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Focused microwave irradiation of the brain preserves in vivo protein phosphorylation: comparison with other methods of sacrifice and analysis of multiple phosphoproteins

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Abstract

At any point in time, net protein phosphorylation represents the contribution of protein kinase and protein phosphatase activities affecting a specific site on a given substrate. Preservation of phosphorylated proteins in neural tissues has traditionally included flash-freezing or fresh tissue processing following tissue isolation. Rapid heat inactivation of protein kinases and phosphatases by focused microwave irradiation sacrifice represents another method to preserve, in vivo, brain protein phosphorylation state. In this study, we compared preservation of the phosphorylation state of a variety of phosphoproteins in the brain following sacrifice of mice by decapitation, decapitation into liquid nitrogen and focused microwave irradiation. We found that microwave irradiation generally provided the highest and most consistent levels of protein phosphorylation, regardless of the substrates examined in striatum and hippocampus. In general, flash-freezing resulted in the least preservation of phospho-state with ERK1/2 and CREB showing almost complete dephosphorylation. When regions of freshly decapitated brains were homogenized and incubated on ice for 30 min, ERK1/2 phosphorylation was completely lost, whereas it was well preserved in microwaved samples left at room temperature for 2 h. Loss of ERK1/2 phosphorylation in the fresh samples could not be attributed to substrate proteolysis. Our results indicate that focused microwave irradiation sacrifice may be required to achieve biologically relevant data for the in vivo protein phosphorylation state of many phosphoproteins.
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Keywords: Brain; Cell signaling; Microwave irradiation; Phosphoproteins; Protein phosphorylation

1. Introduction

Sacrificing rats or mice by focused microwave irradiation results in rapid heat inactivation of brain enzymes (Lenox et al., 1976; Stavinoha, 1993). This technique has been used to preserve in vivo levels of a variety of neurotransmitters (Butcher et al., 1976; Ishikawa et al., 1982; Katsura et al., 1992), second messengers, energy metabolites (Delaney and Geiger, 1996; Guattari, 1989) and neuropeptides (Angelucci et al., 2001; Mathe et al., 1990; Theodorsson et al., 1990) that otherwise would fluctuate widely following sacrifice by decapitation. Over the past few decades, microwave irradiator power outputs have increased and "fixation" times have decreased. Brain heating to above 80 °C can now be obtained in <1 s, resulting in more uniform levels of the neurochemical in question, compared to levels obtained with longer fixation times at lower power levels (Schneider et al., 1982). Although rapid freezing may in many instances achieve the same outcome (Veech et al., 1973), this approach either precludes (e.g. Veech et al., 1973) or serves as a physical barrier to subsequent brain dissection, whereas microwave fixation imparts resilience to brain tissue that facilitates brain dissection (O'Callaghan et al., 1983).

Protein phosphorylation represents the dominant mode of post-translational modification through which physiological

Abbreviations: ATF, activating transcription factor; BCA, bicinchoninic acid; CAMK, calcium/calmodulin-dependent kinase; CREB, cyclic AMP response element-binding protein; ERK, extracellular signal-regulated kinase; GFAP, glial fibrillary acidic protein; HRP, horse radish peroxidase; MAPK, mitogen activated protein kinase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; SAPK, stress-activated protein kinase; SDS, sodium dodecyl sulfate; STAT, signal transducer and activator of transcription

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Table 1
Phospho and non-phospho antibodies used for analysis of in vivo protein phosphorylation

Antibody	Phosphorylation site	Source	Vendor	Catalog #
Phospho-tyrosine		Mouse	CST, Beverly, MA	9411
Phospho-tyrosine		Mouse	BDT, San Diego, CA	P11230
Phospho-threonine/proline		Mouse	CST, Beverly, MA	9391
Phospho-serine/threonine		Rabbit	CST, Beverly, MA	9631
Phospho-ERK1/2	Thr202/Tyr204	Rabbit	CST, Beverly, MA	9101
Phospho-SAPK	Thr183/Tyr185	Rabbit	CST, Beverly, MA	9251
Phospho-p70S6K	Thr389	Rabbit	CST, Beverly, MA	9205
Phospho-CAMKII	Thr286	Rabbit	CST, Beverly, MA	3361
Phospho-CREB	Ser133	Rabbit	CST, Beverly, MA	9191
Phospho-ATF2	Thr71	Rabbit	CST, Beverly, MA	9221
Phospho-STAT3	Tyr705	Rabbit	CST, Beverly, MA	9131
ERK1/2		Rabbit	CST, Beverly, MA	9102
STAT3		Rabbit	SCB, Santa Cruz, CA	sc-483
GFAP		Mouse	ORP, Boston, MA	IF03L

CST: Cell Signaling Technology, Inc., Beverly, MA; BDT: BD Transduction Laboratories, San Diego, CA; SCB: Santa Cruz Biotechnology, Inc., Santa Cruz, CA; ORP: Oncogene Research Products, Boston, MA.

processes are mediated (Cohen, 2002; Greengard, 1978, 2001; Rubin and Rosen, 1975). This is achieved through protein kinase catalyzed transfer of phosphate to serine, threonine, or tyrosine residues of a given protein substrate. These protein- and residue-specific signaling events can now be studied using widely available phospho-state specific antibodies. Due to the rapidly reversible nature of protein phosphorylation, any given phosphorylation event often can be quite transient. At any point in time, therefore, net phosphorylation represents the contribution of protein kinase and protein phosphatase activities affecting a specific site on a given substrate. Thus, preserving phosphorylation state becomes a critical issue, especially when analyzed in vivo, because variations in postmortem activities of kinases and phosphatases are likely to affect net protein phosphorylation, unless these enzymes are rapidly inactivated. Indeed, this has been found to be the case for the few phosphorylated targets examined to date following microwave fixation (Gartner et al., 1998; Li et al., 2003). We hypothesized that high-energy focused microwave irradiation sacrifice would prove generally applicable for the preservation of in vivo phosphorylation state. The present investigation compares the phosphorylation state of several phosphoproteins following microwave fixation, rapid post decapitation freezing, decapitation followed by homogenization in hot sodium dodecyl sulfate (SDS) buffer and decapitation followed by homogenization and incubation in physiological buffers. These modes of sacrifice and post-sacrifice tissue preparation represent the range of available approaches to examine in vivo protein phosphorylation reactions. In aggregate, the results demonstrate considerable variations in phosphorylation levels from one substrate to another, but microwave fixation results in the highest and most consistent levels of phosphorylation, compared to the other two methods.

