

Recapitulation of cell signaling events associated with astrogliosis using the brain slice preparation

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Abstract

Astroglial activation constitutes a dominant response to all types of injuries of the CNS. Despite the ubiquitous nature of this cellular reaction to neural injury, a little is known concerning the signaling mechanisms that initiate it. Recently, we demonstrated that astrocytic hypertrophy and enhanced expression of glial fibrillary acidic protein resulting from toxicant-induced neurodegeneration are linked to activation of the janus kinase (JAK)-signal transducer and activator of transcription-3 (STAT3) pathway. These observations implicate ligands at the gp130 receptor as potential upstream effectors of astrogliosis. Here we used the brain slice preparation to examine potential activators of the JAK-STAT3 pathway. Following incubation of freshly cut striatal slices in phosphate-free oxygenated buffer for up to 75 min, we found that slicing the striatum itself was a sufficient stimulus to initiate a rapid activation of the JAK-STAT3 pathway as assessed with immunoblots of

pSTAT3^(tyr705) using phospho-state specific antibodies. The mRNA for the gp130 cytokines, leukemia inhibitory factor, interleukin-6 and oncostatin M or the β -chemokine, monocyte chemoattractive protein (CCL2) also were up-regulated in the slice. Moreover, we could enhance the activation of STAT3^(tyr705) by adding exogenous cytokines to the slice and we could inhibit phosphorylation of STAT3^(tyr705) by addition of tyrosine kinase inhibitors (Lav A and AG490) or neutralizing antibodies directed against leukemia inhibitory factor or oncostatin M. These data suggest that STAT3 activation is an early event in slice-induced glial activation and establishes the brain slice preparation method as a reliable model to examine the signaling mechanisms that underlie glial activation.

Keywords: astrogliosis, signal transducer and activator of transcription-3, glial fibrillary acidic protein, protein phosphorylation, oncostatin M, leukemia inhibitory factor.

J. Neurochem. (2006) 10.1111/j.1471-4159.2006.04321.x

Astrocytic hypertrophy and the concurrent accumulation of glial fibrillary acidic protein (GFAP) are hallmark indicators of astroglial activation (Eng 1982; Norton *et al.* 1992; O'Callaghan 1993; Norenberg 1994; Eng *et al.* 2000; Panicar and Norenberg 2005). This cellular reaction, often termed 'reactive gliosis' or astrogliosis, occurs in response to 'damage' signals that emanate from the site of injury. Astrogliosis occurs in response to all types of CNS injuries, despite the regional, cellular, or molecular basis of a given insult. These observations imply that a common signaling mechanism(s) may underlie the conversion of astrocytes to their reactive state; however, such signaling pathways have yet to be fully elucidated.

Numerous signal transduction pathways have been implicated in astrocytic activation, *in vitro*, but few signaling cascades have been linked to the induction of gliosis, *in vivo* (Guillemin *et al.* 1996; Rajan and McKay 1998; Bajetto *et al.* 2001; Dunn *et al.* 2002). Recently, however, we used the

selective dopaminergic neurotoxicant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), to examine the *in vivo* signaling events associated with glial activation initiated by dopaminergic nerve terminal degeneration in the mouse striatum (Sriram *et al.* 2004, 2006, in press). We combined

Received July 12, 2006; revised manuscript received July 31, 2006; accepted September 7, 2006.

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Abbreviations used: CNTF, ciliary neurotrophic factor; DARPP-32, dopamine and cyclic AMP-regulated phosphoprotein-32; ECL, enhanced chemiluminescence; GFAP, glial fibrillary acidic protein; HRP, horseradish peroxidase; IL-6, interleukin-6; JAK2, janus kinase-2; LIF, leukemia inhibitory factor; MCP-1, monocyte chemoattractive protein; OSM, oncostatin M; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; STAT3, signal transducer and activator of transcription-3.

