Protein Phosphorylation Cascades Associated with Methamphetamine-induced Glial Activation

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ABSTRACT: Reactive gliosis is the most prominent response to diverse forms of central nervous system (CNS) injury. The signaling events that mediate this characteristic response to neural injury are under intense investigation. Several studies have demonstrated the activation of phosphoproteins within the mitogen-activated protein kinase (MAPK) and Janus kinase (JAK) pathways following neural insult. These signaling pathways may be involved or responsible for the glial response following injury, by virtue of their ability to phosphorylate and dynamically regulate the activity of various transcription factors. This study sought to delineate, *in vivo*, the relative contribution of MAPK- and JAK-signaling components to reactive gliosis as measured by induction of glial-fibrillary acidic protein (GFAP), following chemical-induced neural damage. At time points (6, 24, and 48 h) following methamphetamine (METH, 10 mg/kg × 4, s.c.) administration, female C57BL/6J mice were sacrificed by focused microwave irradiation, a technique that preserves steady-state phosphorylation. Striatal (target) and nontarget (hippocampus) homogenates were assayed for METH-induced changes in markers of dopamine (DA) neuron integrity as well as differences in the levels of activated phosphoproteins. GFAP upregulation occurred as early as 6 h, reaching a threefold induction 48 h following METH exposure. Neurotoxicant-induced reductions in striatal levels of DA and tyrosine hydroxylase (TH) paralleled the temporal profile of GFAP induction. Blots of striatal homogenates, probed with phosphorylation-state specific antibodies, demonstrated significant changes in activated forms of extracellular-regulated kinase 1/2 (ERK 1/2), *c-jun* N-terminal kinase/stress-activated protein kinase (JNK/SAPK), MAPK/ERK kinase (MEK1/2), 70-kDa ribosomal S6 kinase (p70 S6), cAMP responsive element binding protein (CREB), and signal transducer and activator of transcription 3 (STAT3). MAPK-related phosphoproteins exhibited an activation profile that peaked at 6 h, remained significantly increased at 24, and fell to baseline levels 48 h following neurotoxicant treatment. The ribosomal S6 kinase was enhanced over 60% for all time points examined. Immunoreactivity profiles for the transcription factors CREB and STAT3 indicated maximal increases in phosphorylation occurring at 24 h, and measuring greater than 2- or 17-fold, respectively. Specific signaling events were found to occur with a time course suggestive of their involvement in the gliotic response. The toxicant-induced activation of these growth-associated

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signaling cascades suggests that these pathways could be obligatory for the triggering and/or persistence of reactive gliosis and may therefore serve as potential targets for modulation of glial response to neural damage.

INTRODUCTION

Acute injury to the central nervous system (CNS) triggers morphologic and metabolic changes, which act both to protect against infectious agents and to repair damaged tissue.[1–7] In an emergency-like state, injured neurons change their own gene expression and stimulate nearby microglia and astrocytes. The astrocyte response to injury, termed reactive gliosis, involves a graded, stereotypic astrocytic hypertrophy, with an accumulation of astrocytic intermediate filaments, of which glial fibrillary acidic protein (GFAP) is the principle protein component. Considered the hallmark of reactive gliosis, GFAP induction occurs following all types of CNS insults (physical and chemical injuries as well as neurological diseases), and can be evoked by damage to any neural cell type in any region of the CNS.[8–13]

The role of protein phosphorylation events in GFAP upregulation is under investigation in our laboratory. As protein phosphorylation represents the dominant post-translational mechanism through which a variety of cellular processes are regulated, their high abundance within the CNS suggests protein phosphorylation to be a critical molecular mechanism through which extracellular signals, such as hormones and growth factors, exert effects on their cellular effectors.[14] As the understanding of protein phosphorylation and its cellular functions has progressed, the study of phosphorylation as a pathological and toxicological variable has begun to attract attention in recent years.[15–18] Therefore, elucidating the relative contribution of phosphoprotein signaling in GFAP expression may provide a framework for modulating the cellular effectors involved in the neural damage response.

The mitogen-activated protein (MAP) kinase cascades are one of the most intensely studied groups of phosphoprotein signaling cascades. MAP kinases can be activated by a complex set of extracellular stimuli and intracellular molecules. These transduction pathways are present in neurons and glia and have been implicated in many physiological processes, including cell growth, differentiation, oncogenic transformation, immune responses and apoptosis.[19–21] The best characterized of these are the extracellular signal-regulated kinase (ERK), *c-jun* N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 MAP kinase pathways. Most proliferative stimuli activate the ERK pathway, primarily through the small GTP binding protein Ras. Active Ras binds the MAP kinase kinase kinase Raf-1, thereby translocating it to the plasma membrane and promoting its activation. Active Raf-1 then phosphorylates and activates the MAP/ERK kinases (MEKs) 1 and 2, which in turn phosphorylate and activate ERK1 and ERK2. In a similar fashion, exposure of cells to cytokines and cellular stresses primarily activates the JNK/SAPK and p38 MAP kinase cascades. Through the activation of intermediary kinases, the Rho family small G protein Rac and Cdc42hs can regulate the activation of JNK/SAPK, and to a much lesser extent the p38 MAP kinase pathways[22,23] refer to FIGURE 1.

