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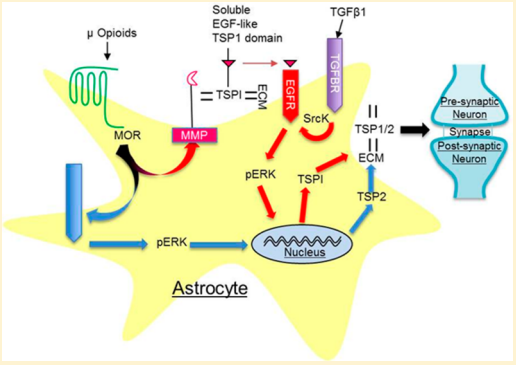
Acute and Chronic Mu Opioids Differentially Regulate Thrombospondins 1 and 2 Isoforms in Astrocytes

Ellen Phamduong, Maanjot K. Rathore, Nicholas R. Crews, Alexander S. D'Angelo, Andrew L. Leinweber, Pranay Kappera, Thomas M. Krenning, Victoria R. Rendell, Mariana M. Belcheva,[†] and Carmine J. Coscia*

E. A. Doisy Department of Biochemistry and Molecular Biology, St. Louis University of Medicine, 1100 S. Grand Blvd., St. Louis, Missouri, 63104, United States

ABSTRACT: Chronic opioids induce synaptic plasticity, a major neuronal adaptation. Astrocyte activation in synaptogenesis may play a critical role in opioid tolerance, withdrawal, and dependence. Thrombospondins 1 and 2 (TSP1/2) are astrocyte-secreted matricellular glycoproteins that promote neurite outgrowth as well as dendritic spine and synapse formation, all of which are inhibited by chronic μ opioids. In prior studies, we discovered that the mechanism of TSP1 regulation by μ opioids in astrocytes involves crosstalk between three different classes of receptors, μ opioid receptor, EGFR and TGFβR. Moreover, TGFβ1 stimulated TSP1 expression via EGFR and ERK/MAPK activation, indicating that EGFR is a signaling hub for opioid and TGFβ1 actions. Using various selective antagonists, and inhibitors, here we compared the mechanisms of chronic opioid regulation of TSP1/2 isoform expression in vivo and in immortalized rat cortical astrocytes. TSP1/2 release from astrocytes was also monitored. Acute and chronic μ opioids, morphine, and the prototypic μ ligand, DAMGO, modulated TSP2 protein levels. TSP2 but not TSP1 protein content was up-regulated by acute (3 h) morphine or DAMGO by an ERK/MAPK dependent mechanism. Paradoxically, TSP2 protein levels were altered neither by TGFβ1 nor by astrocytic neurotrophic factors, EGF, CNTF, and BMP4. TSP1/2 immunofluorescence was increased in astrocytes subjected to scratch-wounding, suggesting TSPs may be useful markers for the "reactive" state of these cells and potentially for different types of injury. Previously, we determined that chronic morphine attenuated both neurite outgrowth and synapse formation in cocultures of primary astrocytes and neurons under similar temporal conditions that μ opioids reduced TSP1 protein levels in astrocytes. Here we found that, after the same 8 day treatment, morphine or DAMGO diminished TSP2 protein levels in astrocytes. Therefore, μ opioids may deter synaptogenesis via both TSP1/2 isoforms, but by distinct mechanisms.



KEYWORDS: *Opioids, morphine, opioid receptors, astrocytes, ERK/MAPK, growth factors*

Astrocytes make integral contributions to neuronal signaling in the brain by releasing molecules that act at synapses during different stages of synaptogenesis. These phases include pre- and postsynaptic development, function, maintenance, and plasticity.[1−7] One family of key astrocyte-secreting molecules includes thrombospondins (TSPs), oligomeric, multidomain glycoproteins that promote synapse formation in vivo and in vitro.[8−14] Yet another molecule is TGFβ that induces excitatory synapses at least in part via a D-serine dependent mechanism.[15] Interestingly, TGFβ and TSPs maintain a reciprocal relationship in astrocytes. Early on, it was recognized that TSP1 activates TGFβ.[16] Alternatively, several independent research groups have reported that in astrocytes TGFβ increases TSP1 gene expression and/or protein levels as shown by immunoblotting, qRT-PCR, and in situ hybridization.[17−20] Both of these processes appear to be initiated in the extracellular matrix (ECM) of astrocytes, wherein TSP levels are far more abundant than in neurons. TSP1/2s are then released from the ECM and interact with $\alpha_2\delta$-1subunits of a voltage-gated calcium channel at neuronal sites to initiate synaptic adhesion and recruitment of scaffolding proteins.[11] Additional evidence supports the existence of an interaction of TSPs with neuronal integrins and neuroligin 1 that is followed by cAMP dependent PKA activation down stream in the cell.[5,8,13,21]

Of the five TSP isoforms that are known to occur in mammalian CNS, TSP1 and TSP2 are detectable in developing brain (P5-8 in the rat) but not in normal adult brain.[9] In vivo studies have been conducted with wild type, TSP1 null, TSP2 null and TSP1/2 double null mice wherein synaptic puncta were measured in cortical fields of postnatal rats of different ages by immunohistochemical staining. Significant deficiencies in synaptic puncta were detected in TSP1/2 double KO mice but neither in TSP1 null nor TSP2 null mice. However, TSP1 but not TSP2 promotes neurogenesis in neural progenitor cells.[22] TSP2 has different roles during development such as potentiating Notch 3 signal transduction.[23,24] Differential