the use of phospho-state-specific antibodies with focused microwave irradiation sacrifice to preserve and examine steady state protein phosphorylation, *in vivo*. Astroglial activation in striatum, subsequent to MPTP-induced dopaminergic neurotoxicity, was linked to activation of the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway. Rapid, but transient phosphorylation of STAT3 was associated with induction of GFAP and astrocytic hypertrophy; STAT3 was activated via phosphorylation on Tyr⁷⁰⁵ and this required gp130 and JAK2 as upstream effectors. We now have documented a key role of the JAK-STAT3 pathway in the induction of astrogliosis in several additional *in vivo* models of toxicant-induced neurodegeneration (Sriram and O'Callaghan 2005; Sriram *et al.* 2006). Nevertheless, further insight into the role of JAK/STAT and other glial pathways must come from an experimental model that facilitates direct analysis of factors that initiate, inhibit, or alter signal transduction through specific signaling cascades.

A major complication of using an *in vivo* method to investigate the signaling pathways responsible for gliosis is that the pathway(s) cannot easily be manipulated within the whole animal. One potential means to overcome this drawback is to examine signaling events using a brain slice preparation. For example, this approach has been highly successful for discovering and characterizing the signaling events that regulate the key dopaminergic phosphoprotein, dopamine and cyclic AMP-regulated phosphoprotein-32 (DARPP-32) (Nishi *et al.* 1997; Bibb *et al.* 1999; Hamada *et al.* 2004). DARPP-32 is contained within medium spiny neurons, cells intrinsic to the neostriatum of rats and mice. As such, slice preparations of striatum can be used to evaluate agonist and antagonists of the various signaling modules that mediate or modulate signaling through the DARPP-32 containing neurons. Given this proven utility of the striatal slice preparation, we reasoned that it would be feasible to use the same preparation to evaluate signaling events associated with activation of the JAK-STAT3 pathway within striatal astrocytes. Discovery and characterization of upstream effectors of the JAK-STAT3 pathway would offer the potential for identifying and modulating signaling mechanisms responsible for astrogliosis, *in vivo*. Here we demonstrate that STAT3 activation can be initiated in the striatal slice and that activation can be modified by cytokines and tyrosine kinase inhibitors. Our data indicate that the brain slice preparation may serve as a useful model for discovery and manipulation of signal transduction events underlying reactive gliosis.

Experimental procedures

Materials

The JAK inhibitor, AG490 (tyrphostin B42), and the protein tyrosine kinase inhibitor, Lavendustin A (Lav A), were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA, USA) and

solutions were prepared in Me₂SO. Recombinant mouse oncostatin M (OSM), anti-mouse leukemia inhibitory factor (LIF), and anti-mouse OSM antibody were purchased from R&D Systems (Minneapolis, MN, USA). Recombinant mouse LIF was purchased from Chemicon International (Temecula, CA, USA). Phospho-state-specific antibodies to STAT3^(Tyr705), and the corresponding non-phospho (pan) antibodies to STAT3, were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG and enhanced chemiluminescence (ECL) immunoblotting substrate were purchased from Amersham Biosciences (Piscataway, NJ, USA). Nitrocellulose membranes were purchased from Schleicher & Schuell (Keene, NH, USA). One step reverse transcriptase-polymerase chain reaction (RT-PCR) kit was purchased from Qiagen (Valencia, CA, USA). All other chemicals and reagents utilized were of analytical grade and purchased from Sigma (St Louis, MO, USA).

