.

Plotting of quantitative data was based on the following considerations. (1) Optical density readings taken from each region of interest varied little in different sections from the same animal. (2) The concentration of RNA probe used produced saturating levels of hybridization signal with maximal signal-to-noise ratio for the four riboprobes used. (3) [^{35}S]UTP-labeled brain paste standards indicated that exposure times were in the linear response range of the film as described [32]. Conditions were of sufficiently high stringency as to rule out cross-hybridization among GluR1(A), GluR2(B), and GluR3(C) [32,33] and more distantly related glutamate receptor subunits (GluR5 to GluR7, KA1, KA2). GluR4 expression is prominent only in the cerebellum [48]. GluR1(A), GluR2(B) and GluR3(C) are 'pan' probes [70] in that they label both 'flip' and 'flop' splice variants. NRI is also a 'pan' probe that labels eight splice variants but not mRNA's encoding NR2 subunits [55].

3. Results

3.1. Summary of physiological variables

Prior to initiating global ischemia, average values (±S.E.M.) were: mean arterial blood pressure, 122±1.5 mmHg; arterial PO_2, 128±3 mmHg, arterial PCO_2, 38.6±0.5 mmHg; arterial pH, 7.40±0.01; plasma glucose, 109±3 mg/dl. At 30 min after the ischemic insult, these values were: mean arterial blood pressure, 115±1.5 mmHg; arterial PO_2, 131±4 mmHg; arterial PCO_2, 39.8±0.7 mmHg; arterial pH, 7.35±0.01. There were no significant intergroup differences for these variables.

3.2. Preservation of CA1 after intraischemic and postischemic hypothermia

Histological assessment of all animals was made on

coronal sections stained with thionin by an experimenter blinded to the three ischemia paradigms used — normothermia, intraischemic hypothermia and postischemic hypothermia — with 1 or 7 days of reperfusion. Microscopic evaluation revealed no obvious differences between the sham-operated control group and the intraischemic hypothermia groups (at 1 or 7 days), indicating full histological protection at early and late time points (not illustrated) consistent with our previous studies [13]. Similarly, at 1 day after postischemic hypothermia, CA1 pyramidal neurons exhibited normal cytoarchitecture and appeared nearly identical to sham-operated control neurons (Fig. 1A,D,G). At 7 days in the postischemic hypothermia group, some disorganization of the CA1 layer was noted, but phase-contrast microscopy at high magnification showed that the overall morphological appearance of cell somata was still preserved, highly similar to the 24-h time point (Fig. 1B,E,H). For the postischemic group at 7 days, one of four rats showed full cellular protection, one showed no protection, and the others were well preserved. Therefore, obvious hippocampal neurodegeneration or cell loss was primarily observed only under conditions of normothermic ischemia and 7 days of recirculation (Fig. 1C,F,I). At this time, CA1 pyramidal neurons were either shrunken, fragmented, or absent, and the layer was uniformly infiltrated with glia (Fig. 1I). Thus, with the exception of rats with normothermia and 7 days of reperfusion, and a single animal in the 7-day postischemic hypothermic group, in situ hybridization experiments for mRNA detection were carried out on highly similar and morphologically intact tissue.