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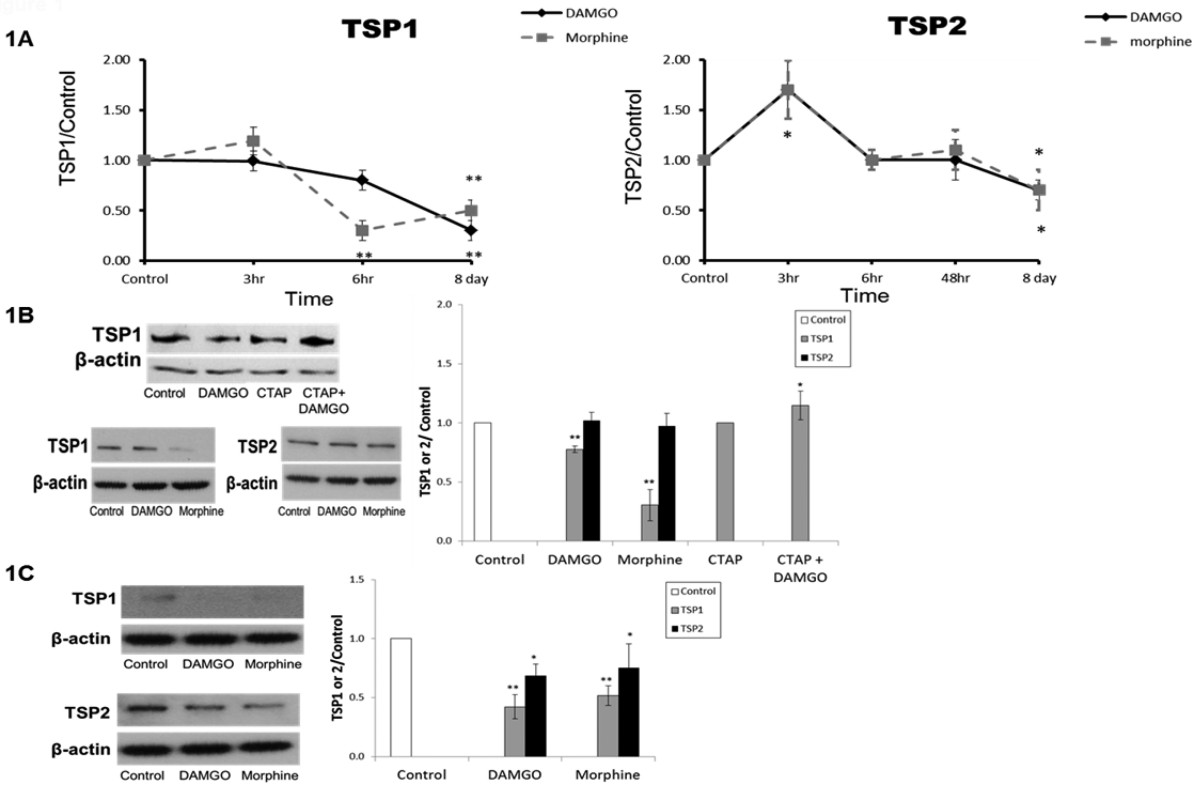

Figure 1. μ opioid modulation of cellular TSP1/2 protein levels in astrocytes. (A) Time course study of μ opioid actions. Oprm1-transfected cells in media devoid of serum were treated with 1 μM morphine or 1 μM DAMGO for 3 h, 6 h, 48 h, or 8 days, cells were lysed, and the lysate subjected to immunoblotting for TSP1/2. N = 12. (B) Cells were preincubated with 1 μM CTAP for 1 h and then treated with 1 μM morphine or 1 μM DAMGO for 6 h before immunoblotting. Left panel: Representative immunoblots. Right panel: Densitometric analyses of TSP1 and TSP2 protein levels. N = 6. (C) Astrocytes were treated daily with 1 μM DAMGO or 1 μM morphine for 8 days in media devoid of serum, and 24 h later TSP1/2 protein levels were monitored by immunoblotting. Left panel: Representative immunoblots. Right panel: Densitometric analyses of TSP1/2 protein levels. N = 6. *p < 0.05, **p < 0.01 vs control.

temporal and neural cell activation of TSP1 and 2 have been detected in adult brain and spinal cord tissue repair following cerebral ischemia, oxidative stress, foreign body reaction and ensuing synaptic plasticity.[25−30] Reactive astrogliosis, the astrocytic response to central nervous system (CNS) injury, has been characterized by increased proliferation and GFAP expression in these cells.[31] An accompanying reactive astrocyte-rich glial scar forms to quarantine the affected area while microglia and macrophages invade and digest cellular debris.[32] In vivo studies of rat spinal cord or brain reveal that after injury, TSP1, TSP2, and TGFβ mRNA and/or protein expression increase at the wound site.[33−37]

The targets of opiate drugs of abuse are μ, κ, and δ opioid receptors (Oprm, Oprk and Oprd, respectively), GPCRs that were found in astrocytes together with endogenous opioid peptides in vitro and in vivo.[38−46] In vivo and in vitro studies revealed that various opioids and especially morphine can negatively influence neurite outgrowth, dendritic spine density, and neuronal morphology.[20,47−51] In prior studies, we characterized the mechanism of opioid modulation of the expression and release of TSP1 in astrocytes in vitro. We also correlated the inhibition of TSP1 by chronic morphine with an abatement of neurite outgrowth and synapse formation in primary astrocyte-neuron cocultures. Our data indicate that the modulation of TSP1 by chronic morphine involves crosstalk between three different classes of receptors and the mediation of ERK/MAPK in immortalized rat cortical astrocytes.[20] Using various selective antagonists, inhibitors, and prototypic ligands, we implicated the Oprm, EGFR, and TGFβR. Oprm initiates matrix metalloprotease (MMP) mediated EGFR transactivation that results in ERK activation.[43,44] TGFβ1 also stimulates TSP1 expression via EGFR transactivation and ERK phosphorylation. Moreover, chronic morphine inhibits TGFβ1-induced TSP1 expression, possibly via a pERK-mediated feedback down regulation of EGFR. Therefore, our studies indicated that EGFR is a signaling hub for opioid and TGFβ1 modulated TSP1 expression. Here we report that in astrocytes TSP2 is up regulated by acute μ opioids via an ERK/MAPK-dependent process and down regulated by chronic μ opioids through a TGFβ1-independent mechanism.