Animals

Female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME, USA) aged 3-4 months were used as a source of tissue for striatal slices. Animals were housed in a temperature (21 ± 2°C) and humidity-controlled (40 ± 10%) colony room maintained on a 12 h light/dark cycle. Animals were allowed *ad libitum* access to chow and water. All animal experiments were carried out in accordance with the Centers for Disease Control and Prevention Guidelines for Care and Use of Laboratory Animals. All procedures were performed under protocols approved by the Institutional Animal Care and Use Committee of Centers for Disease Control and Prevention-National Institute for Occupational Safety and Health (CDC-NIOSH). The CDC-NIOSH animal facility is accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

Preparation and incubation of striatal slices

Mice were sacrificed by decapitation and brains immediately were removed and submerged in oxygenated, ice-cold phosphate-free buffer (124 mmol/L NaCl, 4 mmol/L KCl, 26 mmol/L NaHCO₃, 10 mmol/L D-glucose, 1.5 mmol/L CaCl₂, and 1.5 mmol/L MgSO₄, pH 7.4). Coronal slices (350 μmol/L) were prepared using a Vibratome (Technical Products International, St Louis, MO, USA, Series 3000) and remained submerged in ice-cold oxygenated buffer while striata were dissected from individual sections. Individual striata then were immediately frozen or placed in polypropylene incubation tubes with 2 mL of fresh, oxygenated phosphate-free buffer and incubated at 30°C under constant oxygenation with 95% O₂/5% CO₂ for 15, 30, 45, 60, or 75 min. Incubated slices were treated with kinase inhibitors, cytokines or antibodies, as specified. After incubation, slices were transferred to Eppendorf tubes, frozen on dry ice, and stored at -80°C until assayed for total protein and specific mRNA, protein, or phosphoprotein, as specified below.

Immunoblotting

Frozen samples were placed in boiling 1% sodium dodecyl sulfate (SDS) and sonified before assay for total protein. Total protein concentration of the SDS homogenates was assayed by the method of Smith *et al.* (1985). Bovine serum albumin was used as the standard. Aliquots of the total protein homogenate were assayed for

changes in expression of p-STAT3^(tyr705) using immunoblots. A linear range for the protein load in the immunoblot analysis of these proteins previously has been established in our laboratory (O'Callaghan *et al.* 1999; Sriram *et al.* 2004). Aliquots of the sample homogenate were diluted in sample buffer, loaded onto 10% acrylamide gels, resolved by SDS-PAGE, and electrophoretically transferred to 1.0 mmol/L nitrocellulose membranes. Membranes were blocked with Blotto in PBS-T (phosphate buffered saline including 0.1% Tween-20), washed in PBS-T for 5 min (3X) and incubated overnight at 4°C with primary antibodies (rabbit anti-phospho-STAT3; 1:500). Blots then were washed and incubated with HRP-conjugated anti-rabbit IgG (1:2500) in blocking buffer for 1 h at room temperature (21°C). Antibody binding was determined using ECL immunoblotting detection. Exposed films were scanned and quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). All data were obtained from the linear portion of the densitometry curves.

Reverse transcriptase-polymerase chain reaction

Total RNA was isolated from the frozen slices using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Isolated RNA was quantified by spectrophotometry and 0.2 µg was combined with the following Qiagen One-step RT-PCR reagents (final concentrations): 1X Qiagen One-step RT-PCR Buffer, 400 µM dNTPs, 0.5 µg sense primer, 0.5 µg antisense primer, 1X Q solution, 2 µL Qiagen One-step RT-PCR Enzyme Mix, 5 units RNase inhibitor, and water to a total volume of 50 µL. Semiquantitative RT-PCR was done in a Perkin Elmer (Foster City, CA, USA) GeneAmp PCR System 9700. Reverse transcription was carried out at 50°C for 30 min then DNA polymerase was activated by heating for 15 min at 95°C. Amplification was performed for 35 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 45 s, and extension for 1 min at 72°C with an additional 10 min, 72°C extension added to the final cycle.