3.3. AMPA and NR1 mRNAs after normothermic ischemia

Autoradiograms of control adult rat brain sections

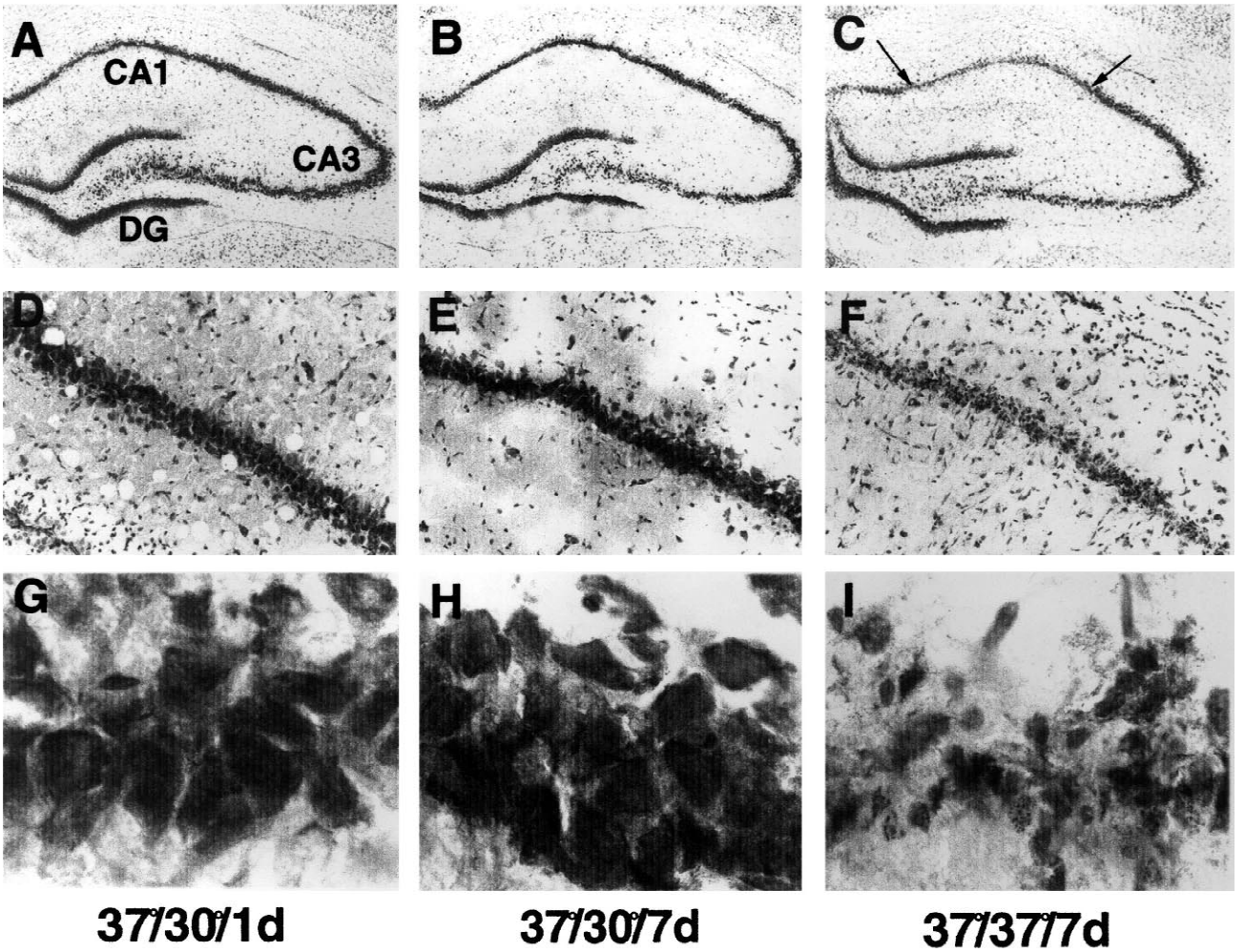

Fig. 1. Photomicrographs of CA1 neurons stained with thionin following postischemic hypothermia or normothermia at low (4×) (A–C), middle (20×) (D–F) and high magnifications (100×) (G–I). At 1 day after postischemic hypothermia (37°/30°/1d), CA1 pyramidal neurons appeared similar to sham-operated controls (A,D). At 7 days (37°/30°/7d), some disorganization of the CA1 layer was noted (B) but high magnification showed the overall morphological appearance of cell somata was still preserved, similar to the 24-h time point (E). After normothermic ischemia and 7 days (37°/37°/7d), CA1 pyramidal neurons were either shrunken, fragmented, or absent, and the layer was uniformly infiltrated with glia (C,F,I).

hybridized with antisense RNA probes directed against GluR1(A), GluR2(B), GluR3(C), and NR1 transcripts showed that mRNA signals were dense and uniform in pyramidal and granule cell layers of the hippocampus, as previously described [32,54] (Fig. 2). Normothermic global ischemia was followed by marked but non-selective

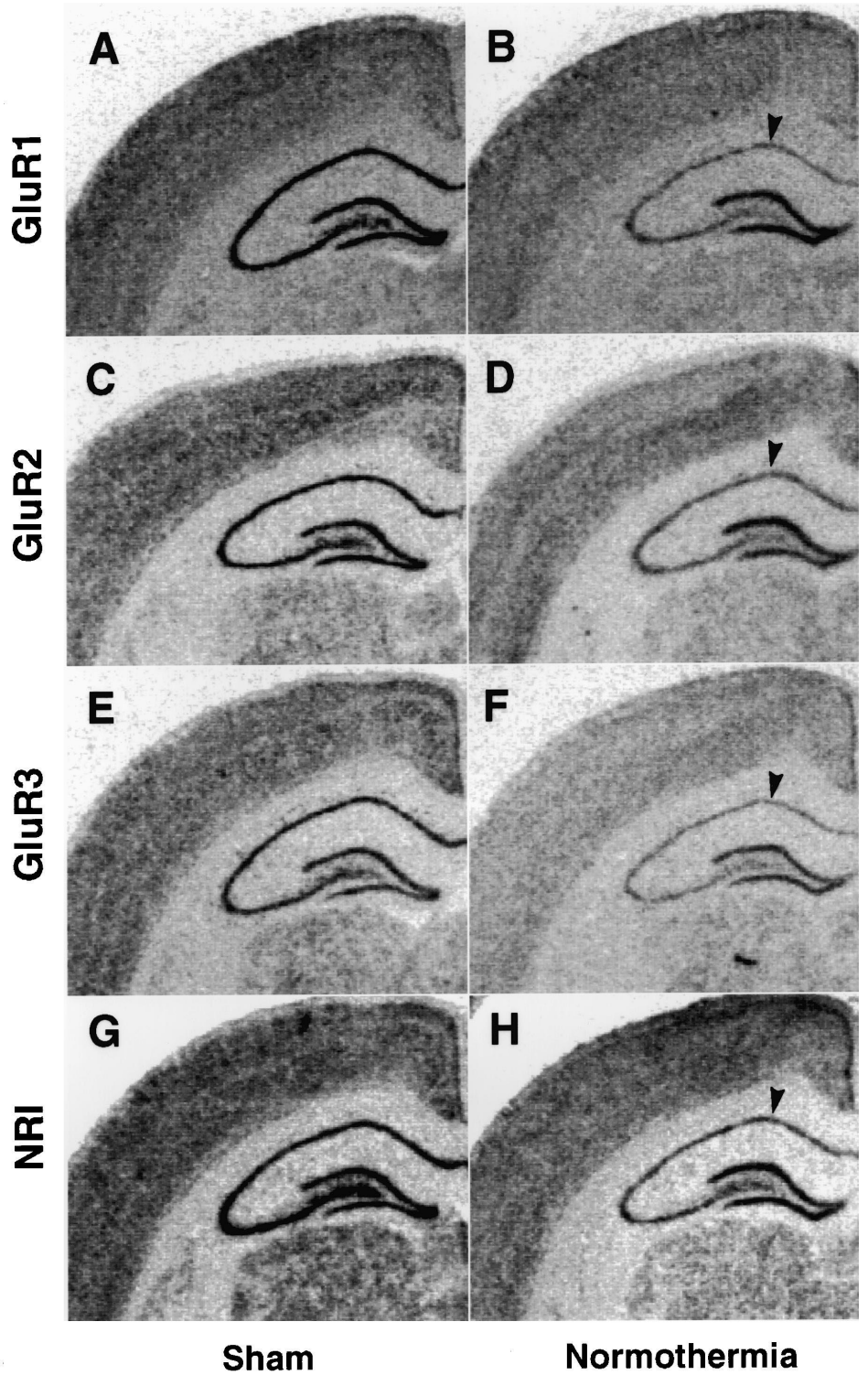

Fig. 2. Autoradiograms depicting the in situ hybridization of AMPA and NR1 receptor RNA probes to adjacent coronal sections from a sham-operated control (left panels) and after 1 day of normothermic experimental ischemia (right panels), at the level of the hippocampus. Normothermic global ischemia (37°C/37°C/1d) induced marked but non-selective decreases in GluR1(A) (A,B) GluR2(B) (C,D), and GluR3(C) (E,F) receptor mRNA expression in CA1–CA3 subfields (arrowhead). NR1 receptor mRNA was decreased significantly in CA1 but to a lesser degree (arrowhead) (G,H).

