

# Post-mortem Interval Effects on the Phosphorylation of Signaling Proteins

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Post-mortem brain tissue provides a unique opportunity to uncover the genes or proteins involved in the pathophysiology of neuropsychiatric disorders. Protein phosphorylation is a common protein modification within intracellular signaling pathways that affects the distribution and function of protein, and has been hypothesized to be of major importance in both the pathophysiology and treatment of major neuropsychiatric disorders. Thus, we were interested in ascertaining the stability of the phosphorylated forms of proteins that are involved in cellular signaling. Antibodies against phospho-tyrosine, phospho-threonine, and phospho-PKA substrates were used to examine the PMI effects on the general amounts of proteins in their phosphorylated form. Phospho-specific antibodies for ERK, JNK, RSK, CREB, and ATF-2 were used to test the effects of PMI on specific proteins whose functioning are known to be regulated markedly by phosphorylation. We found that PMI rapidly decreased the levels of proteins in their phosphorylated states and also decreased the total levels of certain proteins. The PMI effects were observed in the samples stored at both 4°C and room temperature, in both frontal cortex and hippocampus. Thus, it appears that measurements (such as two-dimensional gel electrophoresis and functional assays) that rely on the phosphorylation state of proteins would be extremely sensitive to PMI.

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## INTRODUCTION

Post-mortem brain tissue is increasingly being utilized to study the molecular and cellular pathophysiology of neuropsychiatric disorders. Analysis of post-mortem brains has proven invaluable in helping to uncover the neuropathology of numerous diseases, including Alzheimer's, Huntington's, and Parkinson's disease; indeed, these studies have facilitated the elucidation of the central pathophysiology of these illnesses (Greenfield *et al*, 1997). While these diseases show anatomical changes that are identifiable by gross post-mortem brain examination, there may exist more subtle cellular and molecular changes in the brains of patients with other neuropsychiatric disorders, including bipolar disorder, unipolar depression, and schizophrenia (Gould and Manji, 2002; Harrison, 1996, 1999, 2002; Knable and Webster, 2001; Rajkowska, 2002; Vawter *et al*, 2000). Thus, post-mortem brain specimens represent an invaluable

resource to investigate the pathophysiology of these, and numerous other, neuropsychiatric illnesses.

There are many confounding factors that effect the outcome of post-mortem measurements including the post-mortem interval (PMI), cerebral hemisphere selected, method of sampling and storing tissue, anatomical precision during dissection, histological processing technique, age, gender, general health preceding death (agonal state, which is generally related to brain pH), neurological and psychiatric state, medication, time of day of death (circadian state), and date of death (seasonal state) (Lewis, 2002; Perry and Perry, 1983; Ravid *et al*, 1992).

The PMI is the period from death to freezing of brain specimens for long-term storage at –80°C (Harrison and Kleinman, 2000), and may be one of the more important variables (Dodd *et al*, 1988). Most protocols and brain banks that supply post-mortem brains for analysis attempt to keep the PMI as short, and as uniform, as possible. Unfortunately, given the difficulty in collecting post-mortem brain samples, it is often impossible to avoid long and often variable PMIs. The problem with PMIs may be more pronounced in the study of illnesses like bipolar disorder, depression, and schizophrenia, in which individuals generally die 'unexpectedly', and outside of a well-monitored long-term care facility, thus resulting in a relatively long PMI. Furthermore, it is often difficult to

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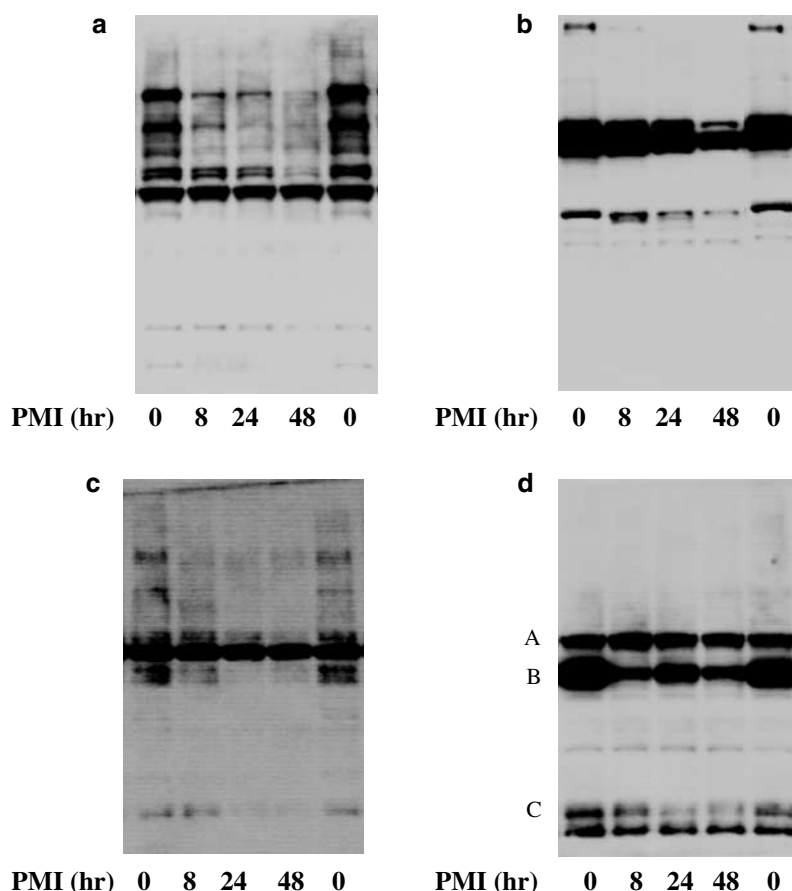
ascertain exactly when expiration occurred, leading to inaccuracies in PMI estimates.