RESULTS

Acute and Chronic Opioid Modulation of TSP2 Expression in Vivo and in Vitro. In prior studies on opiate modulation of TSP1 expression, adult rats were treated with chronic buprenorphine (2.5 mg/kg/day) for 14 days, followed by spontaneous withdrawal for 9 days. Nucleus accumbens tissue was dissected out of brains stereotaxically and subjected to high throughput Affymetrix GeneChip expression array analyses. A total of 302 buprenorphine responsive genes from the complete rat genome array (>30 000 transcripts) were expressed. Verification by RT-PCR revealed that one of the buprenorphine responsive genes, TSP1, was down-regulated 4-fold.[20] Here we report that additional microarray analyses of the data revealed that TSP2 mRNA levels were also down-regulated 4-fold (α = 0.01; N = 3).

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Originally, we discovered that TSP1 protein expression was diminished upon exposure to morphine for 6 h, 2 days, or 8 days in immortalized rat cortical astrocytes.[20] In Figure 1A, we determined by immunoblotting acute and chronic effects of the prototypic Oprm ligand, DAMGO as well as morphine, on both TSP1 and TSP2 protein levels in the same line of immortalized astrocytes. After 3 h, μ opioids up-regulated TSP2 by 70% but did not affect TSP1. In contrast, 6 h μ opioid exposure resulted in down-regulation of cellular TSP1, as seen before for morphine, but TSP2 protein levels were unchanged. The highly selective Oprm antagonist, CTAP, completely reversed this inhibitory action of both opioid ligands on TSP1 protein levels thereby implicating this receptor in the signaling mechanism (Figure 1B). Alternatively, 8-day morphine and DAMGO down-regulated both TSP 1 and 2 protein levels to a similar extent (Figure 1C). Under the same chronic morphine treatment, we found that primary neurons cocultured with primary astrocytes displayed a 40−50% reduction in neurite outgrowth and synaptic puncta formation.[20]

In prior studies, we demonstrated that acute and chronic morphine induced modulation of TSP1 expression via an ERK dependent mechanism that entailed EGFR transactivation.[20] Preincubation of astrocytes with U0126, the highly specific MEK inhibitor that blocks ERK phosphorylation, abolished acute μ opioid up regulation of cellular TSP2 levels (Figure 2A). Interestingly, in these experiments, basal levels of TSP1 were also reduced, suggesting the existence of a constitutively active ERK pathway that induces TSP1 expression by endogenous factors being generated by astrocytes. When the population of TSP1/2 in astrocyte media was measured under the same 3h conditions, we found that U0126 also blocked basal levels of both secreted isoforms (Figure 2B). Agonists had no detectable effect on secreted TSP2 consistent with the lack of acute effects on secreted TSP1 levels in our prior experiments (see Figure 3B in ref 20). There we had to treat astrocytes with agonist for 24 h before seeing a statistically significant change in TSP1 media levels. Therefore, it appears that acute opioid regulation of TSP1/2 is mediated by ERK. Moreover, it is possible that autocrine TGFβ1stimulation of TSP1 that we previously demonstrated to be ERK-dependent may account for the loss of basal levels of TSP1 in the presence of U0126. To determine whether EGFR transactivation was involved in the ERK pathway associated with TSP regulation, astrocytes were treated with EGF for 24 h and both cellular and secreted TSP1/2 protein levels were measured (Figure 2C). Only secreted TSP1 levels were significantly elevated and the response was robust (>6-fold). Based on our previous evidence, at shorter time intervals EGF would be expected to up regulate cellular TSP1 significantly (see Figures 3B and 6 in ref 20). Here it appears that 24 h EGF promoted secretion of TSP1 at the expense of cellular levels.