decreases in GluR1(A), GluR2(B), and GluR3(C) mRNAs in CA1 and CA3 subfields following 1 day of reperfusion, similar to the observations of Frank et al. [28]. NR1 mRNA expression was also decreased in CA1 (Fig. 2G,H), which is in corroboration of others [38]. Quantification of densitometric readings demonstrated that non-selective mRNA downregulations in CA1 were highly significant after 1 or 7 days of reperfusion (Table 1). At 1 day, GluR1(A), GluR2(B), and GluR3(C) mRNAs declined throughout the CA1 subregion to a similar degree (to 54.6±3.9%, 65.5±6.9%, and 50±8%, respectively, $n=5$, $P<0.0001$ vs. sham controls). In CA2 and CA3, pyramidal cells resistant to neurotoxicity, significant reductions in AMPA and NR1 mRNA levels were also observed (Fig. 2 and Table 1). At 7 days, NR1 mRNA levels of the CA1 subregion were sustained to approximately one-third of that observed in sham controls (to 33.9±7.2%), whereas in adjacent sections AMPA mRNAs were nearly absent (Fig. 4 and Table 1).

Decreases in GluR3(C) mRNA persisted in surviving regions of the hippocampus (i.e. CA2, CA3, and dentate gyrus), whereas expression levels of GluR1(A), GluR2(B) and NR1 mRNA at 7 days were not significantly reduced (Fig. 4 and Table 1). Within the resistant dentate gyrus, AMPA and NR1 mRNA levels were not significantly altered at either time point (1 or 7 days). Densitometry confirmed stable expression of all four glutamate mRNAs in this region at both time points. Although both granule cells of the dentate gyrus and CA2–3 pyramidal cells survived after global ischemia, CA2–3 layers underwent prolonged decreases in expressing several of the GluR mRNA subtypes under normothermic and certain hypothermic conditions, whereas the dentate gyrus was virtually unaffected by any treatment. This suggests that pyramidal cell mRNA synthesis was differentially affected by the ischemic episode compared with granule cells despite their common resistance to neurodegeneration after global ischemia.

3.4. AMPA and NR1 mRNAs after intraischemic hypothermia

Intraischemic hypothermia (30°C, followed by 37°C during recirculation), which is neuroprotective for approximately 2 months [24], was induced to determine whether possible formation of toxic assemblies of Ca^{2+}-permeable AMPA receptors within the hippocampus under normothermic conditions [43,60,62] could be prevented. At 1 and 7 days of recirculation, autoradiograms of intraischemic hypothermic rats showed no significant changes in the four

Table 1
Percentages of hippocampal AMPA and NR1 mRNAs at 1 and 7 days after global ischemia under controlled temperatures[a]

		CA1	CA2	CA3	CA4
Normothermic ischemia					
GluR1(A)	1 day	54.6±8.7[c]	64.2±17[b]	72.5±15[a]	98±2.9
	7 days	9.0±6.5[c]	71.4±26[a]	98.3±7	94.5±6.6
GluR2(B)	1 day	56.5±15[c]	62.5±13[c]	65.6±12[c]	99.2±2.3
	7 days	6.4±7.8[c]	86.2±20	87.4±9.5	88±7.4
GluR3(C)	1 day	50±5.4[c]	55.8±6[c]	64±5.2[c]	99.8±3.1
	7 days	6.9±2.1[c]	59.8±38[a]	88.6±9.6	101±6.5
NR1	1 day	78.3±17[a]	78.5±19[a]	77.7±9.4[a]	92±10.5
	7 days	33.9±15[c]	102±5	102±1.6	107±3.7
Intraischemic hypothermia					
GluR1(A)	1 day	90.7±5.3	95±7	98±4.4	97±2.5
	7 days	93.2±17	97.6±9.3	91.3±6.6	104 ±4.4
GluR2(B)	1 day	92±3.6	96.8±7	101±5.2	97.7±2.8
	7 days	105±4.4	100 ±10	96±13.1	103 ±7.5
GluR3(C)	1 day	96.8±6	99±6	107±6.6	104 ±4
	7 days	98.6±16	97.1±18.5	98.6±14	80.8±26
NR1	1 day	101±6.3	99±7	98.4±4	101±6
	7 days	103 ±8.1	97.5±10.7	101±9.6	97±9.4
Postischemic hypothermia					
GluR1(A)	1 day	42.7± 2.6[c]	72 ±13.3[a]	73.8±11[a]	103±2.3
	7 days	48.5±12	75.2±12[a]	74.4±14[a]	94.6±3.8
GluR2(B)	1 day	61±7[b]	80.3±4.4[b]	78.3±0.3[c]	104.3±4.5
	7 days	54±15[b]	69.5±12[b]	90±4.2	95.4±2.7
GluR3(C)	1 day	42±4.3[c]	44.4±3[c]	48±6.1[c]	91.3±9.2
	7 days	38.7± 20[c]	70.1±12[a]	75.9±13[a]	87.2±14
NR1	1 day	77±9.8[c]	91±12.7	95.6±6.8	101±6.7
	7 days	80.8±15	103±5.9	100.8±4.4	93.6±6.5

[a] Values are expressed as % control mRNA and represent the mean±S.E.M. of in situ hybridization autoradiograph densitometry measurements quantified from control and experimental hippocampal subfields. [a]$P<0.05$; [b]$P<0.001$; [c]$P<0.0001$, Two-way ANOVA with post hoc Fisher Protected Least Significant Difference test (PLSD) was carried out on O.D. raw data whereas Student's unpaired *t*-test was used to compare percentages.

glutamate transcripts relative to controls; non-selective downregulations in CA1 and CA3 subfields were fully blocked (Figs. 3–7 and Table 1). Similarly, emulsion-dipped sections showed in situ hybridization grains were abundant and uniform across the pyramidal cell layers after 1 day of recirculation (Fig. 7A,C) and 7 days (not illustrated). Therefore, we observed full preservation of glutamate receptor mRNA expression by the lowered body temperature. This persisted at 7 days, therefore, the effects of intraischemic hypothermia on gene expression and CA1 cell survival were long-lasting.