The phosphorylation of cellular signaling proteins has been implicated in the pathophysiology of numerous neuropsychiatric diseases (Gould *et al*, 2002; Jope, 1999; Konradi and Heckers, 2001; Lenox and Hahn, 2000; Maccioni *et al*, 2001). Indeed, the phosphorylated form of a protein is clearly of major biological interest; it often affects the function and/or subcellular distribution of enzymes, receptors, neurotransmitter transporters, ion channels, and transcription factors. Thus, the ability to measure phosphorylated proteins in their native state could facilitate post-mortem investigations of neuropsychiatric disorders. Phosphorylation also effects the migration of proteins during two-dimensional gel electrophoresis (Sickmann and Meyer, 2001), a technique that may be useful for analyzing disease-specific changes in post-mortem brain specimens (Edgar *et al*, 1999; Fountoulakis *et al*, 2001; Johnston-Wilson *et al*, 2000). For these reasons, we investigated the effects of PMI on protein phosphorylation state in mouse brain tissue, where we can control exactly the PMI and are assured minimal genetic/tissue heterogeneity by utilizing inbred strains. We chose proteins based upon their putative involvement in the pathophysiology and

treatment of mood disorders (Gould *et al*, 2002), and their specificity for different phospho-amino acids (see Materials and methods section). We found that PMI produced profound and relatively rapid effects on the phosphorylation of proteins involved in intracellular signaling.

## MATERIALS AND METHODS

Male C57/BL6 mice 6–7 weeks of age from Harlan (~20 g) were housed 4–5 per cage in a 12 h light/dark cycle with free access to water and food. All mice were killed by decapitation between 9 and 10 am. Heads of animals were covered or placed in a small plastic bag, and stored at either room temperature or at 4°C. Frontal cortex and hippocampus were dissected on ice immediately, 4, 8, 14, 24, 36, 48 h (or 8, 24, and 48 for the room temperature storage) following decapitation. Immediately after dissection, brain specimens were rapidly frozen in dry ice and stored at –80°C until further analyses. The frontal cortex and hippocampus samples were homogenized by sonication in a protein extraction buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerophosphate, protease inhibitor cocktail