MAP kinases are localized within neuronal cell bodies and dendrites[24] and effect a diverse array of cellular processes including neurotransmission, cytoplasmic signaling, cytoskeletal dynamics and ion channel activities at synapses.[25,26] Most of

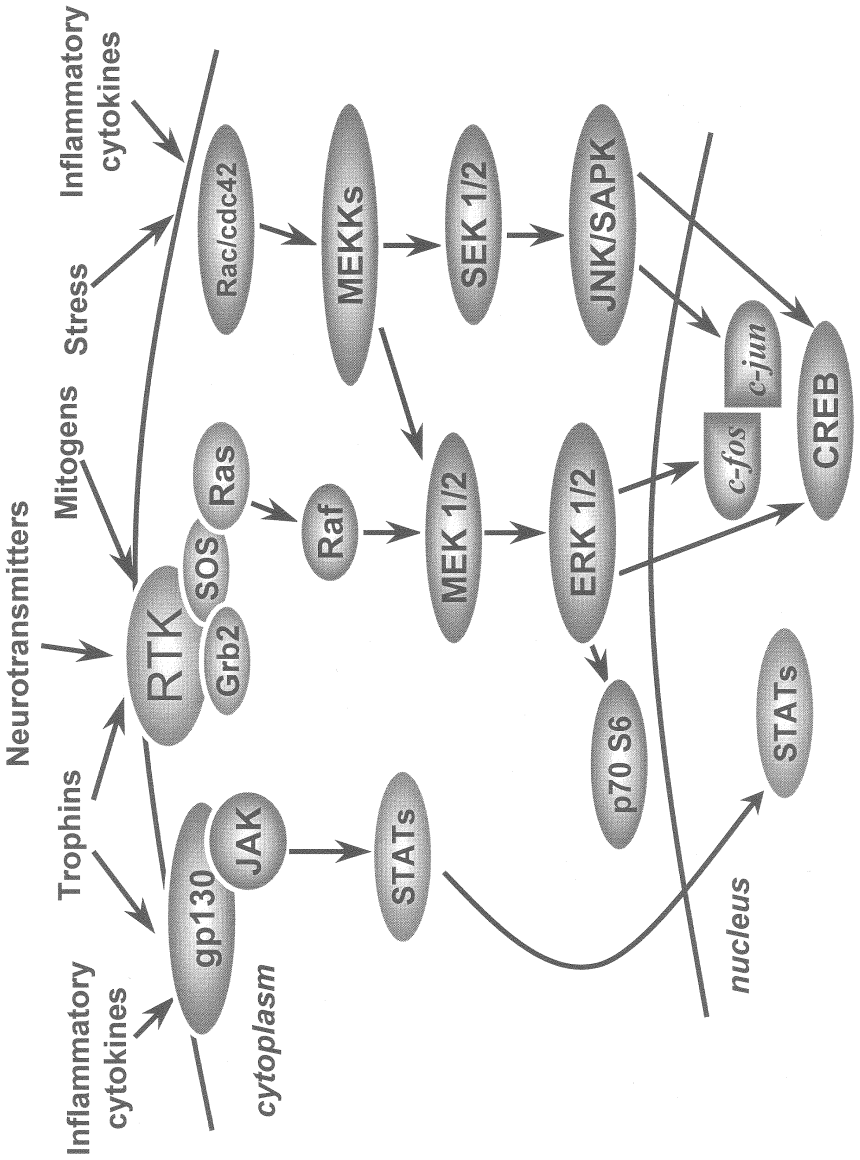

FIGURE 1. *See following page for caption.*

MAPK's physiological effects involve the induction or inhibition of gene expression, but some have been attributed to physical interactions with other signaling proteins.[27,28] ERKs constitute one family of MAPKs that are downstream effector kinases in a signaling pathway activated by a number of extracellular ligands.[29–35] ERK1 and ERK2, also known as MAPK p44/42, are two predominant MAP kinase isoforms expressed throughout the brain,[36,37] and are recognized for their critical role in the regulation of neural cell growth and differentiation.[38–42]

The ERK cascade, stimulated by both receptor tyrosine kinases and G protein-coupled receptors, which classically involves Shc tyrosine phosphorylation, recruitment of the Grb2-Sos complex, and the subsequent sequential activation of Ras, Raf kinase, MEK, and ERK.[43–45] Phosphorylated ERK 1/2 (T202/Y203) translocates to the nucleus where it activates, directly or through kinases of the Rsk family, transcription factors such as c-Myc, *c-fos*, *c-jun*, Elk1 or CREB.[46–52] ERK 1/2 also has cytosolic, cytoskeletal, and membrane-bound substrates that include cytoplasmic phospholipase A2, the microtubule-associated proteins MAP2 and tau, midsized and heavy molecular weight neurofilaments, epidermal and nerve growth factor receptors.[46,49,53,54]

Participation of the ERK 1/2 pathway in the neural response to injury has been suggested. Enhanced phosphorylation of ERK 1/2 has been found in brain tissues following chemically induced or electroconvulsive shocks,[36,55,56] or cerebral ischemia.[57–61] PD098059, a selective inhibitor of the ERK 1/2 pathway was found to reduce neuronal death in a cell-culture model of seizure activity.[62–64]

Involvement of the ERK 1/2 pathway in directly triggering and/or maintaining persistence of reactive astrogliosis has also been suggested. Phosphorylated ERK 1/2 has been detected *in vivo* in reactive astrocytes from Alzheimer patients.[65,66] Investigations in our laboratory have shown increases in phospho-ERK 1/2 at time points coinciding with the earliest phase of reactive gliosis in a mouse model of 1-methyl-4-phenyl-1,2,3,6-tetrahyrdorpyridine (MPTP)-neurotoxicity.[67] Immunoreactivity of activated ERK 1/2 was also revealed in reactive astrocytes from a series of human neurosurgical specimens, including infarct, mechanical trauma, chronic epilepsy, and progressive multifocal leukoencephalopathy.[68]

Upstream of MAP kinases are an array of MAP kinase activators, which regulate the phosphorylation and autophosphorylation of the MAP kinases, and they are in turn mostly phosphoprotein in nature.[69–71] An important activator immediately upstream of MAP kinases is MAP kinase/ERK-activating kinase (MEK1/2), which has dual specificity and is capable of phosphorylating both tyrosine and serine/threonine

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FIGURE 1. Selected components of the MAPK and JAK-STAT cascades. *c-fos*, immediate early gene; *c-jun*, immediate early gene; CREB, cAMP response element-binding protein; ERK 1/2, extracellular signal-regulated kinase 1/2; gp130, signal-transduction receptor component glycoprotein 130; Grb2, an adapter protein in the Ras pathway; JAK, Janus kinase; JNK/SAPK, *c-jun* NH_2 -terminal kinase/stress-activated protein kinase; MEK 1/2, mitogen-activated protein kinase kinase or extracellular signal-regulated kinase kinase 1/2; MEKKs, mitogen-activated protein kinase kinase kinase; SOS, Son of Sevenless, a Ras guanine nucleotide-releasing factor; p70 S6, ribosomal protein S6 kinase; Rac/cdc42, small GTPase complex; Raf, a mitogen-activated protein kinase kinase kinase; Ras, a small GTPase; RTK, receptor tyrosine kinase; SEK 1/2, stress-activated protein kinase kinase; STATs, signal transducers and activators of transcription.