Emulsion-dipping of postischemic brains at either 1 or 7 days showed in situ hybridization grains of GluR1(A) and GluR2(B) mRNAs were downregulated within CA1–CA3 pyramidal layers to a similar degree, times when cyto-architechture was still intact (Fig. 6). Emulsion-dipping of ischemic brains with normothermia also demonstrated that GluR1(A) and GluR2(B) mRNAs expressed per CA1 neuron were equally downregulated, leaving the GluR1(A)/GluR2(B) ratio unaffected at the single cell level (Fig. 7B,D). This pattern differed from the patchy, non-uniform pattern of reduced GluR2(B) mRNA labeling of CA3 neurons reported by us after status epilepticus [33] or in striatum and cortical neurons after focal cerebral ischemia [37]. In the dentate gyrus, glutamate transcripts were not affected significantly at either time point under normothermic or hypothermic conditions.

3.5. AMPA and NR1 mRNAs after postischemic hypothermia

After postischemic hypothermia and 1 day of recirculation, autoradiograms could not be distinguished from those

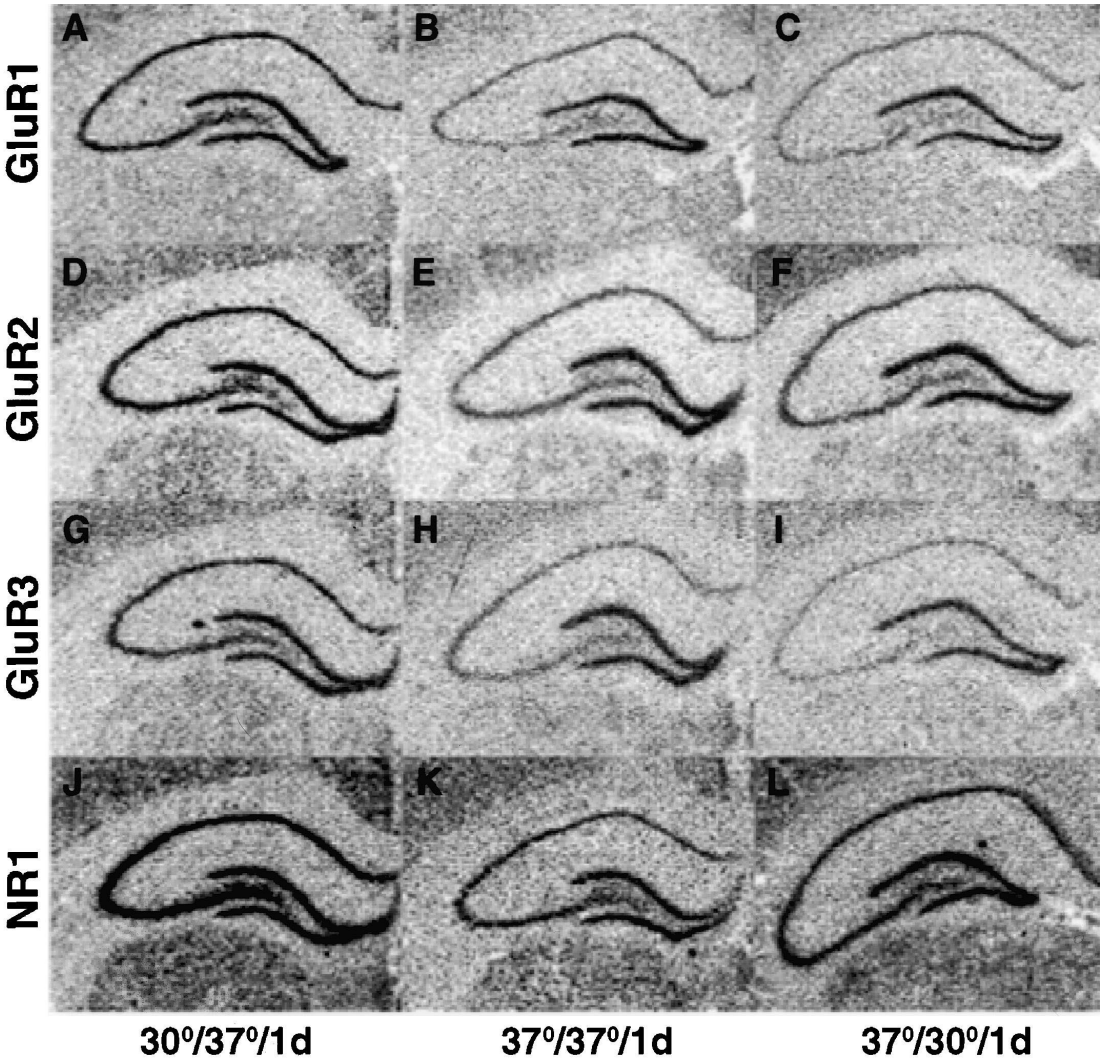

Fig. 3. Autoradiograms of AMPA and NR1 receptor RNA probes to adjacent coronal sections at the level of the hippocampus in rats with intraischemic hypothermia (30°C/37°C/7d) (left panels), normothermic ischemia (37°C/37°C/1d) (middle panels), and postischemic hypothermia (37°C/30°C/1d) (right panels). After 1 day of recirculation, intraischemic but not postischemic hypothermia fully prevented the non-selective loss of GluR1(A) (A–C) GluR2(B) (D–F), GluR3(C) (G–I) and NR1 (J–L) receptor mRNA expression in the vulnerable CA1 subfield.