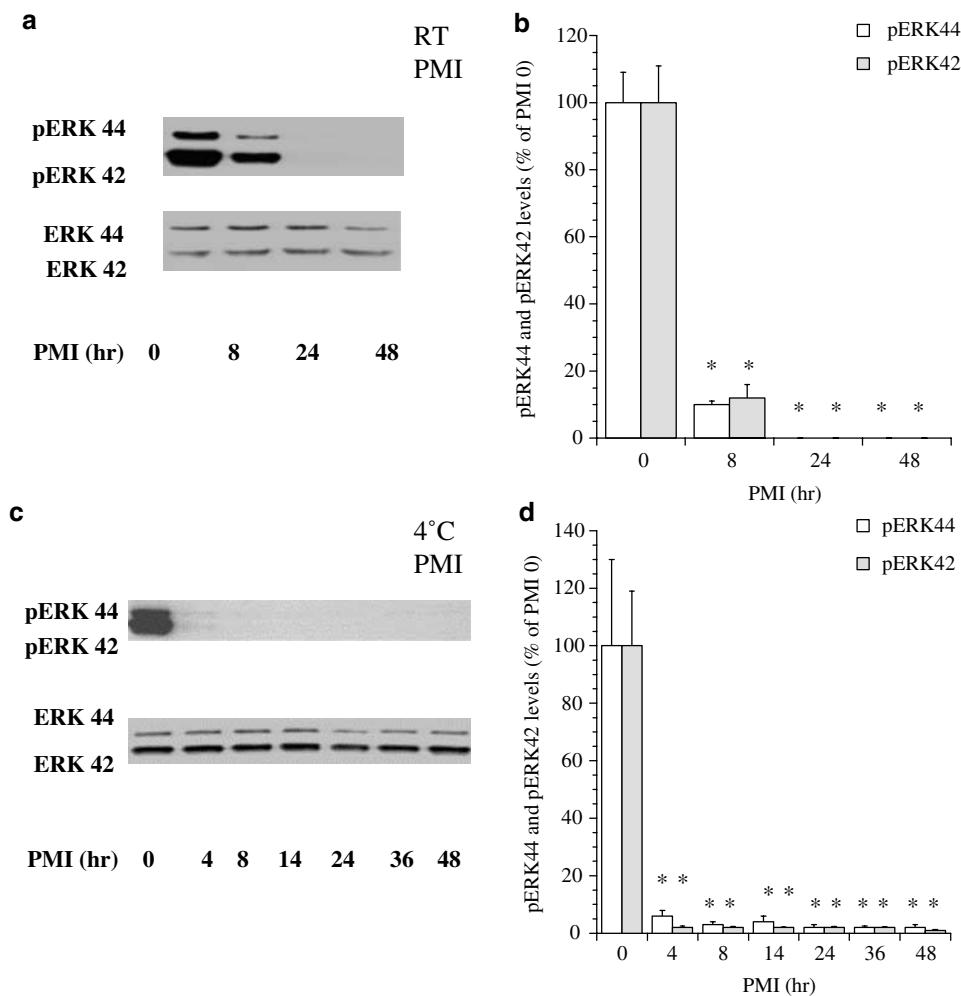


**Figure 1** PMI decreases phosphoprotein levels in the mouse frontal cortex. 40  $\mu$ g of protein was loaded on 10% gel and the immunoblot was stained with an antibody that recognizes (a) phospho-tyrosine, (b) phospho-threonine, (c) phospho-PKA substrate, and (d) acetylated lysine in a fashion largely independent of the surrounding amino-acid sequence. Similar results are also obtained from two additional groups of mice. Similar results were obtained from mouse hippocampus samples. The acetylated lysine antibody identifies three primary bands (d). Among these number bands, an increasing PMI reduced levels of band C, and altered the levels of band B in a tri-phasic manner.

(SIGMA), and phosphatase inhibitor cocktail I and II (SIGMA). The homogenates were then centrifuged at 14000g for 10s to remove undissolved debris. Protein concentrations were determined using the Bio-Rad protein assay kit (Bradford, 1976). The linearity of the protein concentration for immunoblotting for each protein was ascertained by resolution of selected concentrations of protein.

Subsequent protein immunoblotting was performed using previously described methods (Yuan *et al*, 2001). In brief, samples were subjected to SDS-PAGE. The amount of protein loaded was 40 µg per lane, except for p-ERK, ERK, Bcl-2, and Bcl-x, for which 10 µg was loaded. Proteins thus resolved were then electrophoretically transferred to nitrocellulose membranes. Nonspecific binding on the nitrocellulose was blocked with 5% nonfat dry milk/TBST, and then incubated with a primary antibody. Primary antibodies were either from Cell Signaling Technology (p-PKA substrate (catalog 9621), p-tyrosine (9411), p-threonine (9381), acetylated lysine (9681), p-CREB (9191; site Ser 133), CREB (9192), p-ATF2 (9221; Thr 71),

ATF2 (9222), ERK1/2 (9102), JNK1/2 (9252), c-Jun (9162)) or Santa Cruz Biotechnology, Inc. (p-ERK1/2 (SC-7383; Tyr 204), p-JNK1/2 (SC-6254; Thr 183/Tyr 185), Bcl-2 (SC-492)). The antibodies for immunoblots were diluted either 1:1000 (for Cell Signaling Technology) or 1:200 (Santa Cruz Biotechnology, Inc.). After blotting with secondary antibody (anti-rabbit IgG, Cell Signaling Technology, 1:2000; anti-mouse IgG, Amersham Biosciences, 1:3000), the immunocomplex was detected with an ECL or ECL plus kit (Amersham Biosciences). Quantitation of the immunoblots was performed by densitometric scanning of the film using an Image Analysis System (with NIH 1.55). An aliquot of pooled 'standard' appropriate rat brain was run on one lane of each gel. Data were normalized against the 'standard' to minimize between blot variability. Statistical analysis was performed by analysis of variance, followed by Fisher's PLSD.  $P < 0.05$  was considered significant. Data are expressed as the means  $\pm$  SE. Reported statistical significance is between 0 h PMI and the PMI of interest.