Table 2
Microwave irradiation does not alter brain protein content

Brain region	Total protein in fresh tissue (μg/μl)	Total protein in microwaved tissue (μg/μl)
Striatum	13.13 ± 3.23	12.34 ± 1.97
Hippocampus	13.54 ± 2.94	12.69 ± 1.70
Cortex	12.66 ± 2.40	13.03 ± 2.03

Mice were sacrificed by decapitation (fresh) or by focused microwave irradiation (microwave) and the brain regions (striatum, hippocampus and cortex) were dissected free-hand. The tissue samples from fresh and microwave sacrificed animals were homogenized in 10 volumes of 1% hot (85–95 °C) SDS. Total protein content in the samples was estimated by the bicinchoninic acid (BCA) method using bovine serum albumin as standard. The protein levels were calculated as μg total protein/μl of homogenate and are expressed as mean ± S.D.

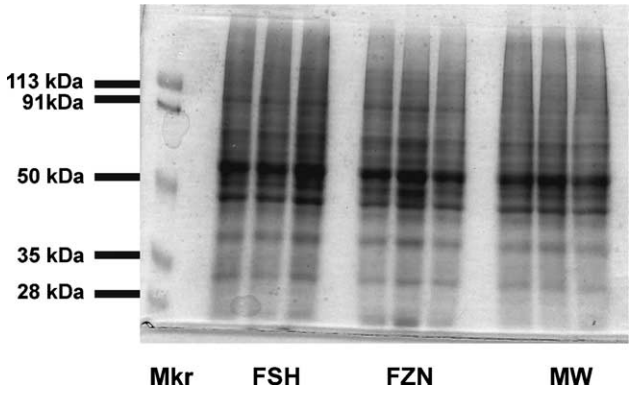

Fig. 1. Microwave irradiation does not alter the electrophoretic pattern or integrity of proteins in the mouse brain. Mice ($n = 3$ per group) were sacrificed by decapitation (fresh; FSH), decapitation into liquid nitrogen (frozen; FZN) or focused microwave irradiation (microwave; MW). The striatum was rapidly dissected and homogenized in hot 1% SDS. Total protein (20 μg) from the striatal homogenates was separated by SDS–PAGE and stained with Coomassie Brilliant Blue R-250. MKR: low range protein molecular weight marker (28–113 kDa).

2. Materials and methods

2.1. Animals

Female C57BL/6J mice, 8–12 weeks of age, were obtained from Jackson Laboratories (Bar Harbor, ME) and housed in a temperature (22 ± 2 °C) and humidity (30–40%) controlled colony room maintained on a 12 h light–dark schedule. Some mice received a single (12.5 mg/kg, s.c.) injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Aldrich Chemical Co., Milwaukee, WI) or vehicle (saline) alone. These mice were sacrificed at 12 h post dosing. All procedures were performed under protocols approved by the Institutional Animal Care and Use Committee of the Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health. The animal facility was accredited by the American Association for Accreditation of Laboratory Animal Care.

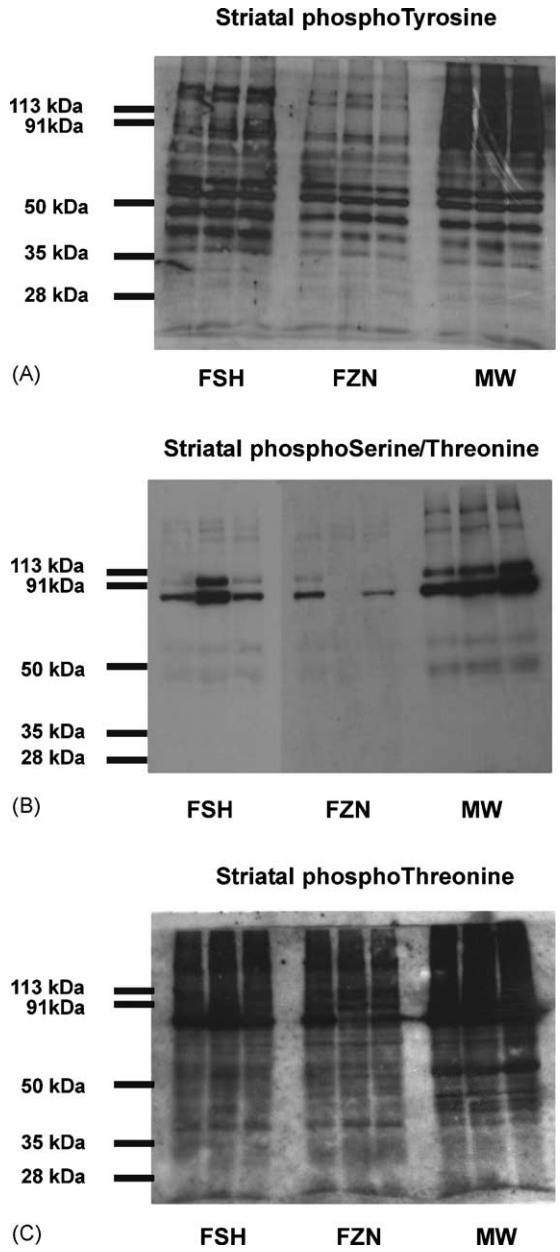

Fig. 2. Microwave irradiation preserves protein phosphorylation in the brain: analysis of striatal phosphoproteins. Mice ($n = 3$ per group) were sacrificed by decapitation (fresh; FSH), decapitation into liquid nitrogen (frozen; FZN) or focused microwave irradiation (microwave; MW). The striatum was rapidly dissected and homogenized in hot 1% SDS. An aliquot of the total protein (20 μg) was separated by SDS–PAGE, transferred onto nitrocellulose and immunoblots with antibodies to: (A) phospho-tyrosine; (B) phospho-serine/threonine; and (C) phospho-threonine were performed. Following appropriate secondary antibody incubation the signals were detected using an ECL chemiluminescent substrate.