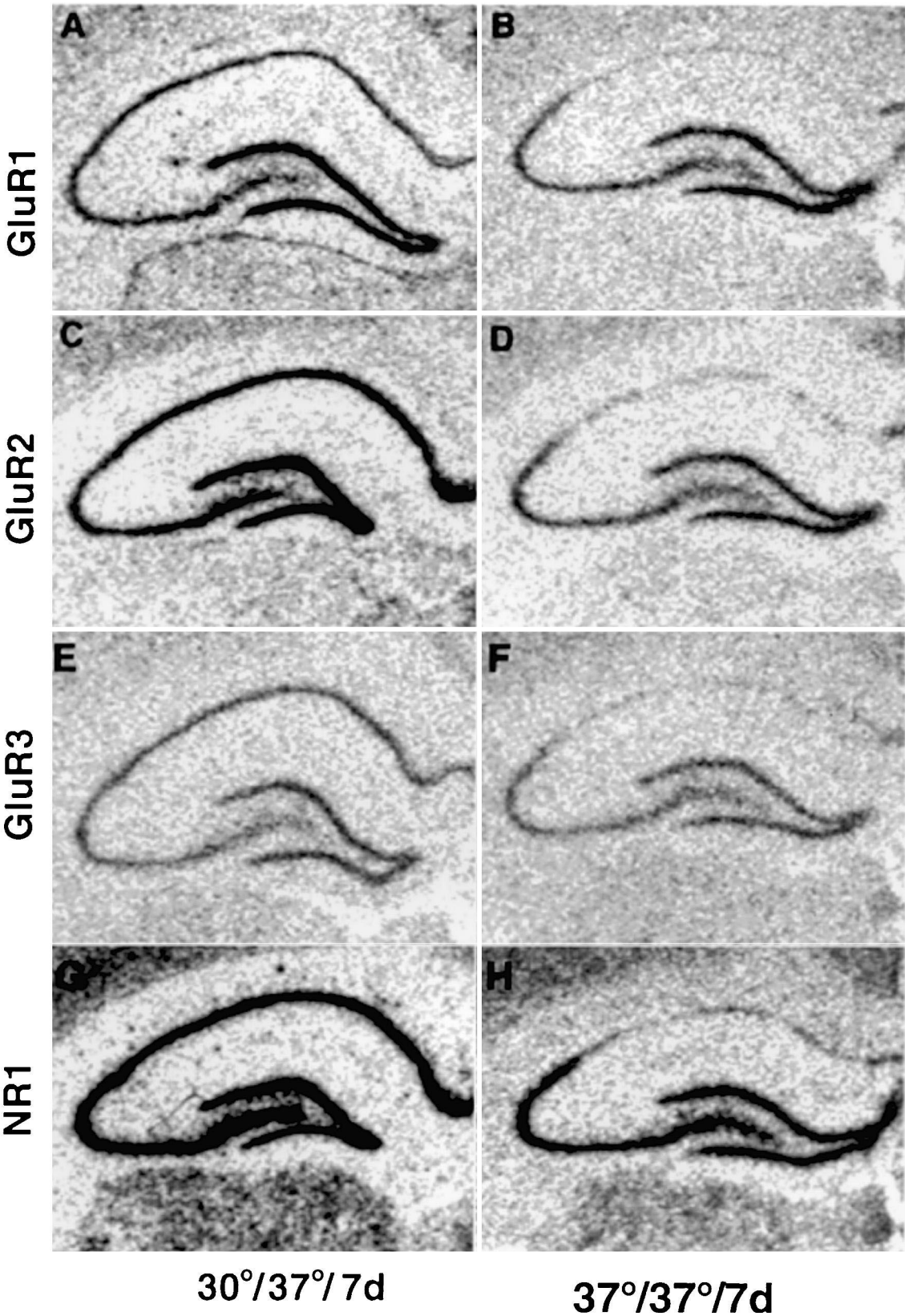

Fig. 4. Autoradiograms of AMPA and NR1 receptor RNA probes at the level of the hippocampus, 7 days after postischemic hypothermia (37°C/30°C/7d). After 7 days of recirculation, intraischemic hypothermia fully rescued the non-selective loss of GluR1(A) (A,B) GluR2(B) (C,D), GluR3(C) (E,F) and NR1 (G,H) receptor mRNA expression in the vulnerable CA1 subfield. GluR2(B) and GluR3(C) mRNAs were deceased in CA2 but not CA3 subfields at this time.

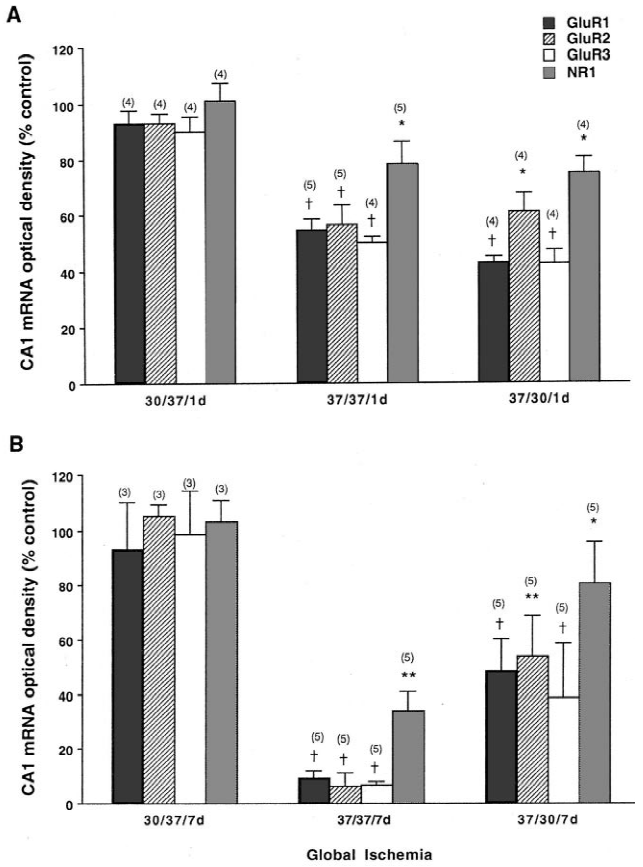

Fig. 5. Quantification of CA1 downregulation of glutamate receptor gene expression at 1 and 7 days after intraischemic hypothermia. Error bars represent the standard error of the mean of values from three to five animals. * $P<0.05$; ** $P<0.001$; $^{\dagger}P<0.0001$; Two-way ANOVA.