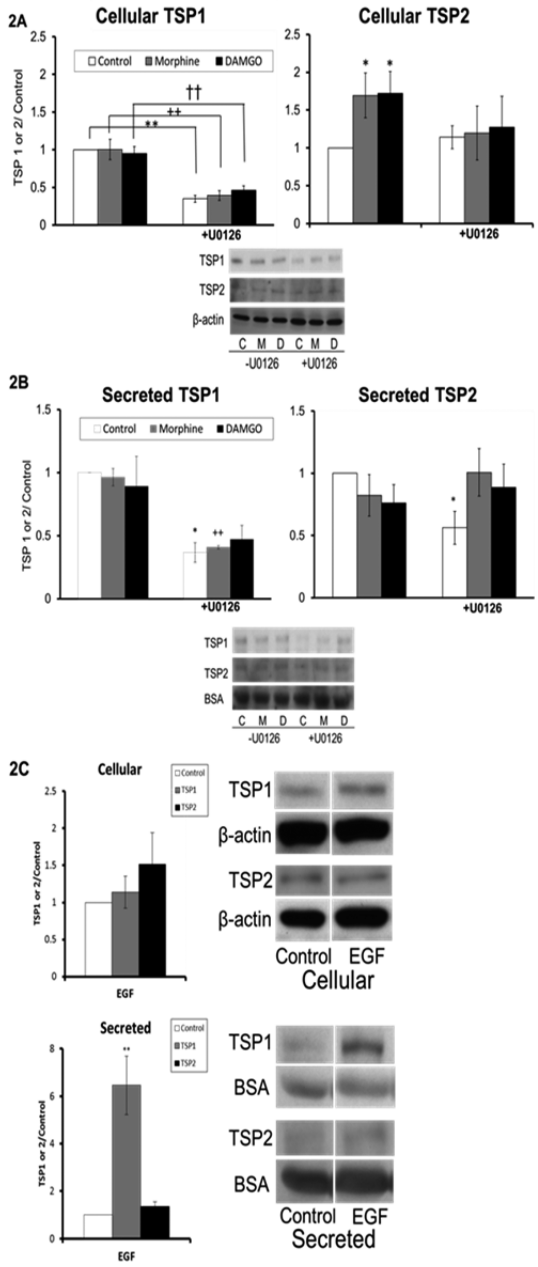

Figure 2. Acute μ opioid modulation of cellular and/or secreted TSP1/2 protein levels in astrocytes is ERK/MAPK dependent. (A) Oprm1-transfected astrocytes were preincubated with 1 μM U0126 for 1 h and then treated with 1 μM morphine or 1 μM DAMGO for 3 h. Top panels: Representative gels of cellular TSP1/2 bands Bottom panel: Densitometric analysis. $^{**}p < 0.01$ control vs treated. $^{++}p < 0.01$ agonist treated vs untreated. $N = 4-9$. (B) Astrocyte-secreted TSP1/2 levels were also measured after the treatment in the (A) panel. Left panels: Representative gels. Right panels: Densitometric analysis. $^{*}p < 0.05$ control vs treated. $^{++}p < 0.01$ agonist treated vs untreated; $N = 3-6$. (C) Cells were treated with 5 ng/mL EGF for 24 h, and TSP1/2 levels were measured for both cellular (top) normalized to β-actin and secreted pools (bottom) normalized to BSA. Left panels: Representative gels. Right panels: Densitometric analysis. $^{**}p < 0.01$ vs control; $N = 6-9$.

TGFβ1 Stimulated TSP1 but Not TSP2 Expression. To determine whether TGFβ1 stimulated cellular TSP2 as it did TSP1, we began with a time course study in which astrocytes were treated with 2−5 ng/mL of TGFβ1 for 2, 3, 6, and 24 h followed by measurements of cellular TSP2. Under these conditions, TSP2 levels remained unchanged (data not shown). Figure 3A shows a study in which TGFβ1, CNTF, and serum (positive control) were administered to astrocytes for 24 h and cellular and secreted TSP1/2 levels were measured. Although TGFβ1 up-regulated both cellular and secreted TSP1, only serum increased cellular TSP2 levels. Two important astrocytic neurotrophic factors, BMP4 (a member of the bone morphogenetic subclass of the TGFβ family of proteins, data not shown, $N = 6$) and CNTF, failed to stimulate cellular and/or secreted TSP2 protein expression after 24 h. Alternatively, SB-431542, a TGFβR antagonist, abolished TGFβ1 stimulation

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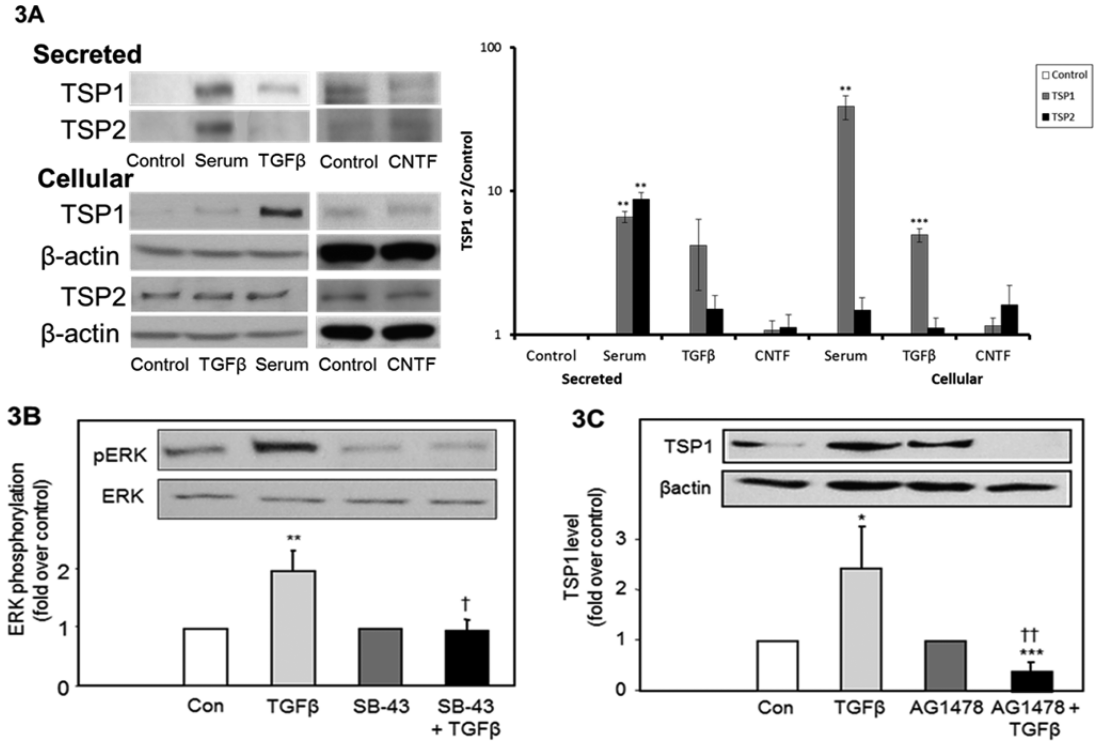