residues of MAP kinases. MEK1/2 is activated by a wide variety of growth factors, cytokines, and also by membrane depolarization and calcium influx.[38,42,72] MEK1/2 is located primarily in the cellular cytosol, following mitogenic stimulation; it is massively translocated to the nucleus.[73] Under abnormally high activation/hyperstimulation conditions, MEK 1/2 complexes with dephospho-ERK 1/2 and transports it out to the cytoplasm, perhaps for reactivation and transduction of signal to downstream effectors.[74] At present, ERK 1/2 are the only known substrates for MEK1/2.[49]

c-jun NH2-terminal kinases/stress-activated protein kinases (JNK/SAPKs) belong to a more recently discovered MAPK pathway as compared to extracellular signal-regulated kinases (ERKs). JNK/SAPK cascade is stimulated by stressful signals, such as osmotic stress, ultraviolet and ionizing radiation, heat shock, and reperfusion after ischemia.[75–77] It is also activated by physiological stimuli, such as hematopoietic cytokines[78]and G protein-coupled receptors.[22] The activation of JNK/SAPK pathway has been associated with many final cellular responses, some of which generate opposite outcomes. Depending on cell or tissue type and experimental conditions, JNK/SAPK stimulation has been reported to induce apoptosis,[79] rescue from apoptosis,[80–84] proliferation,[85,86] and differentiation.[87]

Within the CNS, studies demonstrate that JNK/SAPK is involved in the development of ischemic brain injury[88–93] and suggest that these kinase signaling pathways may play a role in tissue injury after ischemia/reperfusion. Compounds that selectively inhibit JNK/SAPK have been shown to attenuate loss of nigrostriatal dopaminergic (DAergic) neurons[94] following administration of the prototypical dopaminergic neurotoxicant MPTP, and to prevent injury-induced neuronal dedifferentiation *in vivo.*[95]

Among the substrates of ERK are the family of ribosomal S6 kinases (RSKs), which were among the first substrates of ERK to be discovered and which have proven to be a ubiquitous and versatile mediator of ERK signal transduction. RSKs are key players in the control of cell size, growth, and proliferation, all of which must be regulated in a coordinated fashion to bring about the normal development of the organism.[96–102] Considered the major physiological S6 kinase, p70 S6 kinase is an important regulator of cell proliferation and a potential target of agents that modify the immune and proliferative responses.[103,104] It physiologically phosphorylates the S6 protein of the 40S ribosomal subunit in response to mitogenic stimuli and is a downstream component of the rapamycin-sensitive pathway.[105] p70 S6 kinase is activated by numerous stimuli, including growth factors, cytokines, phorbolesters, oncogenic products, Ca^{2+}, and inhibitors of protein synthesis.[106,107] *In vitro* characterized substrates, which include *c-fos*, serum response factor (SRF), estrogen receptor, and Nur77, implicate a regulatory role for this kinase in immediate-early gene activation.[46,98,108,109,110,112,113] Activation of p70 S6 requires sequential phosphorylations within the autoinhibitory domain and at Thr389.[114,115] Increased p70 S6 activity has been demonstrated in PC12 cells treated with the toxic metabolite of MPTP, 1-methyl-4-phenylpyridinum (MPP^+), indicating its potential role in response to damage.[116]

The transcription factor cyclic AMP response element-binding protein (CREB) is phosphorylated by kinases within three distinct pathways: ERK, JNK/SAPK, and Ca^{2+}/calmodulin-dependent kinase (CaMK) pathways.[48,52,117–120] CREB is constitutively expressed and is activated by phosphorylation at a serine residue (S133) in

response to increases in cAMP, calcium ion or growth factors.[120–123] Many protein kinases including protein kinase A, calcium/calmodulin-dependent protein kinase-I, -II, and -IV, PKC, RSK-2, MAPKAP2, MSK1, and ERK have been shown to mediate the phosphorylation of CREB.[124,125] Although phosphorylation classically stimulates transcriptional activators by modulating their nuclear transport or DNA-binding affinity, CREB belongs to a class of proteins whose phosphorylation appears specifically to enhance their transactivation potential.[126]

CREB is a plasticity-associated transcription factor that regulates the expression of many downstream genes containing CRE elements, such as *c-fos*.[47,127,128] By regulating new gene expression and protein synthesis, CREB within the CNS can mediate the long-term remodeling of synapses, which is believed to underlie memory consolidation and neuronal plasticity.[129–131] This factor also mediates the cellular responses to a variety of physiological signals including growth factors, depolarization, synaptic activity, mitogenic and differentiation factors, and various stressors.[125,131] Upon phosphorylation, CREB can facilitate transcriptional activation of genes containing the CRE motif such as those coding for *c-fos*,[47] brain-derived neurotrophic factor (BDNF),[132,133] fibroblast growth factor (FGF),[134] tyrosine-hydroxylase (TH),[135] and others.[136]

Evidence suggests that these pathways become activated in response to stimuli that regulate synaptic function such as the influx of extracellular Ca^{2+} and certain neurotrophin growth factors such as BDNF. Inasmuch as CREB has been suggested to play a critical role in mediation of neuronal adaptive responses to transsynaptic stimuli, it should be no surprise that CREB knockout mice die prematurely, before majority of cerebellar granule neurons are generated.[137] Abnormal activation has been demonstrated following ischemia,[138] convulsive seizures,[139] and *in vitro* models of gliosis.[140] In the 6-hydroxydopamine lesion model of Parkinson's disease, a very robust CREB phosphorylation has been measured throughout the hippocampus.[141–143]