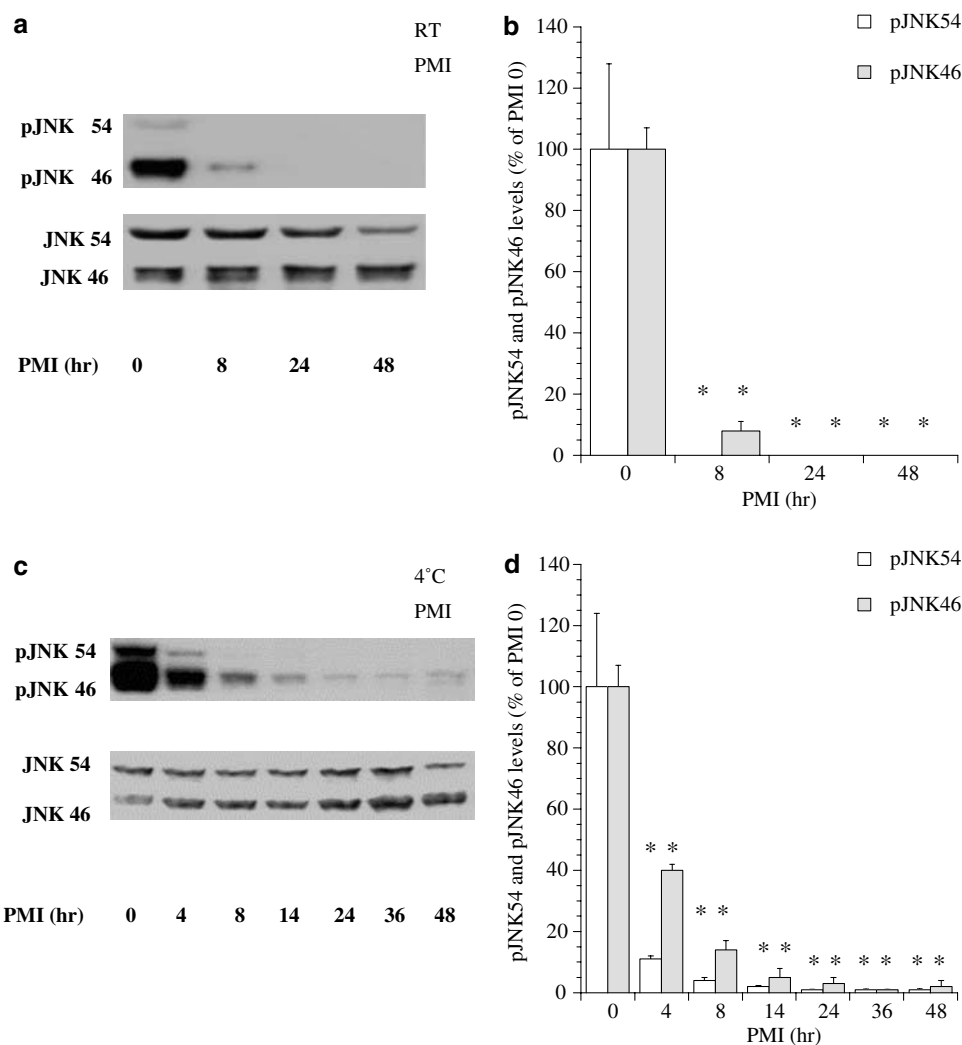


**Figure 2** PMI decreases the phosphorylated forms of ERK (42 and 44 kDa), but does not change the total protein levels. During the PMI, brains were stored at either room temperature (a, b) or 4°C (c, d). The graph represents the mean densitometry reading  $\pm$  SE from a total of three (RT) or four (4°C) mice (\* $P < 0.05$  Fisher's PLSD). p-ERK 44 RT ( $F_{3,8} = 11.2$ ;  $P < 0.0001$ ), 4°C ( $F_{6,21} = 10.6$ ;  $P < 0.0001$ ); p-ERK 42 RT ( $F_{3,8} = 66.1$ ;  $P < 0.0001$ ), 4°C ( $F_{6,21} = 27.2$ ;  $P < 0.0001$ ).