Figure 3. Effects of TGFβ1 on cellular and secreted TSP1/2 protein levels. (A) Cells were treated with 10% FBS, 1−5 ng/mL of TGFβ1 or 5 ng/mL CNTF for 24 h and cells lysates and media TSP1/2 levels were measured. Left panel: Representative immunoblots. Right panel: Densitometric analyses $^{**}p < 0.01$ vs control. $^{***}p < 0.001$ vs control. $N = 6$ (B) Cells were treated with TGFβ (1 ng/mL) ± SB-431542 (3 μM) for 24 h, and lysates were used to measure the induction of ERK phosphorylation. $^{**}p < 0.001$ vs control (con). $^{++}p < 0.01$ vs TGFβ alone. $N = 10$. (C) Cells were treated with TGFβ (1 ng/mL) ± AG1478 (0.1 μM) for 24 h and cellular lysates were analyzed for TSP1 protein levels. $^{*}p < 0.05$ vs control. $^{***}p < 0.001$ vs control. $^{+}p < 0.01$ vs TGFβ alone. $N = 8$.

of ERK activation, suggesting mediation by TGFβR (Figure 3B). A highly selective EGFR tyrosine phosphorylation inhibitor, AG1478, blocked the induction of cellular TSP1 protein levels by TGFβ1, implicating EGFR transactivation in this mechanism (Figure 3C). These data are consistent with the EGF experiments above (Figure 2C) and previous findings.[20]

TSP1/2 Protein Immunofluorescence Is Up-Regulated upon Scratch-Wounding of Astrocytes. As discussed in the introduction, TSP1/2 gene expression increases in different subtypes of reactive astrocytes isolated from brain after ischemic stroke and neuroinflammation.[37] In addition, upon spinal cord injury, TSP1 and TGFβ protein levels were elevated at the wound site as detected by immunoblotting.[35,36] However, all neuroglia contain TSP1/2 so there had been no direct evidence that reactive astrocyte TSP1/2 protein levels increase after wounding. To this end, we compared immunocytochemical staining of TSP1/2, GFAP, and vimentin protein in scratch-wounded astrocytes. GFAP and vimentin are considered prototypical markers for astrogliosis, but it is clear from recent findings that more reliable markers are needed in that not all astrocytes are GFAP$^+$.[31,37,52] As seen in Figure 4, reactive astrocytes generated from scratch-wounding showed a 2-fold elevation in vimentin$^+$ cells and almost a 3-fold increase in the distribution of TSP1/2$^+$ cells, estimated as a percentage of the total number of DAPI$^+$ cells. Only 16% of the total number of immortalized astrocytes cloned were GFAP$^+$, consistent with data previously reported by Radany et al.[53]

DISCUSSION

Here we report that TSP2 like TSP1 is a member of a group of structural and signaling genes associated with synaptic plasticity that are suppressed by chronic opiates in vivo. This is important as the presence of TSP2 in astrocyte media was found to be necessary for astrocytes to stimulate synapse formation when cocultured with neurons.[9] These results are consistent with the notion that earlier reported actions of chronic morphine and other opioids to reduce neurite outgrowth, dendritic spine density, synapse formation, and some morphological features may be mediated by TSP1/2.[20,47−51] Both morphine and DAMGO, the prototypic μ opioid agonist, comparably down-regulated TSP1/2. Since the highly selective Oprm antagonist, CTAP, reversed opioid modulation of TSP1, our data strongly indicate mediation of Oprm in the mechanism. Under the same in vitro conditions of immortalized astrocyte growth wherein chronic (8 days) morphine or DAMGO treatment diminished TSP1/2 protein levels in this study, morphine reduced neurite outgrowth and synaptic puncta formation in primary neurons cocultured with primary astrocytes.[20]

The ability of acute μ opioids, morphine, and DAMGO to display spatiotemporal differential effects on TSP1 and TSP2 may be explained in light of our prior findings. Namely, we demonstrated that acute (5−6 h) actions of morphine increased TSP1 mRNA levels both in vitro (astrocytes) and in vivo.[20] However, at 3 h, no change in astrocyte TSP1 protein levels was detected and at 6 h morphine exposure reduced TSP1 protein levels by an ERK-dependent mechanism in vitro. In a series of earlier studies, we demonstrated that μ opioids activate ERK via a mechanism that entailed MMP mediated