The JAK/STAT pathway connects activation of the receptor complexes directly to transcription of genes. STATs are key transcription factors regulating cell growth and differentiation in systems ranging from *Drosophilia* to mammals.[144–149] Studies of humans and mice, deficient for one of the JAKs or STATs, have revealed crucial roles of these molecules in embryonic development, blood cell formation, and immune responses. Three different types of tyrosine kinases, Janus kinases, receptor-type tyrosine kinases, and certain Src family tyrosine kinases can all activate STAT proteins.[150] STAT transcription factors, normally found in the cytoplasm, and constitutively nonphosphorylated, contain a single src-homology-2 (SH-2) domain and can be phosphorylated on a single tyrosine residue. After tyrosine phosphorylation, STAT proteins dimerize, translocate into the nucleus, and bind to DNA elements, leading to transcriptional activation of target genes.[151–154]

STAT3 induction has been demonstrated to be involved in gliogenesis,[155] as well as neural injury responses, suggesting a possible role in neurotoxicant-induced reactive gliosis. STAT3 is phosphorylated *in vivo* in neurons in the rat superior cervical ganglion after transection of the postganglionic nerves, in microglia after transient focal cerebral ischemia, in cortical glia after excitotoxic lesions, and in the intact retina after exposure to subtoxic bright light and mechanical trauma.[156–161]

Temporal organization of MAPK activities can play an important role in the generation of specific biological responses. Furthermore, the biologic response to

variations in the timing of kinase activation is also cell-type dependent.[44,162] As the neurotoxic response can lead to signal transduction along more than one pathway, factors that modify signaling along each of the pathways may determine the amount that each pathway contributes to the induction of genes in the nucleus. This will then lead to different biological outcomes, depending on which genes are switched on. The presence of activated phosphoproteins following neurotoxic insult may suggest involvement in the astrogliotic process, but the temporal delineation of phosphoprotein activation provides an added dimension with which to investigate respective involvement. MAPK cascades were initially believed to operate predominantly as linear signaling pathways that directly link a specific input signal to a specific biologic response. However, it now seems that communication takes place through a complex network of signaling cascades and that information flows, not only from and to the extracellular environment, but also laterally from one pathway to another. In this way, a minimal number of components, regulated in a binary fashion, can confer specific and appropriate responses to an enormous variety of challenges. By temporally delineating the events involved in glial activation, we hope to elucidate key signaling pathways, which mediate the effects of diverse neurotoxic insults.

Methamphetamine (METH), a widely abused psychostimulant, produces toxicity to striatal dopamine (DA) nerve terminals in mice and hyperthermic rats as exemplified by reductions in DA, the DA transporter (DAT), tyrosine hydroxylase (TH), and the induction of reactive gliosis that is accompanied by silver degeneration staining, reviewed in references 163 and l64. The interpretative power conferred by METH-induced neurotoxicity is often understated. The advantages of this neurotoxicological model include target specificity, method of exposure, and well-defined neurotoxic endpoints. METH selectively damages nigrostriatal terminals limiting the contribution from other pathways in the responses measured. METH, administered systemically, eliminates the confounding factor of physical injury brought upon by local administration of a neurotoxicant (i.e., blood-brain barrier disruption). In clinical conditions such as bacterial infections, head trauma or stroke, the blood-brain barrier can be compromised and the influx of immune cells and cytokines from the periphery could play a significant role in cytokine-associated neuroimmune response and neurological manifestations.[165,166] Furthermore, METH can be administered at dosages that do not result in cell death, thus eliminating the contribution of signaling events related to apoptosis or necrosis in any observed changes. When given systemically to mice in multiple doses it can produce long-lasting changes in DAergic terminals in the striatum. These changes include decreases in DA content, DA release, DA uptake, and TH levels and activity.[167,168] The neurotoxic effects of METH in both the mouse and rat models also involve nerve terminal degeneration as evidenced by reactive gliosis and silver degeneration staining.[163,169,170]

While METH has proven to be a useful and highly selective denervation tool, our studies could not have been performed without the use of focused microwave irradiation. Owing to the rapid reversibility of phosphorylation/dephosphorylation events that occur *in vivo*, investigations of phosphoproteins mandate preservation of steady-state phosphorylation. Following decapitation, during the brief time period of postmortem brain tissue collection, significant changes in neuro-metabolite levels can occur. High-energy-focused microwave irradiation enables researchers to assess molecular biological events in the absence of enzyme-induced confounding changes that occur after decapitation.[67,171–174]

In the present study, quantitative immunolabeling with context-independent and phosphorylation state-specific antibodies was used to assess temporal changes in the activated state of signaling proteins preceding the upregulation of GFAP. Our aim was to elucidate signaling events that may be responsible for the initiation of reactive gliosis. Specific and reproducible activation profiles for MAPK-related and JAK-STAT phosphoproteins were found to temporally correspond with neurotoxicant exposure and induction of GFAP, implicating the involvement of these molecular pathways in the neural injury response.

METHODS

Animals and Tissue Preparation

Female C57BL/6J mice (n = 6–10 per group/time point) were administered d-METH (Sigma Chemical Co., St. Louis, MO) at a dosage of 10 mg/kg subcutaneously (s.c.) or vehicle (saline), every two hours for a total of four injections. At 6, 24, and 48 h following treatment mice were sacrificed by focused microwave irradiation (Muromachi Kikai Inc., Tokyo, Japan; Model TMW-4012C, 10.0 KW, 0.90 s), decapitated, and the brain regions dissected. Striatal and hippocampal regions were dissected free-hand, weighed, and homogenized in 10 vol of hot 1% SDS, frozen on dry ice and stored at −80°C until assayed. Total protein concentration of the SDS homogenates was assayed by the method of Smith *et al.*[175] Bovine serum albumin (BSA) was used as the standard. Tissue samples assayed for DA concentrations were obtained by conventional decapitation method, dissected free-hand, weighed, and stored at −80°C until assayed.