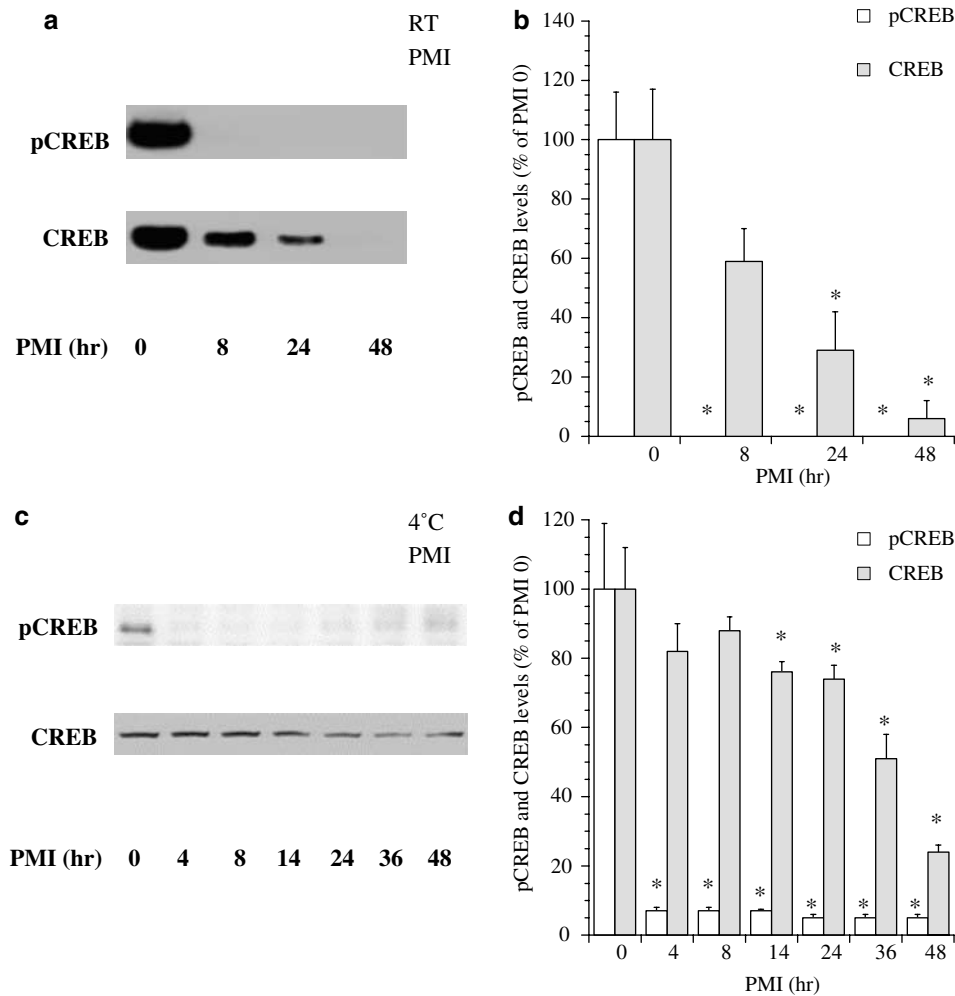
## RESULTS

Mice were sacrificed, and stored at room temperature in 0, 8, 24, or 48 h prior to dissection. We initially examined the effects of PMI on total phosphorylated proteins using antibodies specific for the phosphorylation of specific amino acids, but not specific for individual proteins or the surrounding amino-acid sequence. Antibodies specific for phospho-tyrosine and phospho-threonine containing proteins revealed that PMI reduced the phosphorylation of the majority of these substrates (Figure 1a,b). Similar results were obtained using an antibody for the phosphorylated forms of potential phospho-protein kinase A (PKA) substrates (Figure 1c). We were additionally interested if PMI had an effect on other post-translational modifications. To test this, we used an antibody specific for acetylated-lysine containing proteins. Among a limited number of primary bands, the PMI reduced levels of band C, and altered the levels of band B in a tri-phasic manner (Figure 1d).

We next investigated whether the PMI changes the phosphorylation state of specific protein where phosphorylation is known to affect directly protein function. Protein kinases extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and transcription factors cJun, cyclic AMP response element binding protein (CREB) and activating transcription factor-2 (ATF-2) were selected for the study since alterations in the functions of these molecules have been implicated in the treatment and pathophysiology of mood disorders (Gould *et al*, 2002). In samples stored at room temperature, the earliest PMI (8 h) had a dramatic effect on the levels of all phosphorylated proteins as measured by immunoblotting (Figures 2–5, panels a and b). In contrast to the rather consistent results obtained using phospho-specific antibodies, there was variability of the effect of PMI on the total levels of proteins analyzed (Figures 2–6, panels a and b). Thus, PMI decreased the levels of some proteins as revealed by immunoblotting; however, others were not



**Figure 3** PMI decreases the phosphorylated forms of JNK (46 and 54 kDa), but does not change the total protein levels. During the PMI, brains were stored at either room temperature (A, B) or 4°C (C, D). The graph represents the mean densitometry reading  $\pm$  SE from a total of three (RT) or four (4°C) mice (\* $P < 0.05$  Fisher's PLSD). p-JNK 54 RT ( $F_{3,8} = 12.5$ ;  $P = 0.0022$ ), 4°C ( $F_{6,21} = 15.9$ ;  $P < 0.0001$ ); p-JNK 46 RT ( $F_{3,8} = 155$ ;  $P < 0.0001$ ), 4°C ( $F_{6,21} = 127$ ;  $P < 0.0001$ ).



**Figure 4** PMI decreases the phosphorylated form of CREB. PMI also decreases the total protein levels of CREB (43 kDa). During the PMI, brains were stored at either room temperature (a, b) or 4°C (c, d). The graph represents the mean densitometry reading  $\pm$  SE from a total of three (RT) or four (4°C) mice (\* $P < 0.05$  Fisher's PLSD). p-CREB RT ( $F_{3,8} = 39.1$ ;  $P < 0.0001$ ), 4°C ( $F_{6,21} = 23.9$ ;  $P < 0.0001$ ); total-CREB RT ( $F_{3,8} = 10.5$ ;  $P = 0.0038$ ), 4°C ( $F_{6,21} = 15.5$ ;  $P < 0.0001$ ).