All primers were custom ordered from Biosource International. The specific primers used were CCL-2/MCP-1(303 bp) (sense primer) 5'-GTTGGCTCAGCCAGATGCAGT-3' and (antisense primer) 5'-TTTACGGGTCAACTTCACATTCAA-3', LIF (388 bp) (sense primer) 5'-GCCACGGCAACCTCATGA-3' and (antisense primer) 5'-GACAGGTGGCAGATCCACG-3', interleukin 6 (IL-6, 154 bp) (sense primer) 5'-TGGAGTCACAGAAGGAGTGGC-TAAG-3' and (antisense primer) 5'-TCTGACCACAGTGAGG-AATGTCCAC-3', ciliary neurotrophic factor (CNTF, 243 bp) (sense primer) 5'-ACCTGACTGCTCTTATGGAA-3' and (antisense primer) 5'-GTATGTATTGCCTGATGGAA-3', OSM (266 bp) (sense primer) 5'-AACACTGCTCAGTTTGACCC-3' and (antisense primer) 5'-ACAGTGCTCAGG AAGTGAGG-3' and GFAP (462 bp) (sense primer) 5'-CGAAGCTAACGACTATCGCC-3' and (antisense primer) 5'-TCACATCACACGTCCTTGT-3'. GAPDH (276 bp; sense primer-5'-TGAAGGTCGGTGTGAACGGATT-TGG-3' and antisense primer -5'-ACGACATACTCAGCACCGG-CCTCAC-3') amplification was used as a control for template integrity and normalization of data. PCR products were separated on 2% agarose gels and bands were visualized using ethidium bromide.

Statistical analysis

All results are expressed as the mean ± SEM for each group. Statistical analysis of the difference between groups was assessed using one-way analysis of variance. A *p*-value of <0.05 was

considered statistically significant. Where statistical analyses are not shown, the data are representative of at least three replicate experiments for all time points and conditions.

Results

From our previous *in vivo* experiments, we knew that striatal dopaminergic nerve terminal degeneration because of MPTP initiated a rapid activation of the JAK2-STAT3 pathway that was associated with translocation of STAT3 to the nucleus of astrocytes and a subsequent induction of GFAP. We also knew that, unlike most phosphorylated substrates we have examined, *in vivo* (O'Callaghan and Sriram 2004), the astrocytic phosphorylation of STAT3^(tyr705) was retained post-sacrifice, even in the absence of focused microwave fixation to preserve phosphorylation. These prior *in vivo* findings raised the possibility that, within a striatal slice preparation, STAT3^(tyr705) phosphorylation could be induced or augmented following the addition of putative upstream effectors in the JAK-STAT3 pathway. An increase in pSTAT3^(tyr705) because of the addition of candidate effectors could be taken as evidence for their role in the induction of astrogliosis, *in vivo*. To determine if pSTAT3^(tyr705) could be detected and/or induced in the slice, we began by incubating untreated striatal slices at 30°C in phosphate-free oxygenated buffer for various times (0, 15, 30, 45, 60, or 75 min) and then assaying for STAT3^(tyr705) phosphorylation. Basal phosphorylation of STAT3^(tyr705) is very low, *in vivo*; therefore, we expected to see only a low level of pSTAT3^(tyr705) in untreated slices assayed immediately after dissection. Indeed, these slices (0 time point) had virtually undetectable levels of phospho-STAT3^(tyr705) (Fig. 1). Following incubation in phosphate-free buffer; however, STAT3^(tyr705) phosphorylation was increased in a time-dependent manner over the 75-min incubation period (Fig. 1) and did not increase further with longer incubations. At the final time point examined (75 min), phosphorylation of STAT3^(tyr705) was increased over 20-fold (Fig. 1). Non-phospho-STAT3 levels remained unchanged over the entire incubation period (data not shown). These data suggested that the slice procedure itself served as a stimulus to induce activation of the JAK-STAT3 pathway. This rapid STAT3 activation appeared to be a recapitulation of the damage-induced response observed over several hours *in vivo*. If this was the case, an enhanced expression of upstream effectors in the JAK-STAT3 pathway should be observed in the slice, as they are seen *in vivo* (Sriram *et al.* 2004, 2006). To evaluate this possibility, we examined slices for the expression of mRNA for the gp130 cytokines, LIF, OSM, IL-6, and CNTF, as well as for the chemokine, MCP-1. We found that LIF, MCP-1, OSM, and IL-6 mRNA showed enhanced expression over the 75 min time-course of slice incubation (Fig. 2), mimicking previously described results from *in vivo*