GFAP and TH Analysis

GFAP was assayed be detergent-based sandwich enzyme-linked immunosorbent assay (ELISA).[176] Tyrosine hydroxylase was assayed by a sandwich fluorescent ELISA. Briefly, Immulon-4 96-well plates were coated with anti-TH monoclonal antibody (Calbiochem, La Jolla, CA, No. 657010) at 1:500 in 100 µl/well phosphate-buffered saline (PBS), incubated at 37°C for 1 h, washed with 200 µl/well PBS (4×), blocked with 100 µl/well 5% nonfat dry milk (Blotto) for 1 h at RT, and then washed again with PBS (4×). Standards, ranging from 1–20 µg total protein were prepared from mouse striata homogenized in hot 1% SDS. Standards and samples, diluted in PBS-Triton, were applied 100 µl/well; following a 1 h incubation at RT, plates were washed with PBS-Triton (5 min, 4×). Anti-TH polyclonal antibody at 1:500 (Calbiochem, #657012) and HRP-labeled anti-rabbit antibody at 1:3000 (Amersham, NA934) were diluted in Blotto-Triton, applied at 100 µl/well, for 1 h at RT. Final washes were with PBS-Triton (5 min, 4×). Quantablu substrate (Pierce, Rockford, IL, No. 15169), 100 µl/well, was added for 15 min, bubbles were popped, stop buffer was added, and the plates were read at 320/405 on Fmax Plate Reader (Molecular Devices, Sunnyvale, CA).

HPLC-EC Measurement of Dopamine

Levels of DA in striatal homogenates from control and METH-treated mice were analyzed by high-performance liquid chromatography with electrochemical detection (HPLC-EC). Samples were homogenized in 0.3 ml standard volume of ice-cold 0.2 N perchloric acid, containing dihydroxybenzylamine 1 μM as internal standard. After centrifugation at 10,000 × *g* for 10 min, an aliquot of supernatant (10 μl) was injected using a temperature-controlled (4°C) automatic sample injector (Waters 717 Plus Autosampler connected to a Waters 515 HPLC pump) into a C18 reversed-phase column (Waters SYMMETRY, 4.6 × 250 mm, 5 μm, 100 A) and were electrochemically detected (Waters 464, range 10 nA, potential 700 mV) using Millennium Software 32. The mobile phase consisted of 75 mM dibasic sodium phosphate, 1.7 mM OSA, 25 μM EDTA, 10% v/v acetonitrile, all adjusted to a pH of 3.0 with phosphoric acid, and pumped at a flow rate of 1 ml per min. Quantitation was accomplished by the use of the internal standard (10 pm DHBA per injection) method using standard curves of each analyte (0.5 to 25 pmol per injection). The limit of detection is 1.5 pmol per injection. Levels of DA are expressed as μg/g original tissue weight.

Immunoblot Analysis of METH-induced Changes in Phosphoproteins

Tissue homogenates were resolved on 10% SDS gels and electrophoretically transferred to a 1.0 μM nitrocellulose membrane. Membranes were blocked with Blotto in TBST (TBS including 0.1% Tween-20), washed in TBST for 5 min (3×) and incubated overnight at 4°C with a polyclonal anti-rabbit antibody (1:1000, 5% bovine serum albumin in TBST). Primary antibodies (Cell Signaling Technology, Beverly, MA) were either phosphorylation-state specific, directed against the "activated" state of the phosphoproteins or the context-independent: MEK1/2 (S217/221), ERK 1/2 (T202/Y204), JNK/SAPK (T183/Y185), p70 S6 kinase (T389), CREB (S133), and STAT3 (Y705). After washing, the blots were incubated with HRP-conjugated anti-rabbit (1:2000) amplified with horseradish peroxidase (HRP)-biotin (1:1000) in blocking buffer for 1 h at RT. Detection was accomplished using enhanced chemiluminescence (Lumiglo, New England Biolabs). Exposed films were scanned into Personal Densitometer and quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). All data were obtained from the linear portion of the densitometry curves.

Statistics

Individual variables were evaluated by one-way analysis of variance followed by Tukey-Kramer *post hoc* analysis, $p < 0.05$.

RESULTS

METH-induced Dopaminergic Neurotoxicity

Systemic administration of METH to C57BL/6J mice resulted in the expected neural injury profile, exhibiting regional- and cellular-specific neuronal damage coincident with the more generic injury-dependent astrocytic response. METH-in-