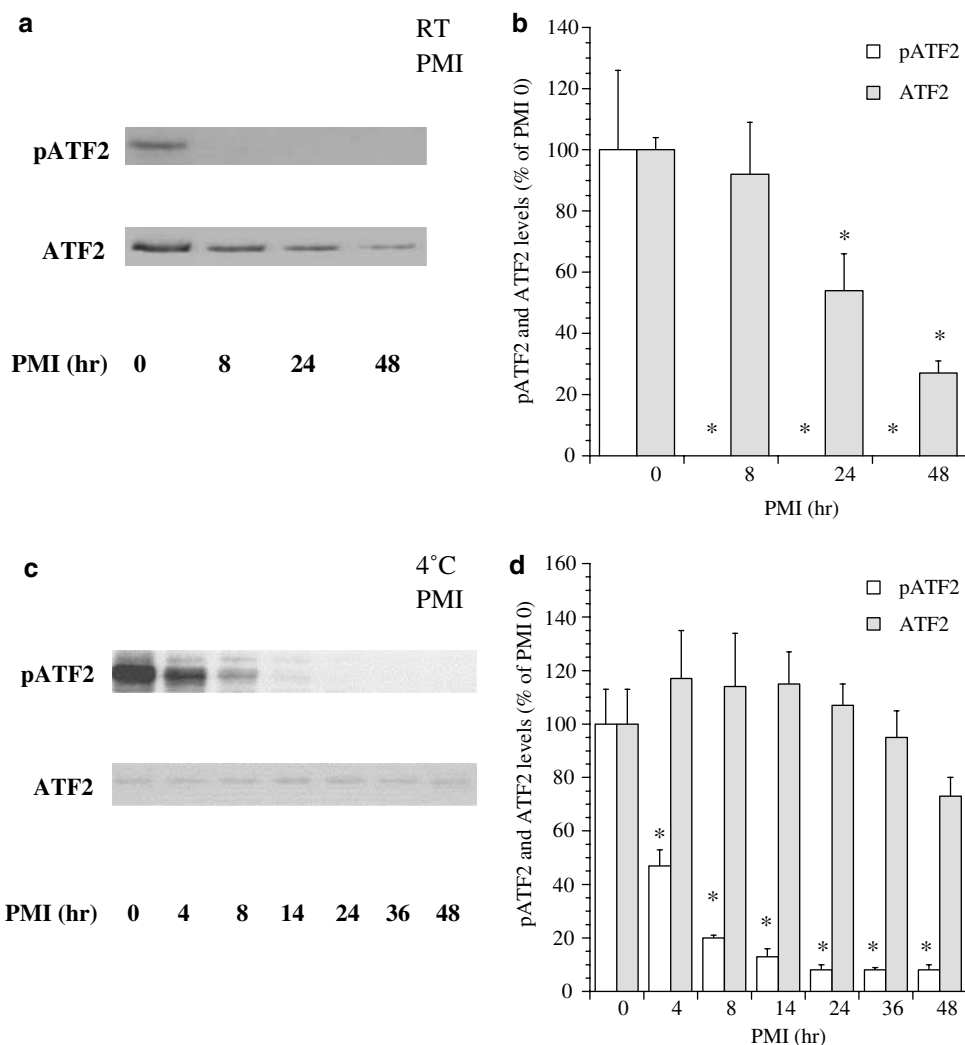
significantly changed using our methods. All experiments were repeated with samples from the hippocampus, with findings similar to those shown in Figures 2–6 (data not shown).

Since the PMI often encompasses time in refrigeration, we repeated the experiments; however, the brains were placed immediately at 4°C after mice were sacrificed, and additional time points were used (0, 4, 8, 14, 24, 36, and 48 h). In general, results at 4°C were similar to those obtained at room temperature. The earliest PMI (4 h) had a dramatic effect on the levels of all phosphorylated proteins (Figures 2–5, panels c and d). Consistent with the results obtained when brains were stored at room temperature, there was variability on the effect of PMI on the total levels of proteins analyzed (Figures 2–6, panels c and d). PMI decreased the levels of many proteins, but other protein levels were unchanged. The proteins that decreased in immunolabeling—or remained stable—after storage at room temperature were similar to those that were stored at 4°C. For example, PMI had an effect on the protein levels of C-Jun (Figure 6) and CREB (Figure 4), but had no significant effect on Bcl-2 (Figure 6) or ERK (42 or 44;

Figure 2). All experiments were repeated with samples from the hippocampus, with findings similar to those shown in Figures 2–6 (data not shown).