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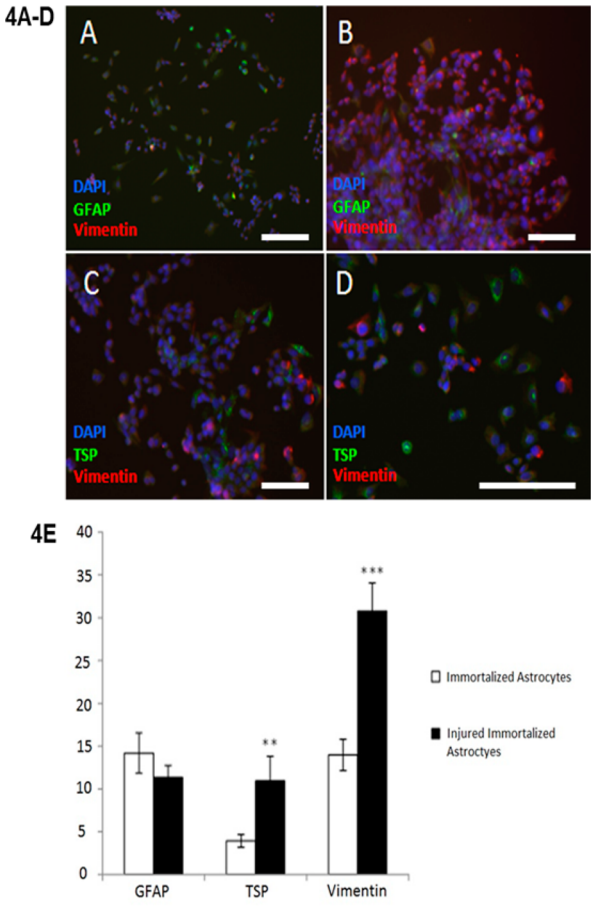

Figure 4. Expression of GFAP, TSP, and vimentin in control and injured immortalized astrocytes. (A) Control cells stained with GFAP. (B) GFAP and elevated vimentin in scratch-wounded cells. (C) Control astrocytes showing TSP and vimentin. (D) Scratch-wounded astrocytes showing elevated TSP and vimentin. (E) Comparison showing the 3-fold increase in TSP and 2-fold increase in vimentin among the injured cells. $^{**}P < 0.01$ vs controls, $^{***}P < 0.001$ vs controls.

limited proteolysis of an unidentified molecule that contains EGF-like ligands thereby inducing EGFR transactivation in astrocytes.[43] Subsequently, we found that acute (6 h) morphine signaling to ERK that entails depletion of TSP1 levels was inhibited by a function blocking monoclonal TSP1 Ab.[20] This raised the interesting possibility that morphine and DAMGO may activate MMPs to degrade TSP1 and release its known EGF-like domains. By this limited proteolysis, TSP1 can serve as a source of EGF-like ligands to activate EGFR either directly or indirectly, as seen in epithelial cells.[54] In contrast, here we found that TSP2 protein levels were up-regulated by μ opioids after 3 h. Therefore, an attractive hypothesis is that 3 h μ opioid exposure up-regulates expression of TSP isoforms, 1 and 2. However at 3 h, TSP1 also serves as a source of EGF-like ligands to activate ERK. As a result, any increase in TSP1 levels was offset in 3 h by their depletion. By 6 h the limited proteolysis of TSP1 was detectable because by that time the induction of TSP1 and TSP2 expression is diminished, as indicated by the lack of any detectable change in TSP2 protein levels. The fact that TSP2 levels were not decreased by 6 h μ opioids as is TSP1 suggests that this isoform is not degraded to EGF-like ligands by MMPs. Confirmation of this possibility awaits the development of a suitable function-blocking TSP2 Ab to use in additional experiments to monitor the mechanism of acute opioid modulation of TSP2 expression.

Additional experiments on the relative efficacies of DAMGO and morphine for the 6 h experiment could also be pursued. The differences between the two ligands in reduction of TSP1 levels is statistically significant, 20% vs 70%, respectively ($p >$ 0.01, Figure 1). This is the only experiment that we conducted in which the outcome appears to entail TSP1 degradation.[20] In contrast, there were no differences in efficacy between DAMGO and morphine observed for the outcomes that may reflect stimulation or inhibition of the synthesis of TSP1/2 (3 h and 8 days, respectively). The apparent functional selectivity in the 6 h TSP1 assays raises the possibility of ligand bias by morphine. In previous studies, we discovered that acute (5 min) DAMGO was almost 2-fold more efficacious than acute morphine in the activation of ERK in Oprm-transfected astrocytes.[58] Therefore, DAMGO may have a distinct bias for acute activation of ERK. In contrast, chronic (6 h) morphine may be a biased ligand for TSP1 degradation that results in PLC2 activation.[54] It would also be interesting to determine whether the functional selectivity arises from differential G protein or β-arrestin signaling. Since DAMGO but not morphine induces Oprm internalization, DAMGO may have a bias for β-arrestin signaling whereas morphine signals through G protein coupling. However, DAMGO stability tests during 6 h cell treatment should be performed to rule out the possibility that DAMGO degradation is responsible for its lower efficacy compared to morphine.

The failure of EGF to stimulate TSP2 protein levels suggests that ERK activation to elevate TSP2 levels is not mediated by EGF activation of EGFR. Precedence exist for pathways involving the transactivation of EGFR by nonconventional ligands or EGFR-independent mechanisms, e.g., transactivation of an isoform of FGFR, that leads downstream to ERK phosphorylation in astrocytes and astrocytic model systems among other cells.[55−57] From this and our previous study, we have learned that EGFR is a signaling hub in the crosstalk between MOR and TGFβR modulation of TSP1 protein levels by an ERK dependent mechanism. Paradoxically, TGFβ1 did not modulate TSP2 protein content. Nevertheless, acute μ opioid up-regulation of cellular TSP2 protein levels is also ERK dependent as are basal levels of secreted TSP2. Therefore, both TSP1 and 2 syntheses can be modulated by ERK phosphorylation despite the fact that their mechanisms of up and down-regulation differ. These results are consistent with our previous findings that suggest differential compartmentalization of multiple μ opioid signaling pathways to ERK in astrocytes.[20,44,58]

The fact that serum promotes TSP2 expression in vitro and TSP2 is induced at different temporal and neural cell dependent modes than TSP1 in vivo suggests that it has other functions and, therefore, other factors may control the regulation of TSP2.[25] It remains to be determined whether the point of convergence for TSP2 modulation by μ opioids and this yet to be identified factor also interacts with EGFR that is driven by nonconventional ligands. Alternatively, TSP2 may be induced via a different receptor and/or another mechanism of crosstalk.