of the normothermic condition (Fig. 3). The decline in mRNA levels within CA1–CA3 subfields resembled the downregulation observed under normothermic conditions, particularly for GluR1(A) or GluR3(C) mRNAs. Therefore, protection by postischemic hypothermia was not afforded at the acute time point when the temperature was dropped just after arterial occlusion was terminated. (Fig. 3). At the later time point of 7 days, the uniform and non-selective reduction in hippocampal expression for AMPA and NR1 mRNAs also mimicked the pattern expressed under normothermic conditions at 1 day of reperfusion, despite the transient shift in the morphological window of cell survival and recovery period (Figs. 4 and 5, and Table 1). Since marked depletion of mRNA and CA1 cell loss occurred after 7 days under the normothermia condition, postischemic hypothermia attenuated the general loss of AMPA and NR1 mRNA expression during the recovery phase but not the acute phase of the ischemia. Densitometry showed that AMPA mRNAs were expressed to approximately 50% and NR1 mRNA to 75% of control values (Fig. 5 and Table 1). Postischemic levels at 7 days indicated that all four transcripts were partially recovered in a rank order of NR1>GluR2(B)>GluR1>GluR3 mRNAs (Figs. 4 and 5, and Table 1).

There were differences in mRNA expression across spared pyramidal regions of the hippocampus. In the CA2, a region highly resistant to neurodegeneration, GluR1(A) and GluR3(C) mRNAs were preferentially decreased not only at 1 or 7 days after normothermic ischemia but also after both survival times after postischemic hypothermia (Table 1). GluR2(B) mRNA was sustained at higher levels, but decreases were significant. Unexpected non-selective decreases in AMPA mRNAs were also observed in CA3/4 areas at both time points, but significant recovery occurred under normothermic but not postischemic conditions after 7 days of reperfusion (Table 1). At 7 days after postischemic hypothermia, NR1 and GluR2(B) mRNAs were expressed at their respective control levels, indicating good recovery of these mRNAs. However, deprivation of the GluR3(C) mRNA persisted, similar to levels observed at 24 h (Figs. 4 and 5, and Table 1).

4. Discussion

The present study examined whether intraischemic or postischemic hypothermia could afford neuroprotection by preventing downregulations of AMPA [28,43,60,62,63] or NR1 mRNA [38] expression that precedes CA1 cell loss following normothermic global ischemia. We found under normothermic and certain hypothermic conditions that GluR1(A), GluR2(B) and GluR3(C) mRNAs decline simultaneously at 1 day after recirculation — a time point preceding cell loss. This suggests that the decreases in AMPA mRNAs in CA1 are non-selective and, therefore, not causally related to the delayed pathology. A different conclusion was reached by Zukin and coworkers [43,60,62] (i.e. select downregulation of GluR2(B) and subsequent formation of toxic assemblies of Ca^{2+}-permeable AMPA receptors in CA1 causes cell death). The reasons for the discrepancy are not due to differences in the in situ hybridization conditions, which were similar. The ischemia model used could also not explain the differences reported herein, since Frank et al. compared AMPA mRNA expression levels in the 2-vessel-occlusion vs. 4-vessel-occlusion models and found similar non-selective decreases in AMPA mRNAs in both models [28].

Intraischemic hypothermia fully blocked the non-selective downregulations at 1 and 7 days, suggesting that gene regulation and protein synthesis recovered at the earlier reperfusion phase and that overshooting of protein synthesis [45] was restored to control levels by 7 days. Under our postischemic condition where neurodegeneration is postponed, non-selective decreases in AMPA mRNAs at 1 or 7 days were indistinguishable from the 24-h normothermic condition, further emphasizing that not only were