2.2. Focused microwave irradiation

A Muromachi Microwave Applicator, Model TMW-4012C (10 kW output), was used to sacrifice unanesthetized mice by exposure of the head to the microwave beam. This irradiator is available through the Stoelting Company (Wood Dale, IL; http://www.stoeltingco.com)

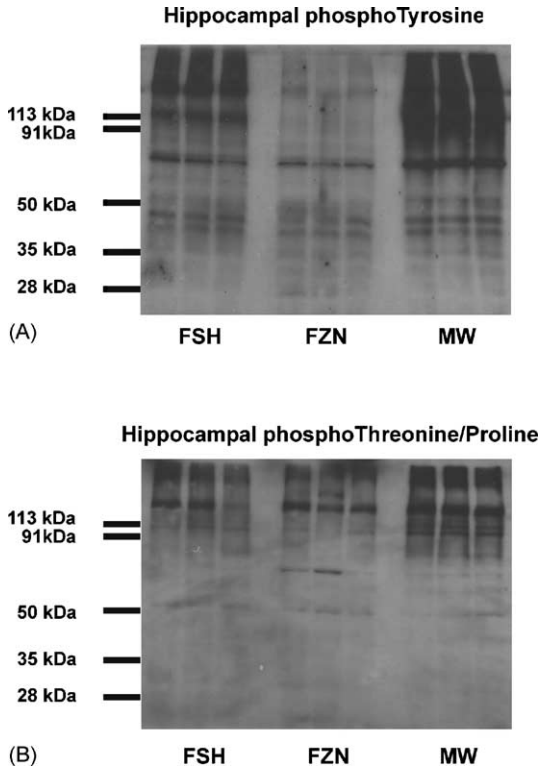

Fig. 3. Microwave irradiation preserves protein phosphorylation in the brain: analysis of hippocampal phosphoproteins. Mice ($n = 3$ per group) were sacrificed by decapitation (fresh; FSH), decapitation into liquid nitrogen (frozen; FZN) or focused microwave irradiation (microwave; MW). The hippocampus was rapidly dissected and homogenized in hot 1% SDS. An aliquot of the total protein (20 μg) was separated by SDS–PAGE, transferred onto nitrocellulose and immunoblots with antibodies to: (A) phospho-tyrosine and (B) phospho-threonine were performed. Following appropriate secondary antibody incubation the signals were detected using an ECL chemiluminescent substrate.

as Model 50047 or http://www.myNeuroLab.com as item # 470001. The results shown in this study for some of the phospho-mitogen activated protein kinase/extracellular signal-regulated kinase (pMAPK 44/42; pERK1/2) data can be achieved with far less powerful (3.5 kW output) irradiators no longer in production (see O'Callaghan et al., 1998), therefore, it is likely that the lower power unit available from Muromachi (5 kW output) will produce results identical to those reported here. In our unit, mice are briefly restrained in a water-jacketed holder prior to insertion into the TAW-174 Applicator Head, a procedure that takes approximately 5 s (with practice). The beam is then activated to sacrifice the mouse and fix the brain. A power setting of 4.0 kW (approximately 4 kW output) and an exposure time of 0.90 s was used. These irradiation conditions result in a core brain temperature of 90 °C as determined by insertion of a thermistor probe (Yellow Springs Instruments, Yellow Springs, OH) into the brain immediately after exposure to the microwave beam. The irradiator settings were chosen empirically with the intent of achieving the most rapid fixation time and the preservation of brain tissue throughout the rostral-caudal axis of the brain, i.e. without causing heating "pockets" while achieving fixation of all structures.

2.3. Tissue preparation

Following sacrifice by decapitation (hereafter referred to as fresh (FSH)), by decapitation following immediate immersion of the head in liquid nitrogen (referred to as frozen (FZN)) or by focused microwave irradiation (referred to as microwave (MW)), brains were removed from the skull and the regions of interest were dissected. In our hands (with practice), free-hand dissection of a region (striatum, hippocampus or cortex) can be achieved within 30–45 s of removing the brain. Brains frozen in liquid nitrogen are removed frozen from the skull, allowed to thaw to approximately 0 °C and then dissected. Microwaved mice must be cooled on ice or at room temperature for 5–10 min prior to dissection to prevent burning the fingers during removal of the brain. The dissected tissue from fresh, frozen or microwaved brains were homogenized by sonification in 10 volumes of hot (85–95 °C) 1% SDS and then stored at −80 °C until use. In addition, another set of striatal tissues were obtained from brains of mice sacrificed by decapitation (fresh) or microwave irradiation (microwave) and homogenized in cold (4 °C) neutral pH buffer (25 mM HEPES; pH 7.4). The fresh samples from this set were placed on ice for 30 min, while the microwaved samples were placed at