As mentioned in the introduction, CNS TSP1/2 are only expressed in developing brain and upon brain injury. In addition to temporal and cell type-dependency variations of TSP 1 and 2 induction, the type of CNS injury is also a contributory factor.[25−30] Interestingly, in response to spinal

cord injury, TSP1, its inducer, TGFβ, and TSP2 are up-regulated.[33−36] This occurs at a time associated with pathological loss of spinal vasculature but may not involve abolition of TSP1-mediated antiangiogenic signaling. Upon dorsal spinal nerve ligation injury, TSP4 but neither TSP1 nor TSP2 were up regulated.[59] TSP4 but not TSP2 promotes SVZ astrogenesis after injury as well.[60] Clearly more studies are needed to elucidate the various roles of overexpressed TSPs upon CNS injury. Although microglia and other neural cells are also sources of TSPs, there is a growing recognition of the importance of reactive astrocytes as providers of physical and chemical barriers in the recovery from CNS injury that disrupt homeostasis between neurons and mature astrocytes.[61,62] From these studies it appears that TSP1/2 may be a useful marker of reactive astrogliosis. Once suitable antibodies (Abs) that are selective for TSP1 and TSP2 become available, it would also be of interest to determine whether immunohistochemical staining of each isoform can be used to distinguish between different types of CNS injury and the individual roles of these isoforms in reactive gliosis.

The GFAP distribution in the immortalized rat cortical astrocytes used in this investigation contrasts markedly with the 90% enrichment of GFAP$^+$ primary astrocytes obtained when prepared from P1 rat pup cortex.[58,63] The diminution of the percentage of GFAP$^+$ immortalized astrocytes may explain the absence of an increase in their distribution upon scratch wounding in this population of astrocytes. Alternatively, the immortalized GFAP$^+$ astrocyte subtype may be reactive astrocytes that are not influenced further by injury. If either of these is the case, the data suggest that vimentin and TSP1/2 are better markers for reactive astrogliosis.

There is rapidly emerging data to suggest that astrocyte dysfunction occurs in neurodevelopmental, psychiatric and neurodegenerative disorders that ensue from synaptic effects.[6,7] For example, astrocytes isolated from post-mortem human fetal Down syndrome brain were found to be TSP1-deficient.[64] When cocultured with rat hippocampal neurons, they generated altered dendrite lengths, spine malformation and reduced functional synapses that were restored to normal spine morphology and density upon addition of exogenous TSP1. The implication of astrocyte-secreted TSP1/2 in the mechanism of opiate tolerance presented here indicates that drug addiction may be yet another disease that features dysfunction of this class of glia.

METHODS

Reagents. Chemicals and Abs were purchased from Sigma Chemical Co. (St. Louis, MO) with the following exceptions: [D-ala^2,mephe^4,gly ol^5] enkephalin (DAMGO), morphine sulfate, D-Phe-Cys-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP), and buprenorphine were from NIDA Drug Supply (Research Triangle, NC); TGFβ from Austral Biologicals (San Ramon, CA); BMP4 from R&D Systems (Minneapolis, MN), and CNTF from PeproTech (Rocky Hills, NJ). EGF and U0126 from Calbiochem (San Diego, CA); trypsin-EDTA solution from Gibco (Carlsbad, CA); DMEM and fetal bovine serum (FBS) from ATCC (Manassas, VA); Alexa Fluor labeled secondary Abs and horse serum were from Invitrogen - Molecular Probes (Carlsbad, CA), anti-phospho-ERK1/2 (directed against phospho Thr202/Tyr204) antibody (Ab), was from Cell Signaling Technology (Beverly, MA); anti-GFAP Ab was from ImmunoStar, Inc. (Hudson, WI, catalog #22522); anti-ERK and anti-TSP2 Abs were from Santa Cruz (Santa Cruz, CA); anti-vimentin Ab was from Novus Biologicals (Littleton, CO), anti-TSP1/2 Ab (immunohistochemistry) and anti-TSP1 Ab, Ab-11 (immunoblotting) were from LabVision (Fremont, CA); AMCA-conjugated anti-chicken IgY was from Jackson ImmunoResearch Laboratories (West Grove, PA); and VECTASHIELD Mounting Medium was from Vector Laboratories, Inc. (Burlingame, CA).

Immortalized Rat Type-1 Cortical Astrocyte Cultures. Rat cortical astrocytes (CTX TNA2; American Type Culture Collection) were established from cultures of primary type 1 astrocytes from 1-day-old rat brain frontal cortex. The cultures were originally transfected with a DNA construct containing the oncogenic early region of simian virus 40 under the transcriptional control of human glial fibrillary acidic protein promoter.[53] The cells have the phenotypic characteristics of type 1 astrocytes. This cell line was maintained in DMEM + 10% FBS at 37 °C in a humidified atmosphere of 95% air, 5% CO_2 for up to 5−35 passages. Later passage cells (for the most part, 10−20) were transfected with MOR1 in our experiments as we found that they did not contain endogenous Oprm or Oprk by Western blotting.[65] Therefore, in experiments with transiently transfected immortalized astrocytes, we observed an interaction of morphine with transfected Oprm1. The amounts of Oprm1 in transfected immortalized astrocytes are higher than levels of endogenous Oprm that we detected in primary astrocytes in Western blots. Nevertheless, in parallel experiments with Oprm1 transfected immortalized astrocytes and untransfected primary astrocytes similar opioid signaling responses were observed in most cases.[20] This supports the notion that the extent of Oprm overexpression in immortalized astrocytes does not exceed the physiological range. Agonists, antagonists or inhibitors were delivered in serum-free media.