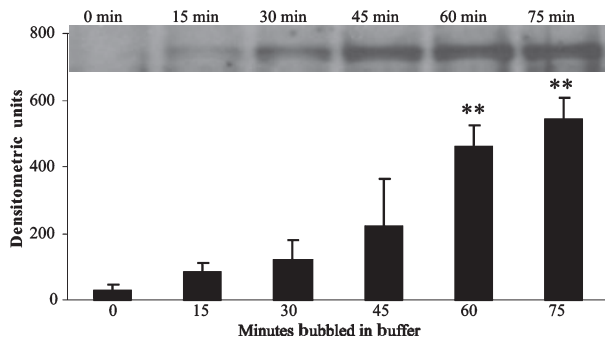


Fig. 1 Phosphorylation of signal transducer and activator of transcription-3 (tyr705) is increased in a time-dependent manner in the striatal slice preparation. Representative immunoblot analysis of phosphorylation-signal transducer and activator of transcription-3 (Tyr705) in striatal slices at 0, 15, 30, 45, 60, or 75 min post-preparation (upper panel) and densitometric scans of immunoblots (lower panel). Graph values represent mean \pm SEM for three independent observations. ** $p < 0.01$ compared with 0 min (untreated) control; One-way analysis of variance (ANOVA). These images were prepared in accordance with the Provisional Guide for Digital images (<http://www.nature.com/nature/authors/submissions/images/index.html>).

studies (Sriram *et al.* 2004, 2006). At 75 min post-incubation, mRNA for the chemokine MCP-1 showed the most dramatic increase in expression (280% of control), while mRNA for the gp-130 cytokines, IL-6, OSM, and LIF, had more modest increases (151%, 140% and 155% of control, respectively). Messenger RNA for CNTF, however, was not enhanced over the duration of the incubation time course (54% of control after 75 min incubation). These data suggest that many of the early events associated with the induction of astrogliosis *in vivo* can be recapitulated in the striatal slice over a 75-min period after the slice-induced 'injury'. We were not able to extend these observations to the induction of mRNA for GFAP; within the 75-min incubation period, there was no detectable change in GFAP mRNA expression (Fig. 2).

To determine if the JAK2/STAT3 signaling pathway could be manipulated in the slice, we examined the effects of incubating slices with tyrosine kinase inhibitors, exogenous LIF, exogenous OSM, or antibodies to LIF or OSM. To inhibit JAK2 phosphorylation of STAT3, slices were incubated with either a JAK2 inhibitor, AG490 (tyrphostin B42), or a general tyrosine kinase inhibitor, Lavendustin A (Lav A). Incubation with either AG490 or Lav A attenuated phosphorylation of STAT3 (36% and 46% of buffer control, respectively) (Fig. 3). Incubation of slices with LIF or OSM, known upstream effectors in the JAK2-STAT3 pathway, augmented the pSTAT3 phosphorylation (141% and 129% of buffer control, respectively) seen over the 45-min incubation period (Fig. 4). This effect of exogenously added cytokine could be blocked by co-incubation with anti-LIF or anti-OSM antibodies (82% and 109% of buffer control) (Fig. 4).