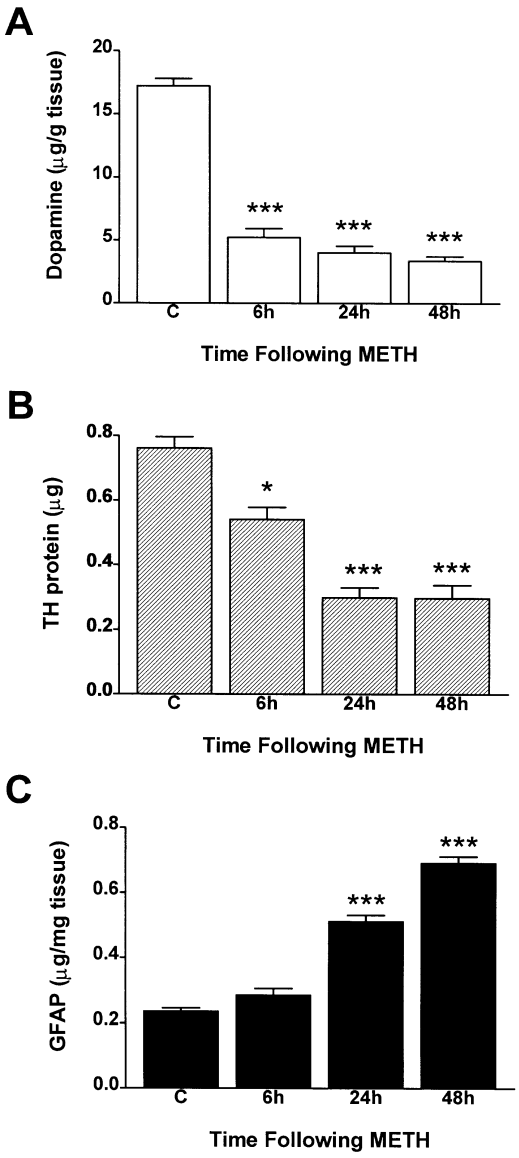

FIGURE 2. Measures of dopaminergic neurotoxicity following administration of METH to the C57Bl/6J mouse. Striatal samples were collected from mice sacrificed by focused microwave irradiation at 6, 24, or 48 h following the fourth injection of METH (10 mg/kg as the base, s.c. every 2 h × 4). (**A**) Dopamine tissue levels as measured by HPLC-EC. (**B**) Tyrosine hydroxylase (TH) protein concentration determined by sandwich ELISA. (**C**) GFAP induction determined by sandwich ELISA. Each value represents the mean ± SEM of at least six mice. $^{***}p < 0.001$, $^{*}p < 0.05$, level of significance difference as compared to control group, C.

duced DAergic neuronal injury was evidenced by severe depletions in both transmitter levels and biosynthetic enzyme concentrations within the striatal homogenates. Gliosis within the same tissue was indicated by significant upregulation of GFAP protein.

Dopamine levels within the terminal fields of METH-treated mice were markedly diminished as early as 6 h following dosing. As seen in FIGURE 2A, HPLC-EC measurements of DA whole-tissue levels at this first time point indicated a 70% reduction as compared to controls (5.21 ± 0.71 vs. 17.22 ± 0.58, μg/g tissue, $p < 0.001$). Transmitter concentration continued to decline in mice treated with METH, resulting in a greater than 80% loss of striatal DA 48 h following toxicant exposure (3.37 ± 0.36 μg/g tissue, $p < 0.001$).

METH-induced DAergic nerve terminal damage was also verified by significant declines in TH, the rate-limiting biosynthetic enzyme responsible for DA production. TH-immunoreactivity, measured by ELISA, revealed a significant attenuation 6 h following METH exposure, at which time TH protein levels were 29% reduced as compared to control (FIG. 2B). TH protein levels continued to decline, with the largest deficit (>60%) measured at the 48 h time point (0.30 ± 0.04 vs. 0.76 ± 0.04 μg protein).

Reductions in DAergic enzyme and transmitter levels within the METH-treated striatum were accompanied by marked astrogliosis as evidenced by enhanced expression of GFAP (FIG. 2C). Increments in GFAP were noted as early as 6 h following treatment (20% increase) with peak effects measured at 48 h post dosing (2.2-fold).

Phosphorylated Forms of MAPKs and STAT3 Were Increased after METH

Striatal homogenates obtained from METH-treated mice exhibited significant increases in activated MAPK-related and STAT3 phosphoproteins. Representative immunoblots of striatal proteins obtained from saline- and METH-treated mice at 6, 24, and 48 h post dosing are shown in FIGURE 3. Quantification of phospho-MEK 1/2, -ERK 1/2, and -SAPK revealed a significant (40–60%) increase in phosphorylation 6 h following neurotoxicant exposure, an effect that declined over time. METH-induced phosphorylation of these proteins persisted for over 24 h, with levels near or at baseline 48 h following dosing. The ribosomal kinase, p70S6, was more than 60% phosphorylated over control at all time points examined. The activation profile for the transcription factors CREB and STAT3 indicated significant toxicant-related changes in these phosphoproteins beginning at 6 h, increasing to maximal levels by 24 h. Injury-related changes in phospho-STAT3 were the most striking, measuring more than 18-fold of control levels at the 24-h time point.

Total MAPK and STAT3 Concentrations Remained Unchanged following METH Exposure

To determine whether the total amounts of MEK 1/2, ERK 1/2, SAPK, p70 S6, CREB, and STAT3 were altered by methamphetamine administration, striatal extracts were subjected to Western analysis (FIG. 4). No significant changes in the amounts of each protein were observed following neurotoxicity as compared with control animals. These results indicated that the induction of immunoreactivity of