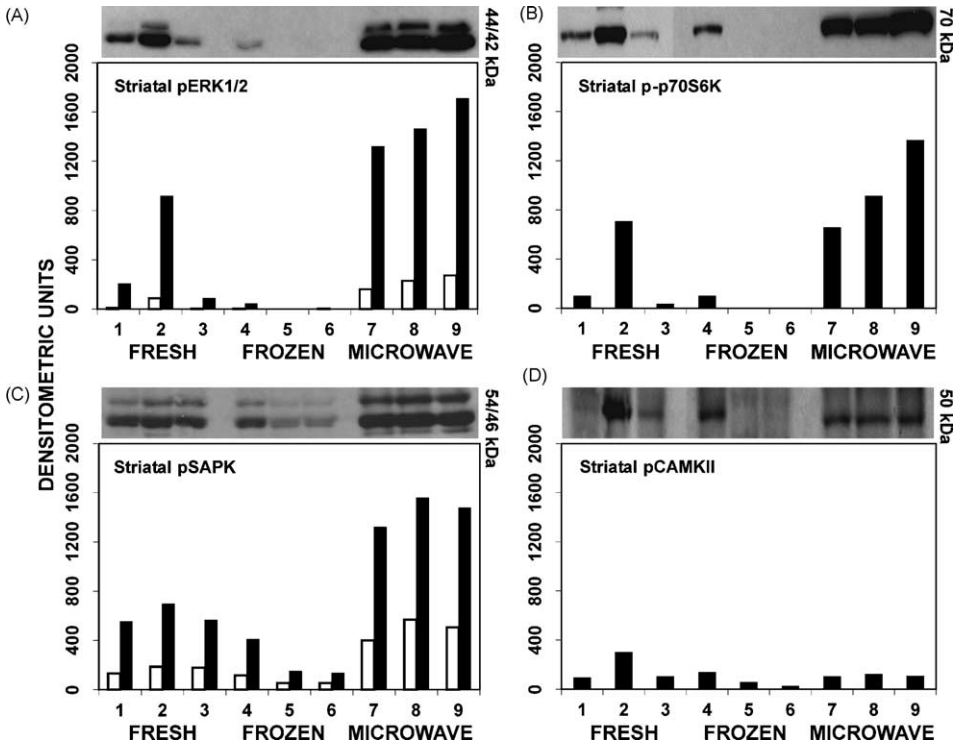

Fig. 4. Microwave irradiation preserves phosphorylation of protein kinases in the striatum. Mice ($n = 3$ per group) were sacrificed by decapitation (fresh), decapitation into liquid nitrogen (frozen) or focused microwave irradiation (microwave). The striatum was rapidly dissected and homogenized in hot 1% SDS. An aliquot of the total protein (20 μg) was separated by SDS–PAGE, transferred onto nitrocellulose and immunoblots with antibodies to: (A) phospho-ERK1/2 (Thr202/Tyr204), (□) pERK1 (44 kDa), (■) pERK2 (42 kDa); (B) phospho-p70S6K (Thr389); (C) phospho-SAPK (Thr183/Tyr185), (□) pSAPK (54 kDa), (■) pSAPK (46 kDa); and (D) phospho-CAMKII (Thr286) were performed. Following appropriate secondary antibody incubation the signals were detected using an ECL chemiluminescent substrate. The band intensities were semi-quantified by densitometric analysis and individual values are plotted.

room temperature for up to 2 h. The samples were subsequently re-homogenized in 10 volumes of hot 2% SDS (1% final concentration) and stored at −80 °C until use. Putamen samples from three human decedents enrolled in the Honolulu-Asia Aging Study had been processed in 1% SDS and stored at −80 °C for use in another study. Aliquots of total protein from these samples were used as positive controls for postmortem degradation of brain proteins. Total protein was estimated by the bicinchoninic acid (BCA) method using bovine serum albumin as standard (Smith et al., 1985).

2.4. SDS–PAGE and Immunoblot analysis

Aliquots of striatal or hippocampal homogenates (2–3 μg total protein from murine or human samples for immunoblot of glial fibrillary acidic protein, GFAP; 20 μg for other proteins) were diluted in sample buffer, boiled and loaded on either 7.5 or 10% sodium dodecyl sulfate–polyacrylamide (SDS–PAGE) gels (Laemmli, 1970). Proteins then were electrophoretically resolved and stained with Brilliant Blue R-250 (Coomassie stain) or transferred to 0.1 μm nitrocellulose membranes (Towbin et al., 1979). Following transfer, immunoblot analysis was performed using a variety of phospho-state-specific antibodies directed against a diverse array of phosphoproteins. All steps were carried out at room temperature. Briefly, membranes were blocked for 1 h in 5% non-fat dry milk prepared in phosphate buffered saline containing 0.1% Triton-X-100 (PBS-T), washed (1 × 15 min; 2 × 5 min) with PBS-T and incubated for 2 h with primary antibodies directed against phospho-serine/threonine, phospho-threonine or phospho-tyrosine, or the phospho (activated) form of the following protein kinases and transcription factors: mitogen-activated protein kinase 44/42 (MAPK p44/42; ERK1/2), p70 S6 kinase (p70S6K), stress-activated protein kinase (SAPK; JNK), calcium/calmodulin-dependent kinase (CAMKII), cyclic AMP response element-binding protein (CREB), activating transcription factor-2 (ATF-2) and signal transducer and activator of transcription 3 (STAT3). In addition, immunoblots using antibodies to non-phospho ERK1/2, STAT3, and GFAP were performed. A list of all the antibodies used in this study is tabulated in Table 1. All primary antibodies were used at a dilution of 1:500 except anti-phosphotyrosine which was used at a dilution of 1:1000. Following incubation with primary antibodies, blots were washed with PBS-T (1 × 15 min; 2 × 5 min) and subsequently were incubated with anti-rabbit IgG-HRP conjugate (1:2500) for 1 h. Followed by incubation in