Transient Transfection. Immortalized astrocytes were transfected with rat Oprm1 cDNA (in pCMV-neo expression vector) using FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN) following the manufacturer's instructions and using 1 μg of cDNA and 3 μL of transfection reagent. After 24 h of incubation, drug treatments were begun with serum-deprived media as vehicle. In some cases, cultures were cotransfected with pcDNA3 (for mock transfections), rat Oprm1 cDNA (pCMV-neo expression vector) using FuGENE 6 as described above.

Thrombospondin1/2 Protein Assays. Cells were treated with morphine (1 μM), DAMGO (1 μM) and/or U0126 (1 μM) as described in the figure legends. After treatment, media was collected and cells were washed with cold phosphate-buffered saline and then lysed with buffer containing 50 mM Tris hydrochloride, 0.25% sodium deoxycholate, 150 mM sodium chloride, 1 mM EGTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin. After 15 min, lysates were spun at 14 000g for 10 min at 4 °C and protein concentration of the supernatants was measured. Collected media was concentrated through 30 kDa molecular weight cutoff centrifuge concentrator (Millipore, Bedford, MA) following the manufacturer's instruction and then protein concentration was determined. Cell lysates (30 μg protein/lane) and concentrated media (100 μg protein/lane) were separated by 7.5% SDS-polyacrylamide gel electrophoresis. Proteins were blotted on Immobilon P Polyvinylidene difluoride membranes (Millipore). Nonspecific sites were blocked with 5% milk in Tris-buffered saline +0.2% Tween 20 (TBST). Blots were then washed three times with TBST and incubated with anti-TSP1 Ab diluted 1:200 in 5% milk/TBST for 2.5 h at room temperature. After three washes with TBST, blots were incubated with 1:2000 diluted goat anti-mouse-IgG for 1 h at room temperature. Bands were visualized using an ECL chemiluminescence detection system from GE Health Care (Piscataway, NJ) and exposure to Classic Blue sensitive X-ray film (Molecular Technologies, St. Louis, MO). Band intensities were measured by densitometric analysis using a digital camera and imaging software.

Measurement of ERK Activity. Upon treatment with opioids, cellular ERK phosphorylation was measured by immunoblotting as described.[43] Blots were incubated with a phospho-ERK Ab (1:2000) overnight at 4 °C, followed by incubation with HRP conjugated IgG (1:2000, Sigma) for 1 h at RT. Blots were also reprobed with an ERK Ab (1:1000) and the densities of the bands were used as loading controls. Band intensities were determined by densitometry as described above. ERK stimulation in opioid- and/or and growth

factor-treated cells was expressed as fold change over basal levels of phospho-ERK in control cells.

Scratch-Wounding Assay. Cells were scratch-wounded following the protocol described by Etienne-Manneville and Hall.[66] Briefly, cells were grown on 8-well chamber slides to confluency, and their growth medium with serum was changed at least 16 h prior to scratching. Next, a sterilized glass Pasteur pipet tip was forcefully dragged across the surface of the cell monolayer. Cells were incubated for another 6–8 h following scratch-wounding and prior to fixation and immunocytochemistry.

Immunocytochemistry. Astrocytes were washed, fixed for 20 min in 4% paraformaldehyde and permeabilized with a 0.4% Triton-X solution for 5 min. After washing, they were incubated in blocking buffer (10% FBS/0.4% Triton-X) for 30 min to reduce nonspecific binding. Cells were then incubated with primary Ab solution for 2 h at 24 °C or overnight in 4 °C. Abs were diluted as follows: anti-GFAP: 1:1 (Immunostar, Inc.), anti-vimentin: 1:2000 (Novus Biologicals), anti-thrombospondin (TSP1/2): 1:125 (LabVision). Cells were incubated in secondary Ab solution for 1 h in the dark at 24 °C. Secondary Abs (Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 594 goat anti-chicken IgG, Alexa Fluor 594 goat anti-mouse, Alexa Fluor 488 goat anti-mouse, all from Invitrogen) were diluted at 1:1000 with DAPI at 1:250 (Sigma). Cells were then washed and mounted on slides with antifade Vectashield mounting medium and topped with coverslips. Slides were stored in the dark at 4 °C until viewing them with an Olympus AH3 microscope and its associated program cellSens.

Cell Counting. Cells within a range of 300–400 μm from the scratch were counted using the computer program ImageJ (NIH) and its plugin, Cell Counter. Cells were counted as the number of $GFAP^+$ or $TSP1/2^+$ or $vimentin^+$/total $DAPI^+$ cells to obtain a percentage of cells expressing these proteins out of the total number of cells present. Average number of cells and fields counted were a total of 2000/experiment and 10/experiment, respectively. Two independent counts of the same micrographs, one blinded, gave similar significant differences.

Statistical Analysis. Statistical analysis of densitometry and cell counting data was performed using Graph Pad Prism software. These mean values were compared to the means of other experiments using Student's *t* test to determine significance values.

AUTHOR INFORMATION

Corresponding Author

*Tel.: +1 314 977 9254. Fax: +1 314 977 9205. E-mail: cosciacc@slu.edu.

Present Address

[†]M.M.B.: Department of Developmental Biology, Washington University School of Medicine, 660 S. Euclid Ave., Box 8103, St. Louis, Missouri, 63110 USA.

Notes

The authors declare no competing financial interest.

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