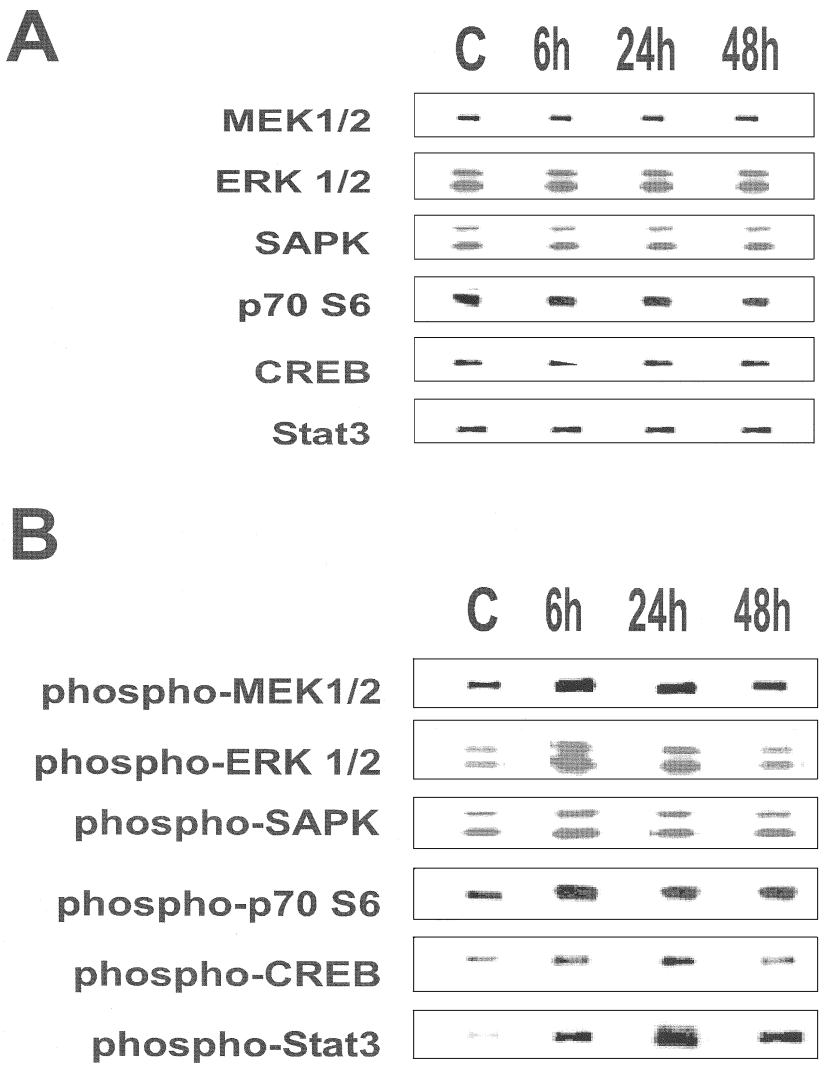

FIGURE 3. Phosphoproteins are activated following METH exposure. Each lane represents immunolabeling of phosphorylated protein in homogenates of striatum from individual saline-control or METH-treated mice sacrificed by focused microwave irradiation at time points 6, 24, and 48 h after dosing regimen. Proteins from these blots were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western blots were performed using antibodies that recognized total phosphoprotein (**A**) or activated phosphoprotein (**B**). Thirty micrograms of total protein were loaded for every determination. Phosphoprotein levels were quantified by ECL analysis of immunoblots probed with antibodies specific for the activated state of each phosphoprotein whereas total protein levels were determined by probing with context independent antibodies. Detection is by enhanced chemiluminescence (ECL).

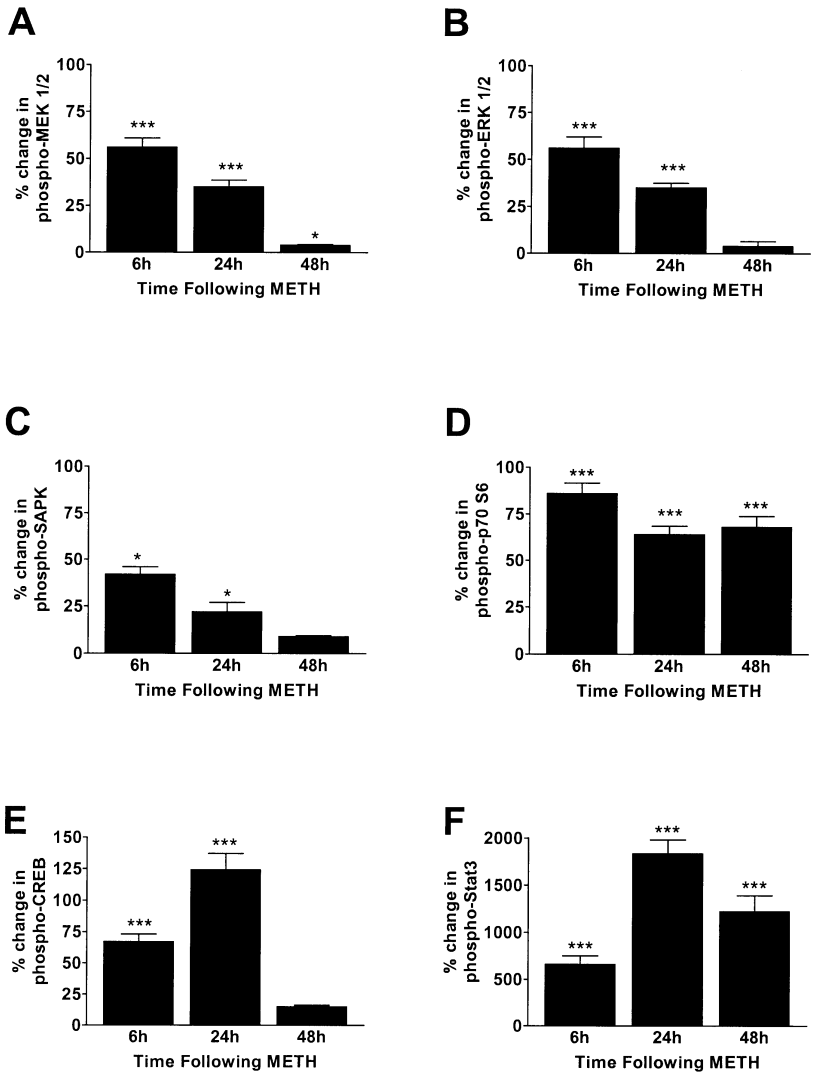

FIGURE 4. Time course of phosphoprotein activation following METH administration. Each value represents the mean ± SEM of at least 10 mice. Phosphoprotein levels were quantified by ECL analysis of immunoblots (represented in FIG. 3) probed with antibodies specific for the activated state of each phosphoprotein. The values are expressed as percentage change from control (saline-treated) levels. (**A**) phospho-MEK 1/2 (S217/221), (**B**) phospho-ERK 1/2 (T202/Y204), (**C**) phospho-JNK/SAPK (T183/Y185), (**D**) phospho-p70 S6 kinase (T389), (**E**) phospho-CREB (S133), and (**F**) phospho-Stat3 (Y705). $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, level of significance difference as compared to control group, C.

active forms of the phosphoproteins analyzed were not attributable to the increase of total protein amounts.

DISCUSSION

The identification and characterization of transduction events in response to chemical-induced neurotoxicity is an important step in understanding how the brain responds to and recovers from traumatic insult. Correlation of the time course of induction with intracellular events evoked by neurotoxicant exposure can provide important clues as to possible roles for these factors, in addition to furthering our understanding of the molecular basis for the pathology and recovery from neural injury that is essential for designing future therapeutic interventions.

Systemic administration of METH resulted in dramatic overexpression of GFAP, which was found to be associated with substantial activation of MAPK- and JAK-related signaling components. Phosphoproteins appeared to be activated in a wave-like fashion, with those closer to the cell membrane (phospho-MEK 1/2, -ERK 1/2, -SAPK) exhibiting maximal activation at an earlier time point (6 h) when compared to the transcriptional-phosphoproteins (CREB and STAT3), which peaked 24 h after METH treatment. Taken together, these results suggest that neurotoxicant exposure enhances the phosphorylation of MAP- and JAK-family members at time points that precede the maximal gliotic response.