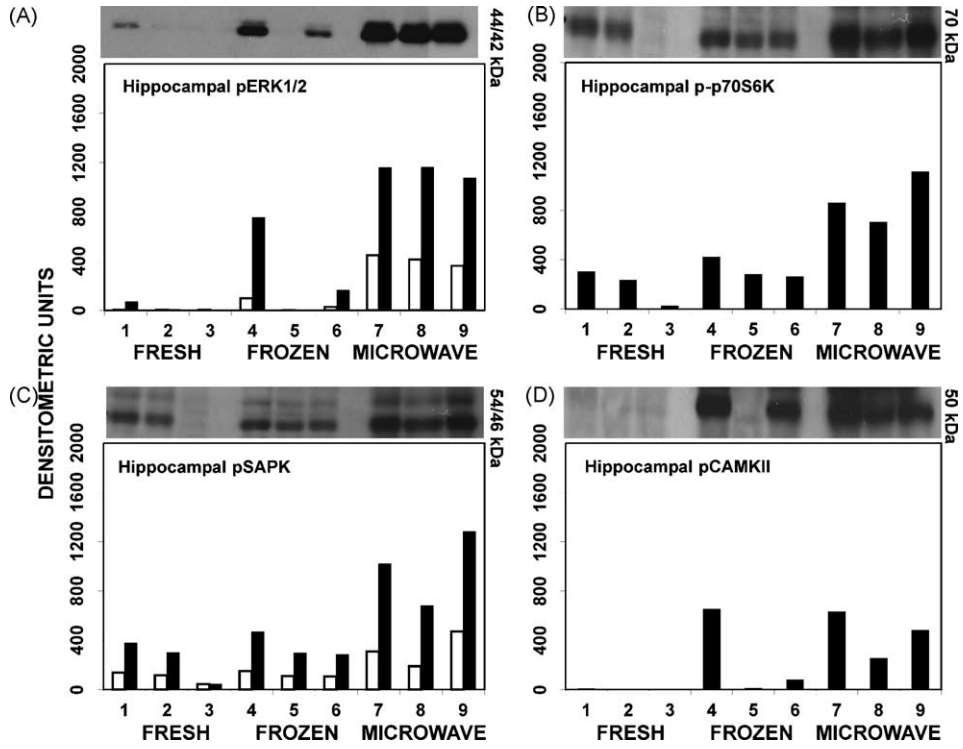

Fig. 5. Microwave irradiation preserves phosphorylation of protein kinases in the hippocampus. Mice ($n = 3$ per group) were sacrificed by decapitation (fresh), decapitation into liquid nitrogen (frozen) or focused microwave irradiation (microwave). The hippocampus was rapidly dissected and homogenized in hot 1% SDS. An aliquot of the total protein (20 μg) was separated by SDS–PAGE, transferred onto nitrocellulose and immunoblots with antibodies to: (A) phospho-ERK1/2 (Thr202/Tyr204), (□) pERK1 (44 kDa), (■) pERK2 (42 kDa); (B) phospho-p70S6K (Thr389); (C) phospho-SAPK (Thr183/Tyr185), (□) pSAPK (54 kDa), (■) pSAPK (46 kDa); and (D) phospho-CAMKII (Thr286) were performed. Following appropriate secondary antibody incubation the signals were detected using an ECL chemiluminescent substrate. The band intensities were semi-quantified by densitometric analysis and individual values are plotted.

appropriate secondary antibodies, membranes were washed (1 × 15 min; 4 × 5 min) in PBS-T and the signals detected using an ECL chemiluminescent substrate (Amersham Biosciences, Piscataway, NJ). Signals were captured on X-ray film (Fuji Medical Systems, Stamford, CT), typically by exposure for 10 s to 1 h depending on the signal intensity. An approximately linear relationship between the protein load and signal obtained has been previously established for GFAP and some of the phosphoproteins examined in this study (O'Callaghan et al., 1999).

3. Results

3.1. Focused microwave irradiation of the brain does not alter total protein content or resolution of total protein by SDS–PAGE

Conceivably, the rapid elevation in brain core temperature (to >80 °C in <1 s) and resulting fixation of proteins could alter the protein concentration of brain samples or their resolution by SDS–PAGE. To address this possibility, total protein content of striatal, hippocampal or cortical tissue homogenates (10% (w/v) in hot 1% SDS) prepared from fresh and microwaved samples was estimated by the BCA method (Smith et al., 1985). The total protein content did not vary significantly between fresh (non-microwaved) and microwaved samples from any of these brain regions (Table 2). When homogenates of striatum or hippocampus prepared from fresh, frozen or microwaved brains were subjected to SDS–PAGE, the protein profiles observed did not differ across the three preparations (Fig. 1). In general, gels stained with Coomassie Brilliant Blue R-250 showed qualitatively similar staining profiles for all conditions) no fixation-related degradation/accumulation pattern was observed in any of these samples (Fig. 1). Microwave irradiation did, however, have the tendency to leave a slight amount of stained material in the wells of the stacking gel (data not shown), observations suggestive of a heat-generated insoluble material that would not enter the gel. When present, such staining represented a very small percentage of the total staining of a given lane.