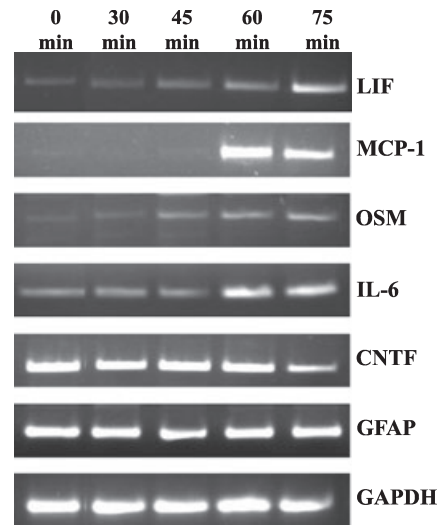


Fig. 2 Relative mRNA expression levels for various cytokines and chemokines in the striatal slice preparation. Striatal mRNA expression of monocyte chemoattractive protein-1 (MCP-1), leukemia inhibitory factor (LIF), oncostatin M (OSM), interleukin-6 (IL-6), ciliary neurotrophic factor (CNTF), and glial fibrillary acidic protein (GFAP) was examined by reverse transcriptase-polymerase chain reaction using template specific primers with GAPDH serving as an internal control. Agarose gel analysis shows a band of expected size in each case (LIF 388 bp, MCP-1 303 bp, OSM 266 bp, IL-6 154 bp, CNTF 243 bp, GFAP 462 bp, GAPDH 276 bp). Lanes were loaded with RNA prepared from slices as follows: 0 min (frozen immediately), 30, 45, 60, and 75 min post-preparation. Representative gels from at least three independent experiments are displayed. mRNA expression levels for MCP-1, OSM, and IL-6 at 60 min were significantly increased over 0 min control ($p < 0.05$ – 0.01 , one-way ANOVA). mRNA expression for LIF at 75 min was significantly increased over 0 min control ($p < 0.01$; one-way ANOVA). CNTF and GFAP did not differ significantly from 0 min control at any time point. These images were prepared in accordance with the Provisional Guide for Digital images (<http://www.nature.com/nature/authors/submissions/images/index.html>).

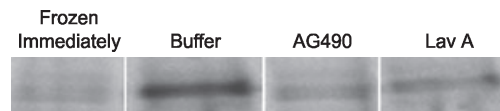


Fig. 3 Phosphorylation of STAT3^(tyr705) is attenuated by addition of tyrosine kinase inhibitors to the striatal slice preparation. Immunoblot analysis of P-STAT3^(tyr705) in striatal slices following a 45 min incubation with 100 μ mol/L AG490 or 100 μ mol/L Lavendustin A. These images were prepared in accordance with the Provisional Guide for Digital images (<http://www.nature.com/nature/authors/submissions/images/index.html>).

Discussion

We have demonstrated that incubation of fresh slices of mouse striatum, *in vitro*, results in the activation of many cell-signaling events associated with the induction of astro-

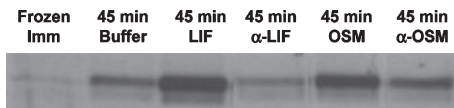


Fig. 4 Phosphorylation of STAT3^(tyr705) is increased by addition of the gp130 cytokines, leukemia inhibitory factor (LIF) and oncostatin M (OSM), to the striatal slice preparation. These effects are antagonized by addition of neutralizing antibodies directed against these cytokines. Immunoblot analysis of P-STAT3^(tyr705) in striatal slices following incubation in buffer alone, 100 $\mu\text{mol/L}$ leukemia inhibitory factor (LIF), 100 $\mu\text{mol/L}$ OSM, or co-incubation with 50 μg $\alpha\text{-LIF}$ or 50 μg $\alpha\text{-OSM}$ for 45 min. These images were prepared in accordance with the Provisional Guide for Digital images (<http://www.nature.com/nature/authors/submissions/images/index.html>).

gliosis, *in vivo*. Phosphorylation of STAT3^(tyr705) and enhanced expression of mRNA for ligands for the gp130/JAK2-STAT3 pathway can be observed within 30–60 min of slice incubation. These astroglial activation events, known to be associated with the induction of striatal astrogliosis, *in vivo*, can be antagonized by tyrosine kinase inhibitors, augmented by exogenous cytokines, and inhibited by addition of antibodies to the same cytokines. These findings imply that many of the early cell-signaling events associated with the induction of astrogliosis over several hours, *in vivo*, can be recapitulated, *in vitro*, with the slice ‘injury’ serving as the initiating damage stimulus. A schematic representation of our findings appears in Fig. 5.