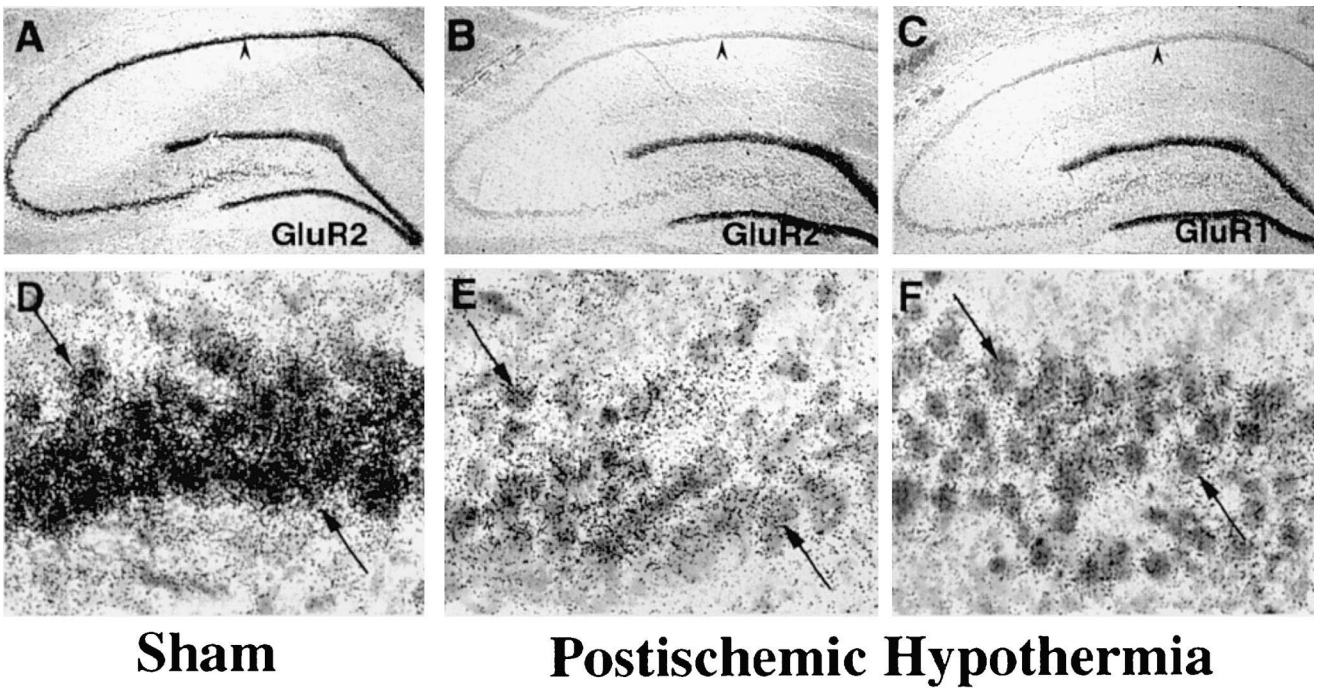

Fig. 6. Emulsion-dipped sections at low and high magnifications (arrow heads) following postischemic hypothermia at 7 days of recirculation. (A,D) Sham-controls and 7 days recirculation show rich and uniform expression of GluR2 mRNA throughout the hippocampus (arrows). Postischemic hypothermia and 7 days show in situ hybridization grains of GluR1(A) (C,F) and GluR2(B) (B,E) mRNAs were uniformly downregulated throughout the hippocampal pyramidal layers, a time when cytoarchitechture was intact (arrows).

downregulations of GluR1(A), GluR2(B) and GluR3(C) non-selective, but also that the pattern and extent of mRNA expression lost was highly reproducible under two separate conditions. Since postischemic hypothermia partially rescued the recovery phase and not the acute downregulation phase in a pattern that was nearly the same as that observed under normothermia at the early time point, suggests that temperature-dependent processes limit pre- or posttranslational mechanisms. There also appears to be a shift in the expression pattern that may correspond to the delayed window of neuronal vulnerability provided by postischemic temperature regulation. Non-selective decreases of AMPA but not NR1 mRNAs extended to CA2 and CA3/4 areas, suggesting that there are temperature-dependent processes that arise late in the recovery period in both vulnerable and resistant pyramidal cells and that they are under differential regulation.

There are still little data as to whether the severe loss of oxygen and substrate supply, and the depression of protein synthesis produced by ischemia models [6,71] allow for acute postischemic translation of glutamate receptors to occur. In the dentate gyrus, a region resistant to protein synthesis inhibition, immediate early genes are rapidly transcribed but postischemic translation of these genes in vulnerable CA1 and in CA3/4 subfields is not prominent until 24–48 h after reperfusion [45]. Kjoller and Deimer recently demonstrated that GluR2(B) protein synthesis is decreased in a non-selective manner in both vulnerable (CA1) and resistant regions (CA3/DG) at 24 h after global ischemia [49]. By 72 h, when neurodegeneration can first be detected under normothermic conditions, Gotlieb and Matute showed marked but similar decreases in GluR1(A) and GluR2(B)/3(C) immunoreactivities in CA1 [42]. In a different model of retinal ischemia after 48 h of reperfusion, AMPA and NR1 proteins are sustained or upregulated in the inner plexiform layer of the retina and not decreased in vulnerable cell populations (Clarke et al. [17] and in preparation). Together, a subsequent switch in subunit composition of AMPA receptors in CA1 is not supported by quantitative mRNA and protein studies in global ischemia models.

At 24 h, we have unpublished immunohistochemical observations of decreased immunoreactivity in CA1 with a polyclonal GluR2(B)/3(C) antibody (Chemicon) after normothermic ischemia but not with a more specific GluR2(4)(3A11) monoclonal antibody (donation of Dr J. Morrison, Mount Sinai Medical Center, NY) or a GluR1(A) antibody (Chemicon). The present study showed under both normothermia and postischemic hypothermia conditions that GluR3(C) mRNA was highly depleted throughout all pyramidal subfields to a greater extent than the other mRNAs tested under all conditions, which is also corroborated by two other reports of mRNA in global ischemia [28,60]. Therefore, it cannot be ruled out that the GluR3(C) subunit may be the AMPA-subtype that is predominantly depleted in CA1 early on and that this loss