3.2. Focused microwave irradiation preserves brain protein phosphorylation

Having established that resolution of total protein homogenates of striatum and hippocampus by SDS–PAGE was apparently unaffected by microwave fixation, we subjected fresh, frozen and microwaved samples of these

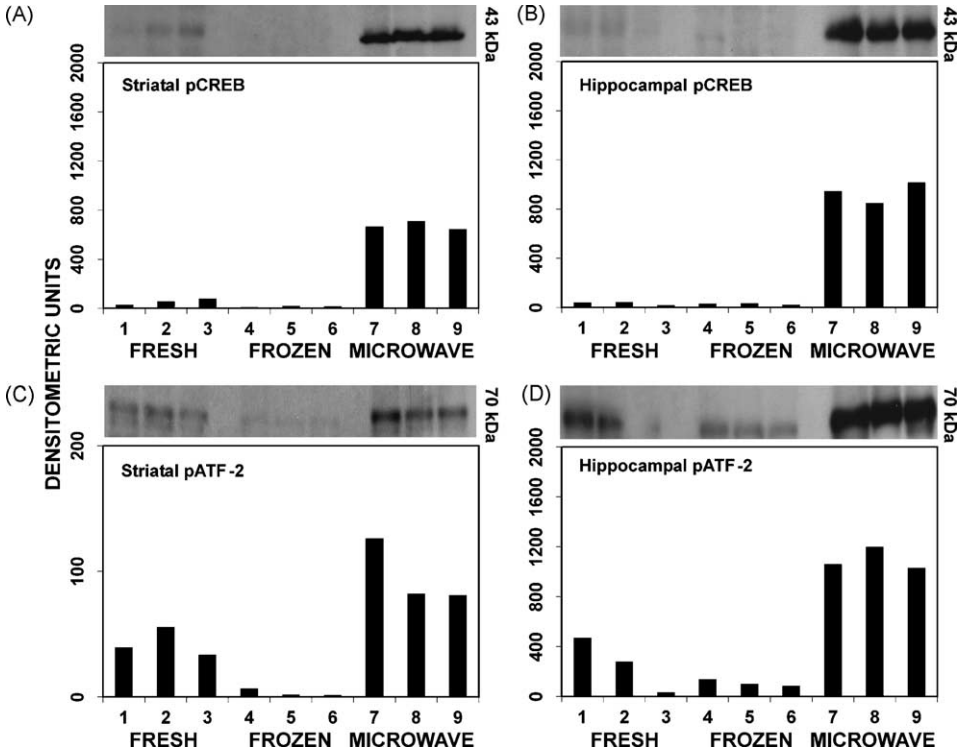

Fig. 6. Microwave irradiation preserves phosphorylation of transcription factors in various brain regions. Mice ($n = 3$ per group) were sacrificed by decapitation (fresh), decapitation into liquid nitrogen (frozen) or focused microwave irradiation (microwave). The striatum and hippocampus were rapidly dissected and homogenized in hot 1% SDS. An aliquot of the total protein (20 μg) was separated by SDS–PAGE, transferred onto nitrocellulose and immunoblots with antibodies to: (A) and (B) phospho-CREB (Ser133); and (C) and (D) phosphoATF-2 (Thr71) were performed. Following appropriate secondary antibody incubation the signals were detected using an ECL chemiluminescent substrate. The band intensities were semi-quantified by densitometric analysis and individual values are plotted.

brain regions to immunoblot analysis using antibodies to phospho-tyrosine, phospho-threonine, phospho-threonine/proline or phospho-serine/threonine. The results show that protein phosphorylation is generally better preserved in microwaved samples than in fresh or frozen tissue samples (Figs. 2 and 3). Overall, microwave fixation sacrifice also resulted in less animal-to-animal variability in comparison to the other modes of sacrifice. With respect to the effects of flash-freezing in liquid nitrogen (frozen samples), a decrease in phosphorylation was seen in nearly all immunoblots performed (Figs. 2 and 3), while rapid dissection and homogenization (fresh samples) preserved phosphorylation of certain proteins (Figs. 2 and 3).

3.3. Focused microwave irradiation preserves the phosphorylation state of protein kinases and transcription factors in various brain regions

Having established, in general, that microwave irradiation sacrifice results in the best preservation of brain protein phosphorylation state, we then examined specific phosphoproteins using phospho-state-specific antibodies directed toward the activated (phospho) form of the various kinases and transcription factors (Table 1) in homogenates prepared from fresh, frozen or microwaved samples. The general trend remained the same; phosphorylation of a given substrate was generally the highest and most consistent for microwaved samples prepared from homogenates of striatum (Fig. 4) or hippocampus (Fig. 5). The relative degree of preservation, however, varied markedly from one phosphoprotein to another among the fresh or frozen samples as well as by brain region examined. For example, in comparison to results obtained from microwaved samples, ERK1/2 phosphorylation was almost absent in frozen striatal (Fig. 4A) or in fresh hippocampal (Fig. 5A) homogenates. Moreover, in the case of CREB (Fig. 6), phosphorylation was almost abolished in either fresh or frozen samples from striatum or hippocampus; while microwave irradiation preserved the phospho-state exceptionally well in both of these brain regions (Fig. 6A and B). On the other hand, phosphorylation of the transcription factor STAT3, induced by treatment with the dopaminergic neurotoxicant MPTP (Sriram et al., 2002), was preserved in all samples, albeit to a lesser degree in the frozen samples in comparison to fresh or microwaved striatum (Fig. 7B). We note that STAT3 phosphorylation was undetectable in saline-treated animals (Fig. 7A), i.e., it was not constitutively phosphorylated, unlike the other proteins discussed above (Figs. 4–6). The fact that phosphorylation of STAT3 was at least partially retained after all methods of sacrifice suggests that preservation of protein phosphorylation may be highly substrate dependent. Semi-quantification of the band intensities by densitometric analysis suggested that possible differences exist in the amounts of phosphoprotein among various brain regions (Figs. 4–7). Such differences are expected owing to the regional and cell-type distribution of a given phosphoprotein (De Camilli et al., 1983).