Previous work from our laboratory demonstrated chemical injury-related phosphorylation of ERK 1/2 coincident with GFAP upregulation in mouse striatal homogenates.[67] We have confirmed and extended these findings by (1) using a structurally and mechanistically different compound to injury the same brain region, METH versus MPTP, (2) measuring the activation of components within other pathways as well as up- and downstream of ERK 1/2, and (3) examining more time points. Taken together, our studies suggest that activation of MAP- and JAK-family phosphoproteins as part of the neurotoxic response is temporally reproducible, coincident with GFAP induction, and may be a universal response—not dependent upon specific chemical toxicants.

Possible mechanisms responsible for MAPK- and JAK-related protein activation following neural damage include an array of upstream interactions of other signaling events resulting from receptor activation. Pathway components may be transiently or persistently activated, depending on the specific program(s) activated by the cell surface receptor/receptor complex, presence of complementary or antagonistic signals, and cell type in question. Thus, the biological context of a signal plays a determinative role in the way that receptor activation is interpreted. For example, although ERKs generally regulate cell growth and cell differentiation and JNK/SAPKs participate in a stress response, this is not always the case and in certain cell types activation of JNK/SAPKs can induce proliferation.[177] Interaction between ERK and JAK-STAT pathways results in a synergistic activation of certain genes;[178] ERKs can also antagonize JAK-STAT signaling in several systems.[179]

Consequently, the regulation of reactive gliosis and/or GFAP induction is most probably not dependent on the activation of one member of a signaling pathway, but rather on the convergence of a number of pathways, with the overall fate determined by the sum of these signals (FIG. 5). Delineation and correlation of these activation

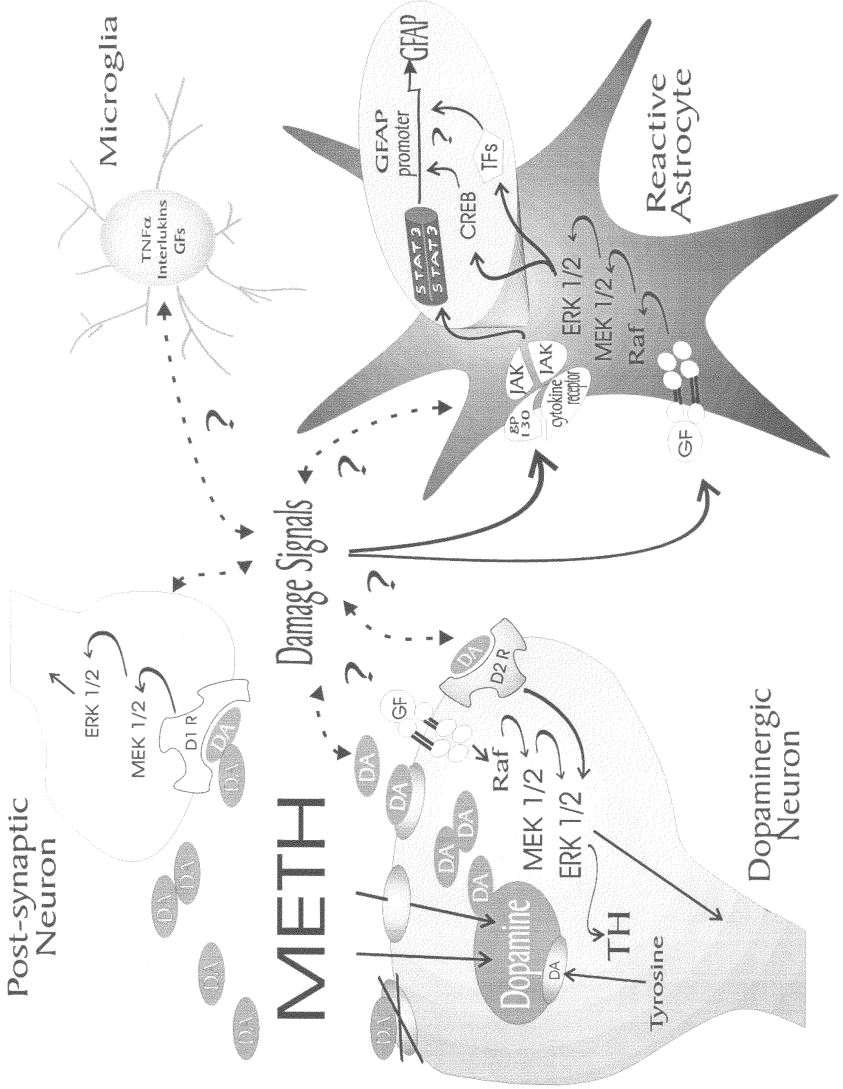

FIGURE 5. *See following page for caption.*

profiles not only provides a framework for understanding the mechanism of phosphoprotein signaling in neurotoxicant exposure and GFAP induction, but also serves to link specific input signal(s) to a universal biologic response to neural injury.

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FIGURE 5. Scheme illustrating a possible mechanism by which METH exposure activates specific MAP kinase signaling components in the mouse striatum. CREB, cAMP response element-binding protein; DA, dopamine; D_1 R, dopamine receptor type 1; D_2 R, dopamine receptor type 2; ERK 1/2, extracellular signal-regulated kinase 1/2; GFAP, glial fibrillary acidic protein; GF(s), growth factor(s); gp130, signal-transducing receptor component glycoprotein 130, JAK, Janus kinase; JNK/SAPK, *c-jun* NH_2_terminal kinase/stress-activated protein kinase; MEK 1/2, mitogen-activated protein kinase kinase or extracellular signal-regulated kinase kinase 1/2; Raf, a mitogen-activated protein kinase kinase kinase; STAT3, signal transducer and activator of transcription 3; TFs, transcription factors; TH, tyrosine hydroxylase; TNFα, tumor necrosis factor-α.

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