## DISCUSSION

PMI has a major impact on the levels of proteins in the phosphorylated (often functional) state as detected by immunoblot (Figures 1–5). Furthermore, PMI decreased the total level of some proteins (Figures 2–6). Storage of brains at 4°C did not appear to affect the decrease in phospho-protein levels; however, it did appear to delay the degradation of some total proteins (Figures 2–6). Phosphorylation of the tau protein has been investigated in a number of studies; these studies have shown that while phosphorylation of most sites on tau are highly unstable during the PMI, some appear not to be affected to the same degree (Burack and Halpain, 1996; Gartner *et al*, 1998; Matsuo *et al*, 1994; Song *et al*, 1997). Our data are generally consistent with—and extends—this series of findings, suggesting that it may be difficult to measure the level of most phosphoproteins that are involved in intracellular

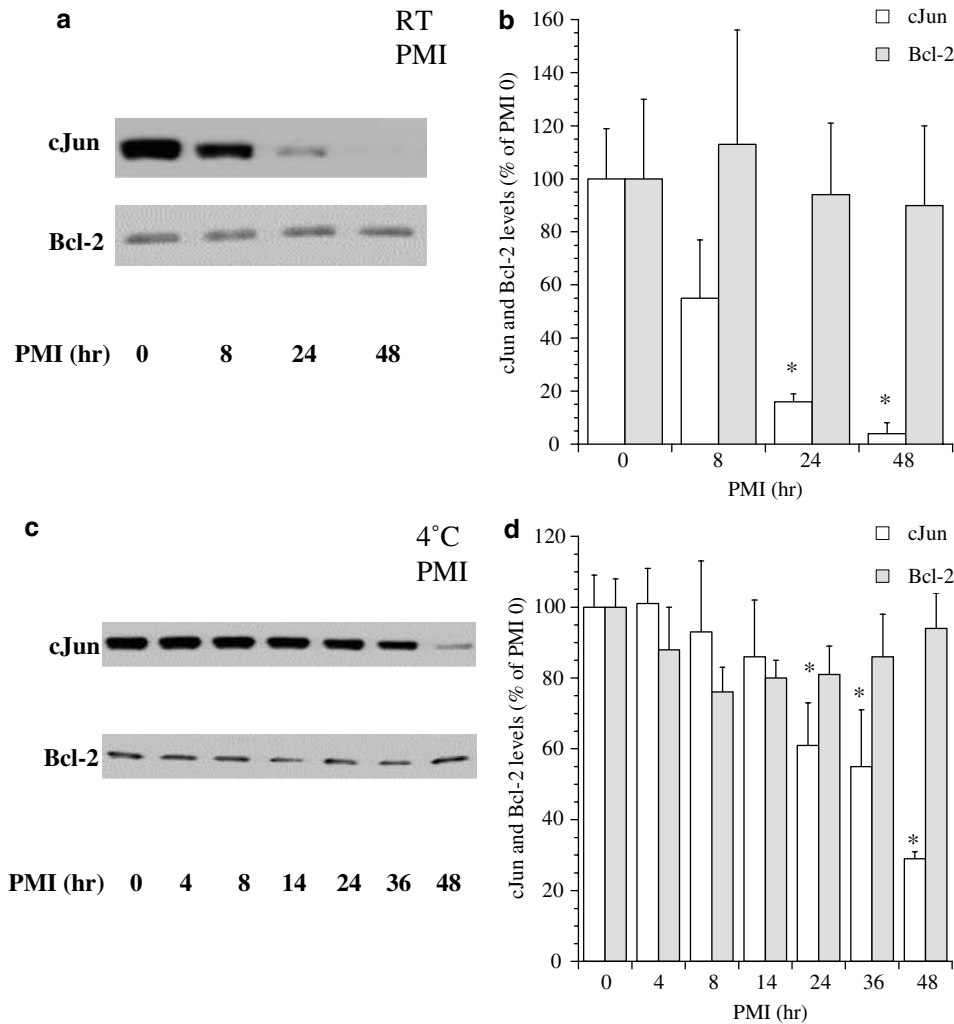


**Figure 5** PMI decreases the phosphorylated form of ATF2 (65 kDa). PMI also decreases the total protein levels of ATF2 during storage at room temperature (a, b). However, total protein levels do not change significantly when brains are stored at 4°C (c, d). The graph represents the mean densitometry reading  $\pm$  SE from a total of three (RT) or four (4°C) mice (\* $P < 0.05$  Fisher's PLSD). p-ATF2 RT ( $F_{3,8} = 15.0$ ;  $P = 0.0012$ ), 4°C ( $F_{6,21} = 37.6$ ;  $P < 0.0001$ ); total-ATF2 RT ( $F_{3,8} = 9.52$ ;  $P = 0.0051$ ), 4°C ( $F_{6,21} = 1.35$ ;  $P = 0.28$ ).

signaling in post-mortem human brain specimens (where the average PMI is often longer than 24 h). However, considering that nonprotein-specific phospho-antibodies appeared to identify some phosphorylated proteins unaffected by PMI (Figure 1), it is possible that not all phosphorylated proteins are affected to such a degree (Walaas *et al*, 1989). Furthermore, as many enzymes are regulated by phosphorylation, it may be difficult to measure reliably the *in vitro* activity (as a correlate of *in vivo* activity) of many enzymes from the post-mortem brain. Indeed, numerous studies have found that the activities of certain enzymes are sensitive to PMI (Bowen *et al*, 1976; Puymirat *et al*, 1979; Roytta *et al*, 1980; Spokes and Koch, 1978); our data suggest a possible mechanism underlying these findings.