3.4. Postmortem incubation of brain tissue results in substrate-dependent dephosphorylation that can be prevented by microwave irradiation sacrifice

Of the phosphoproteins examined, ERK1/2 appeared to be the most susceptible to postmortem dephosphorylation and STAT3 appeared to be the least affected. We further explored this question by incubating homogenates of freshly decapitated (fresh) tissue on ice for 30 min and homogenates of microwaved tissue on the bench for 2 h (Fig. 8). Phosphorylation of ERK1/2 was abolished by incubation for 30 min on ice, whereas only one of three microwaved samples appeared to be slightly decreased by 2 h of incubation on the bench (Fig. 8A). In contrast, phosphorylation of STAT3 appeared to be only slightly reduced by 30 min of incubation on ice and microwaved tissue was not affected by 2 h

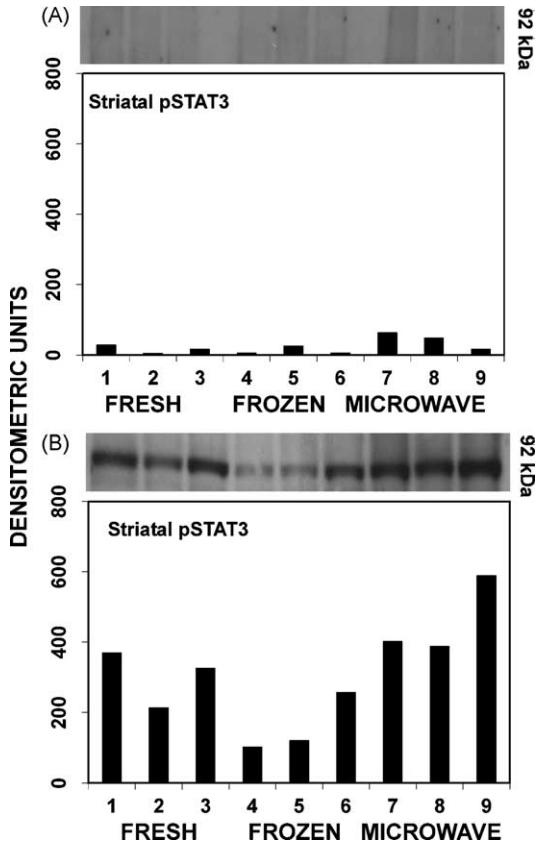

Fig. 7. Microwave irradiation preserves phosphorylation of the transcription factor STAT3 induced by the neurotoxicant MPTP. Mice ($n = 3$ per group) were administered either saline or MPTP (12.5 mg/kg, s.c.) and were sacrificed 12 h later by decapitation (fresh), decapitation into liquid nitrogen (frozen) or focused microwave irradiation (microwave). The striatum was rapidly dissected and homogenized in hot 1% SDS. An aliquot of the total protein (20 μg) from: (A) saline-treated; or (B) MPTP-treated animals was separated by SDS–PAGE, transferred onto nitrocellulose and immunoblots with phospho-STAT3 (Tyr705) were performed. Following appropriate secondary antibody incubation the signals were detected using an ECL chemiluminescent substrate. The band intensities were semi-quantified by densitometric analysis and individual values are plotted. Note that phosphorylation of pSTAT3 is undetectable in saline-treated animals.