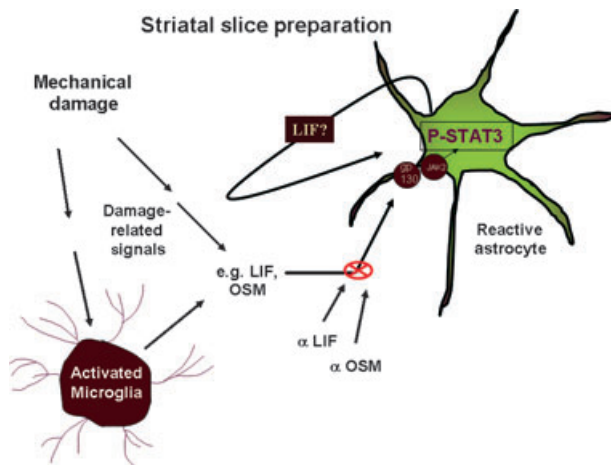


Fig. 5 Mechanical damage associated with preparation of striatal brain slices results in recapitulation of cell signaling events associated with astrogliosis. For example, slice-induced or exogenously applied leukemia inhibitory factor (LIF) or oncostatin M (OSM) results in the enhanced phosphorylation of STAT3^(tyr705), an event known to be associated with activation of astrocytes. LIF, OSM and other mediators may emanate from the damaged cells, activated microglia or even the astrocyte itself. The effects of LIF and OSM on phosphorylation of STAT3^(tyr705) can be blocked by addition of neutralizing antibodies ($\alpha\text{-LIF}$ or $\alpha\text{-OSM}$) to the slice.

Before undertaking the present study, we established the key role, *in vivo*, for the gp-130/JAK2-STAT3 signal transduction pathway in mediating the astroglial reaction to dopaminergic nerve terminal damage instigated by administration of MPTP. Because astrogliosis is a generalized reaction to neural damage, irrespective to the type or location of the insult, we now have begun to establish the role of JAK-STAT3 signaling in glial activation that occurs following diverse insults affecting multiple areas of the brain. As suggested by our extensive use of MPTP as a striatal denervation tool; however, most of the data we have compiled on injury-related glial signaling events has been associated with agents and conditions known to cause or mitigate damage to the striatum. This fact, combined with the long-standing use of striatal slices for analysis of dopaminergic signaling in this structure, led us to utilize this preparation in the current study. We have no reason to believe that the results we obtained for glial signaling in slices of striatum will not apply to slices prepared from other brain structures, but that possibility remains to be demonstrated.

Using phosphorylation of STAT3 as a means to assess activation of astroglial signaling pathways *in vitro*, we expected to see low basal levels of STAT3^(tyr705) as we did, *in vivo*, and we planned to add candidate upstream effectors to the slice to activate the pathway. However, we abandoned this initial strategy when we found increasing levels of STAT3^(tyr705) with increasing slice incubation times, findings suggesting that the slice itself served as an injury stimulus for pathway activation. That this indeed was the case was supported by the observed time-dependent increases of the mRNA for multiple upstream effectors in the JAK2-STAT3 pathway. The up-regulation of the gp130 cytokines, IL-6, LIF and OSM, as well as the enhanced expression of the beta chemokine, MCP-1, all had previously been observed, *in vivo*, before the activation of STAT3^(tyr705) in multiple models of toxicant-induced astrogliosis (Patterson 1994; Banner *et al.* 1997; Gadiant and Patterson 1999; Sugiura *et al.* 2000; Little *et al.* 2002; Sriram *et al.* 2004; Sriram and O’Callaghan 2005). The lack of induction of CNTF mRNA in the slice was not unexpected because, *in vivo*, its enhanced expression occurs several hours after the up-regulation of other gp-130 cytokines (Sriram *et al.* 2004). As with CNTF mRNA, we failed to observe an increase in GFAP mRNA in the slice. While GFAP expression (mRNA and protein) is a key feature of astroglial activation, it is downstream from STAT3 activation, *in vivo*, and an increase in GFAP mRNA expression is not seen until several hours after IL-6, LIF, and OSM mRNA levels are up-regulated (Sriram *et al.* 2004). Together, these observations raise the possibility that extending slice incubation times beyond the 75-min period used in the present study might result in increased expression of CNTF and GFAP mRNA. This