Clearly, as witnessed by the relative stability of the total level of some proteins compared to others among our data, there is considerable variability of the PMI stability of total protein levels. This finding is consistent with previous studies addressing the stability of other biological products such as protein levels (Akil and Lewis, 1994; Fountoulakis *et al*,

2001; Geddes *et al*, 1995; Li *et al*, 1996; Schwab *et al*, 1994), mRNA (Burke *et al*, 1991; Finger *et al*, 1987; Pardue *et al*, 1994; Schramm *et al*, 1999), ligand binding (Kornhuber *et al*, 1988), monoamine levels (Spokes and Koch, 1978), and enzyme activity (Bowen *et al*, 1976; Puymirat *et al*, 1979; Roytta *et al*, 1980; Spokes and Koch, 1978) in brain specimens; while findings for a specific measure are generally reproducible among studies, there is variability among members of the same class (ie different mRNAs, enzymes, etc). Thus, it cannot be assumed that because one particular protein, mRNA, etc is stable, all are stable during a certain PMI. In addition to PMI and storage temperature, other factors such as agonal state at death (Bowen *et al*, 1976; Butterworth *et al*, 1983; Harrison *et al*, 1991; Lewis and Akil, 1997; Perry *et al*, 1977a, 1982; Spokes, 1979; Yates *et al*, 1990), pH (generally related to agonal state) (Barton *et al*, 1993; Eastwood and Harrison, 2000; Harrison *et al*, 1995; Johnston *et al*, 1997; Kingsbury *et al*, 1995), freezing time (Roytta *et al*, 1980), and time or season of death (Bucht *et al*, 1981; Carlsson *et al*, 1980; Perry *et al*,



**Figure 6** PMI decreases the total level of c-Jun (39 kDa), but does not significantly change the total level of Bcl-2 (26 kDa). During the PMI, brains were stored at either room temperature (a, b) or 4°C (c, d). The graph represents the mean densitometry reading  $\pm$  SE from a total of three (RT) or four (4°C) mice (\* $P < 0.05$  Fisher's PLSD). c-Jun RT ( $F_{3,8} = 8.95$ ;  $P = 0.0062$ ), 4°C ( $F_{6,21} = 4.38$ ;  $P = 0.0051$ ); Bcl-2 RT ( $F_{3,8} = 0.099$ ;  $P = 0.96$ ), 4°C ( $F_{6,21} = 0.853$ ;  $P = 0.544$ ).

1977a–d), among many others, have been found to influence the degradation—or level—of biological products in post-mortem brain specimens. Thus, it remains critical to ascertain the post-mortem stability, and the stability under other conditions, of the biological product.

While important, *post hoc* correlational analysis may not always be indicative of the stability of proteins. There is likely a greater relative variation in post-mortem human specimens—as opposed to animal models—because of the factors discussed in this manuscript, and/or the relative genetic and environmental heterogeneity found in humans. Furthermore, as has recently been articulated, it is quite possible that the biological measure had already changed substantially from the *in vivo* value and that within the PMI of post-mortem brain specimens (12–48 h) there is little variation (Lewis, 2002). As previously suggested (Lewis, 2002; Lewis and Akil, 1997; Perry and Perry, 1983; Spokes and Koch, 1978), it may be advisable to test—in experimental animal models—the stability of the specific biological products of interest during various PMIs and under different post-mortem conditions prior to perform-

ing a similar analysis in human post-mortem brain specimens. Importantly, it must be kept in mind that there exists the possibility that protein and protein phosphorylation in the human brain may not degrade at the same rate or at the same manner as in the mouse brain. A valuable resource may be the availability of human post-mortem brain tissue with experimentally defined post-mortem storage times—and other post-mortem factors—from the same individual (to limit human heterogeneity). The availability of such a repository would allow for more scientifically rigorous testing of the effects of postmortem factors than is currently available when utilizing *post hoc* correlational analyses.

Clearly, the human post-mortem brain represents a tremendously important resource for the study of neuropsychiatric disorders (Duman, 2002). However, care must be taken to ascertain the effect of PMI on protein levels, or post-translational forms of proteins under investigation. While PMIs generally found in most studies may be adequate to quantitate the total levels of many proteins, extreme caution is likely required in the interpretation of

phosphoprotein levels, two-dimensional gel electrophoresis, and the activity of enzymes regulated by phosphorylation.

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