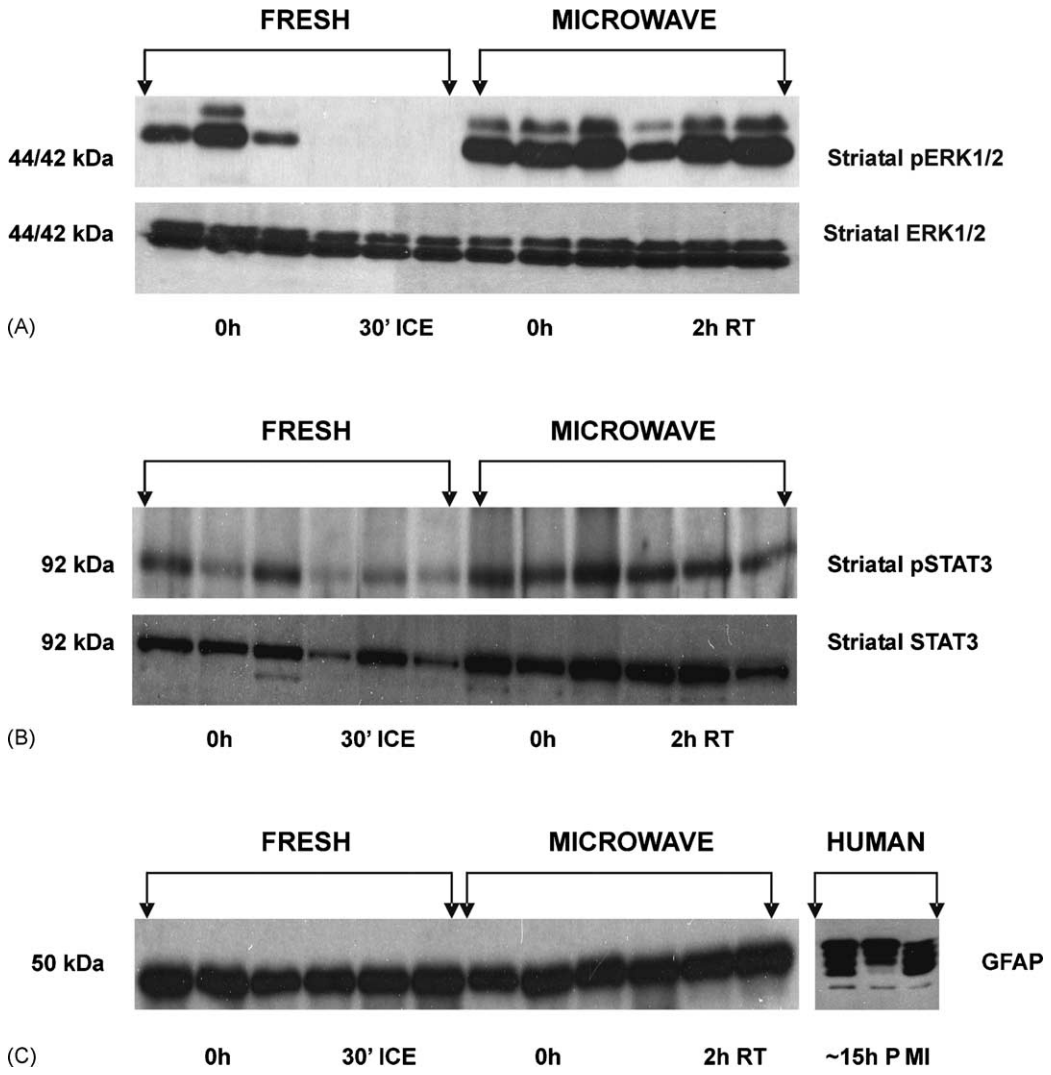

Fig. 8. Microwave irradiation sacrifice can prevent the loss of protein phosphorylation due to post-mortem delay. Mice ($n = 3$ per group) were administered either saline or MPTP (12.5 mg/kg, s.c.) and were sacrificed 12 h later by decapitation (fresh) or focused microwave irradiation (microwave). The left striatum and was rapidly dissected and homogenized in hot 1% SDS (0 h group). The right striatum from the fresh group was homogenized in HEPES buffer and placed on ice for 30 min while that from the microwave group was homogenized similarly, but left at room temperature for 2 h. Following appropriate incubations (30 min or 2 h) both sets of samples were re-homogenized in hot 2% SDS (1% final concentration). Tissues from saline-treated animals were processed in an identical manner. (A) Striatal total protein (20 μg) from saline-treated animals was separated by SDS–PAGE and immunoblots with phospho (Thr202/Tyr204) and non-phospho antibodies to ERK1/2 were performed. (B) Striatal total protein (20 μg) from MPTP-treated animals was separated by SDS–PAGE, transferred on to nitrocellulose and immunoblots with phospho (Tyr705) and non-phospho antibodies to STAT3 were performed. Note the loss of phosphorylated ERK1/2 and STAT3 in samples stored on ice for 30 min, compared to microwaved samples stored at room temperature for 2 h. The non-phosphorylated forms of these proteins did not significantly differ between various conditions. (C) Total protein from mouse striatum (3 μg) and human putamen (2 μg) homogenates were separated by SDS–PAGE, transferred to nitrocellulose and immunoblot with antibody to GFAP was performed. The signals were detected using an ECL chemiluminescent substrate. Note the degradation of GFAP in human post-mortem (~15 h) samples, which indicates post-mortem related proteolysis.

incubation at room temperature (Fig. 8B). Thus, susceptibility to postmortem changes in phosphorylation is highly substrate-dependent. The results observed for ERK1/2 are not likely the result of generalized proteolysis because, not only is total ERK1/2 not affected by postmortem incubation for up to 30 min (Fig. 8A) but, GFAP, the proteolysis susceptible intermediate filament protein of astrocytes, was not affected by incubation up to 30 min on ice (Fig. 8C). These latter results stand in contrast to the proteolysis of GFAP observed after a greater than 15 h postmortem interval prior to preservation of a sample of human brain tissue (Fig. 8C).

4. Discussion

We have demonstrated that high-energy focused microwave irradiation sacrifice of mice preserves in vivo brain

protein phosphorylation of serine, threonine and tyrosine residues, in general, and a variety of kinases and transcription factors, in particular. The degree of preservation of phosphorylation was highly dependent on the substrate examined. Regardless of the substrate analyzed, however, microwave fixation generally resulted in the highest and most consistent level of phosphorylation in comparison to results obtained from brain tissue prepared from mice sacrificed by decapitation or from brains that had been flash frozen in liquid nitrogen. Our results suggest that preservation of in vivo phosphorylation state by microwave irradiation may be required to achieve biologically meaningful results for many brain phosphoproteins. This conclusion is in agreement with other data obtained for a limited number of individual phosphoproteins (Hebert and O'Callaghan, 2000; Hossain et al., 1994; Jope et al., 1991; Mobley and Gonzalez, 1991; O'Callaghan et al., 1998; Snyder et al., 2000).

Both the variability and reduced level of phosphorylation observed for proteins examined in brain homogenates prepared from mice sacrificed by decapitation suggest that this mode of sacrifice will not prove suitable for most phosphoprotein analyses. Moreover, because we made every attempt to reduce the time interval between sacrifice and homogenization of brain tissue in denaturing detergent, it is highly unlikely that our results obtained with "fresh" tissue can be improved upon. Our data for proteins analyzed after flash-freezing brains in liquid nitrogen were even more disappointing, primarily due to the low level or the absence of phosphorylation compared to results obtained with the other modes of sacrifice. When we thawed these brains to 0 °C to dissect the two areas examined (striatum and hippocampus), it could be argued that this step in the analyses led to the observed dephosphorylation. Given the marked regional distribution of any given phosphoprotein, dissection of the brain would seem to be a necessity. We took all due caution not to elevate brain temperature above 0 °C during the dissection procedure, but the possibility exists that the temperature of the tissue was elevated enough to cause a postmortem dephosphorylation. Protein phosphorylation also can be analyzed, in vivo, with phospho-state-specific antibodies using an immunohistochemical approach (e.g. Ferrer et al., 2003). In such studies, it is assumed that the perfusion fixation preserves the steady state levels of protein phosphorylation. Given the rapid postmortem dephosphorylation we observed for some phosphoproteins in fresh or fresh frozen brains (e.g. ERK1/2 and CREB), it seems likely that the immunohistochemical approach also may be vulnerable to changes in phosphorylation during the postmortem, pre-fixation interval.

In summary, our data suggest that brain protein phosphorylation state, in vivo, can be rapidly fixed by high-energy focused microwave irradiation sacrifice and that phosphorylation state of proteins prepared from fresh or fresh frozen brains often is not well preserved.

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