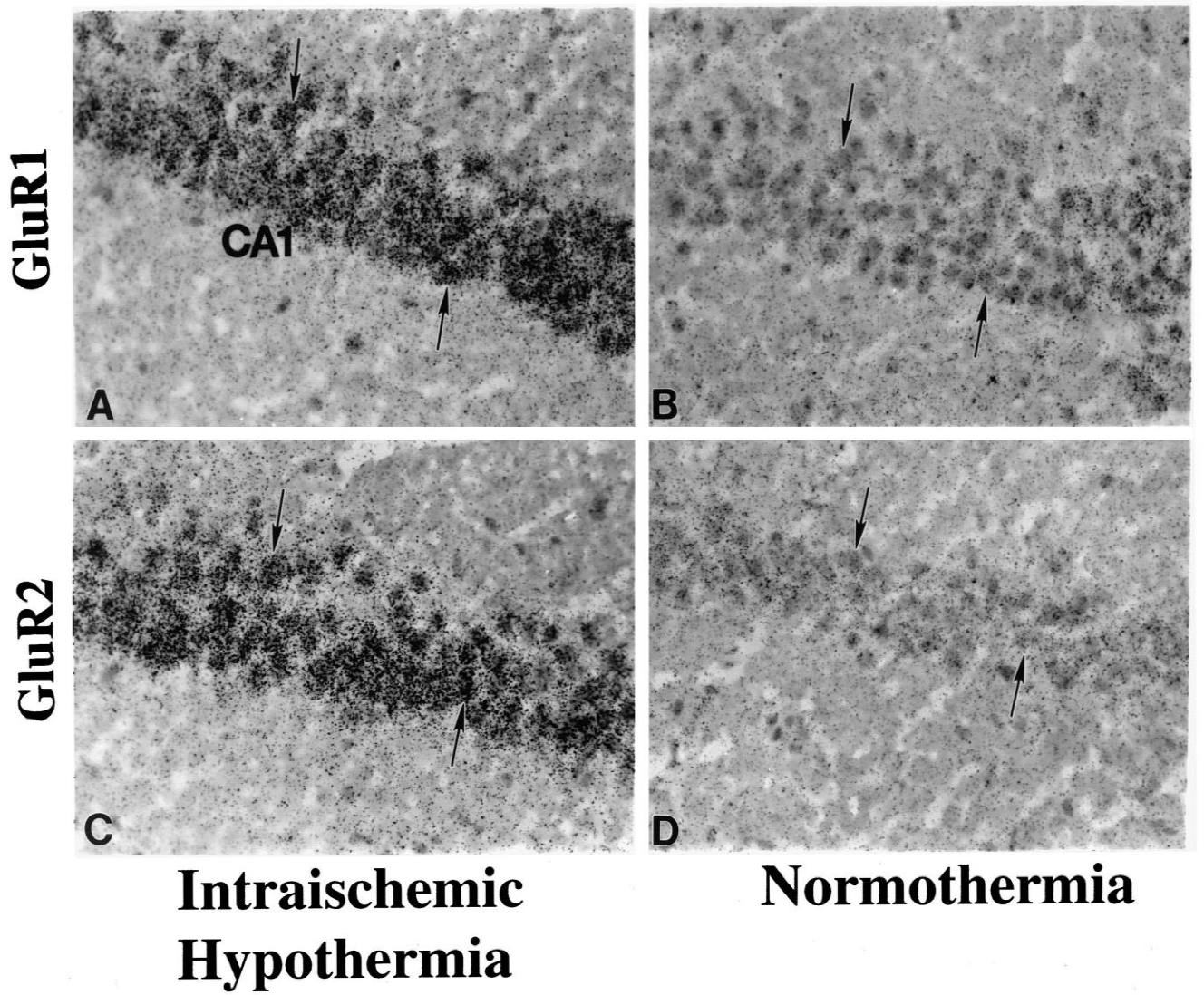

Fig. 7. Emulsion-dipped sections after intraischemic hypothermia at 24 h of recirculation resembled the postischemic groups at either time point examined. (A) GluR1(A) and (B) GluR2(B) mRNAs after intraischemic hypothermia exhibited sham-control levels in CA1 pyramidal cells. (C,D) Normothermic-ischemia sections were depleted of both mRNAs in a uniform pattern across the CA1 cell layers, a time when normal morphology is observed (arrows).

contributes to acute postischemic changes in membrane excitability.

It is not likely that increased formation of Ca^{2+}-permeable AMPA receptor assemblies within the hippocampus alone can precipitate cell death. Genetic studies show that a specific GluR2(B) gene mutant, with 30% less edited GluR2(B) and 2-fold increases in AMPA receptor-mediated Ca^{2+} influx, does not exhibit hippocampal pathology [47]. Therefore, certain elevations of Ca^{2+} influx through AMPA receptors must be achieved to elicit toxicity. Since the efficiency of GluR2(B) mRNA editing at the Q/R site in CA1 is unaltered after global ischemia or relative to CA3, selective rises in Ca^{2+} influx through AMPA receptors have been discredited as being responsible for postischemic neurodegeneration [46,58,65,77]. In contrast, GluR5 and GluR6 subunits may be considered as players in the molecular mechanisms underlying ischemic cell damage since they appear to be differentially regulated and editing of their Q/R site declines in CA1 [77]. The role of the GluR2(B) subunit in neurodegeneration is complicated since increases in the GluR1(A)/GluR2(B) ratio to favor Ca^{2+} permeability have also been implied in other models of hippocampal cell death, including natural aging [57] or recurrent limbic seizures [32,33,36]. For example, GluR2(B) antisense knockdown studies show that increases of the GluR1(A)/GluR2(B) ratio within a single hippocampus is sufficient to promote age-dependent epileptogenesis and premature neuronal vulnerability of the CA3 [34–36], akin to studies in GluR2(B) editing-deficient mutants [8,26]. However, these studies show loss of the GluR2(B) subunit alone is insufficient to kill neurons unless animals succumb to severe seizures.

As hippocampal downregulation of AMPA receptor expression appears to be non-selective after global ischemia in our hands, or decreases may not translate due to suppression of protein synthesis [6,71]. A possible explanation as to why non-NMDA antagonists are neuroprotective against normothermic ischemia damage may be a reduction in injurious ischemic depolarizations [1,52]. It is also possible that generalized loss of AMPA receptor expression observed in the present study may be an adaptive response to reduce fast excitatory neurotransmission and relieve Mg^{2+}-dependent block of NMDA receptors by decreasing postischemic glutamate release. This would occur under normothermia by reducing Na^{+} depolarizing current and presynaptic uptake of Ca^{2+} [78]. While it may be true that Ca^{2+} overload can stimulate certain intracellular cascades that may be neurotoxic, it is still not certain whether the amount of glutamate-evoked Ca^{2+} influx or the route of increased intracellular Ca^{2+} actually modulates neuronal injury [29–31,50,72]. Interestingly, dantrolene, an antispastic drug that prevents Ca^{2+} release from at least two pools of intracellular Ca^{2+} stores [27], can almost completely prevent delayed degeneration of CA1 and CA3 neurons after kainate-induced status epilepticus common to the Wistar rat strain; nimodipine, an L-type Ca^{2+} voltage-dependent blocker, did not afford neuroprotection, suggesting that excess release from intracellular stores may be required for development of delayed neurodegeneration [5].

In summary, the present study demonstrated that hippocampal AMPA receptor mRNAs decline at a relatively similar rate after normothermic global ischemia, leaving the GluR1(A)/GluR2(B) mRNA ratio unaffected. Hypothermia either prevented or attenuated the generalized downregulations. Therefore, neuroprotection by intraischemic hypothermia occurred independently of altered subunit composition of AMPA receptors. Since decreases persist within resistant neurons under the postischemic condition, AMPA receptor-mediated Ca^{2+} currents probably do not contribute to the postischemic pathophysiological sequelae within the CA1 region.

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