option may not be possible, however, due to limitations in the viability of the slice preparation over extended time periods. Whether GFAP mRNA can or cannot be demonstrated in the slice preparation does not detract from the use of this preparation to evaluate signaling events leading to astroglial activation. Our data now clearly demonstrate that a mechanical slice injury recapitulates many of the signal transduction events required to phosphorylate STAT3^(tyr705), an early hallmark of the induction of astrogliosis, *in vivo*, (Sriram *et al.* 2004; Sriram and O'Callaghan 2005).

While our serendipitous finding that the preparation of a brain slice sets in motion the signaling events known to underlie astrogliosis, *in vivo*, we had not achieved our original goal of developing a preparation for use in manipulating glial signal transduction, *in vitro*. To do so, we attempted to activate and inhibit the JAK2-STAT3 pathway at two different levels. We initially utilized two tyrosine kinase inhibitors to inhibit JAK2 and prevent phosphorylation of STAT3. LavA and AG490 both abrogated the phosphorylation of STAT3, even after a 45 min incubation in oxygenated buffer, inhibiting STAT3 phosphorylation to a similar degree. These data suggested that astroglial signaling initiated by the slice could be manipulated. To determine if we could affect JAK/STAT signaling at the ligand level, striatal slices were incubated with the gp130 cytokines, LIF or OSM. Both of these cytokines augmented the phosphorylation of STAT3^(tyr705) and neutralizing antibodies blocked these effects, observations suggesting that these particular gp-130 ligands play a role in astroglial activation responses associated with striatal injury. LIF is known to be expressed in astrocytes and to play a key role in the injury response of the CNS by modulating astrocyte and microglial reactions to CNS damage (Patterson 1994; Banner *et al.* 1997; Gadiant and Patterson 1999). LIF is produced by glia, can act directly on neurons and glia, and is up-regulated in glial cells post-CNS injury (Sugiura *et al.* 2000). The gp-130 cytokine most closely related to LIF (functionally, structurally, and genetically) is OSM (Gearing 1993; Gearing *et al.* 1993). In the central nervous system OSM is produced by microglia (Ruprecht *et al.* 2001; Repovic and Benveniste 2002; Chen and Benveniste 2004). Specifically, within astrocytes, OSM also is known to induce expression of IL-6 and MCP-1 within astrocytes (Van Wagoner *et al.* 2000; Ruprecht *et al.* 2001; Chen and Benveniste 2004) and OSM receptor binding initiates STAT3 phosphorylation (Schaefer *et al.* 2000; Van Wagoner *et al.* 2000; Heinrich *et al.* 2003; Repovic *et al.* 2003; Chen and Benveniste 2004). In addition, OSM mediates astrocytic differentiation and the induction of GFAP through a STAT3 dependent pathway (Yanagisawa *et al.* 1999). Thus, our present observations using the striatal slice are consistent with the known effects of LIF and OSM linked to astrocytic responses.

Taken together, these data indicate that the brain slice preparation can be used to uncover and characterize the components of signal transduction pathways that lead to activation of STAT3-mediated cellular responses that are presumed to underlie astrogliosis. At a minimum, we have demonstrated that the striatal brain slice provides a tool to dissect complex signaling events leading to the phosphorylation of STAT3, knowledge of which will serve to increase our understanding of signaling related to injury-related astrocytic activation, *in vivo*.

Acknowledgements

We acknowledge Brenda Billig and Christopher Felton for excellent technical assistance.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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