



Serum response factor mediates nociceptor inflammatory pain plasticity

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Abstract

Introduction: Chronic metabotropic glutamate receptor activation in nociceptive afferents may upregulate A-Kinase Anchoring Protein 150 (AKAP150) expression and/or function.

Objectives: To quantify transcriptional changes in AKAP150 expression and/or function after long-term mGluR5 agonist exposure, and identify transcriptional elements responsible.

Methods: Dorsal root ganglia (DRG) were dissected from Sprague-Dawley rats and cultured for biochemical analysis of AKAP150 expression after prolonged mGluR5 agonist exposure. Serum response factor (SRF) expression was knocked down through siRNA in cultures to demonstrate significance to AKAP150 upregulation. Serum response factor was also knocked down in vivo through intrathecal injections of specifically targeted oligonucleotides to demonstrate significance to hyperalgesic priming behavior in persistent mechanical hypersensitivity.

Results: Serum response factor and AKAP150 are coexpressed in TRPV1(+) DRG neurons in intact DRG. Prolonged mGluR5 agonist exposure increases SRF-dependent transcription and AKAP150 expression in a manner sensitive to protein kinase C inhibition and SRF knock down. Serum response factor in vivo knock down reduces mechanical hyperalgesic priming.

Conclusion: Serum response factor transcription plays an important role in transcriptional upregulation of AKAP and hyperalgesic priming behavior, and may contribute to the increased role of AKAP150 in the transition from acute to chronic pain.

Keywords: Pain, Plasticity, AKAP, Nociceptor, SRF

1. Introduction

Multiple mechanisms contribute to primary afferent neuroplasticity including modulation (reversible posttranslational changes in sensory neuron excitability) and modification (extended changes in receptor, channel, and/or transmitter expression/function) of sensory neurons that define neuroplastic changes and support persistent pain.³⁰ Although the central nervous system plays a role, primary afferent biochemistry produces the initial peripheral

sensitization responsible for increasing nociceptor responsivity to environmental stimuli. Biochemical signaling pathways downstream of inflammatory mediator-activated receptors drive post-translational modulation of other receptors and ion channels, increasing the likelihood and frequency of nociceptor depolarization and pain sensation. However, transcriptional modulators can also be upregulated after inflammatory injury, thereby modifying gene expression. At this point of transcriptional regulation, we identify a new target for analgesic consideration that could serve to quell inflammatory sensitization of nociceptors that contribute to persistent pain phenotypes.

Serum response factor (SRF) is a member of the MCM1, Agamous, Deficiens, and SRF (MADS) box family of transcription factors and binds the core sequence of CArG (CC [A/T] 6 GG) as a homodimer after Ser162 phosphorylation by protein kinase C (PKC).¹⁶ Serum response factor binds to serum response elements (SREs) located in multiple immediate early genes involved in cell growth, migration, and cytoskeletal organization.¹⁵ Importantly, SRF also controls the phenotypes of embryonic dorsal root ganglia (DRG) neurons²⁹ through nerve growth factor (NGF) signaling. Given that SRF transcription plays a role in adult cardiac and muscle tissue,⁶ we questioned whether SRF also mediates adult DRG transcriptional changes in protein expression. Additionally, we questioned whether tissue inflammation could upregulate transcriptional activity via SRF. Analysis of over 200 genes containing consensus SRE sequences²³ revealed several important genes expressed by DRG neurons,

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including the gene for A-Kinase Anchoring Protein 150 (AKAP150).

AKAP is a scaffolding protein expressed in nervous tissue that has been shown to positively modulate multiple channels associated with inflammatory hyperalgesia. Importantly, AKAP150 exists as the rodent homolog to AKAP79, which is the human homolog of the scaffolding protein. Previous studies have demonstrated that AKAP150 expression is required for protein kinase A- and PKC-mediated phosphorylation and sensitization of transient receptor potential channel family V1 (TRPV1^{9,10,18,31}). Recent work has also identified a role for the scaffolding protein in protein kinase A- and PKC-phosphorylation and sensitization of TRPA1.⁴ As a biochemical integrator of signaling pathways known to sensitize nociceptors, AKAP150 expression and function exists as a barometer of peripheral inflammatory plasticity.

Multiple measures of nociceptor sensitivity were used here to identify whether inflammatory activation of SRF mediates changes in AKAP150 expression and function. Studies on primary cultures, combined with *in vivo* rodent behavior, complement each other to identify that inflammation stimulates SRF-dependent upregulation of AKAP150, and that SRF contributes towards inflammatory hyperalgesic priming to generate persistent mechanical hypersensitivity. Taken together, these findings identify SRF as an important transcriptional mediator in primary afferent neurons that contribute to persistent pain.

2. Materials and methods

2.1. Animals

Procedures using animals were approved by the Institutional Animal Care and Use Committees of The University of Texas Health Science Center at San Antonio (UTHSCSA). Moreover, these procedures were conducted in accordance with policies for the ethical treatment of animals established by the National Institutes of Health and International Association for the Study of Pain.

2.2. Tissue culturing

Male Sprague-Dawley rats (175–200 g; Charles River Laboratories, Wilmington, MA) were used. L4 to L6 DRG were removed bilaterally from male rats, and dissociated by collagenase treatment (30 minutes, Worthington, Lakewood, NJ), followed by trypsin treatment (15 minutes, Sigma, St. Louis, MO). Cells were centrifuged and resuspended between each treatment with Pasteur pipettes. Dorsal root ganglia were then centrifuged, aspirated, and resuspended in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY) with 10% fetal bovine serum (Gibco), 100 ng/mL NGF (Harlan, Indianapolis, IN), 1% 5-fluoro deoxyuridine (Sigma), 1% penicillin/streptomycin (Gibco), and 1% L-glutamine, and then placed on plates coated with poly-D-lysine (Corning, Corning, NY). Cultures were maintained at 37°C, 5% CO₂, and grown in 48-well or 6-well plates for 5 to 7 days for biochemistry experiments.

2.3. Serum response factor siRNA

Small interfering RNA (siRNA) directed against rat SRF (Gene ID: 501099) were custom designed through Dharmacon: SRF siRNA-1 sense 5' GCGUGAAGAUC AAGAUGGAUU, antisense (AS) 5' UCCAUCUUGAUCUU CACGCUU, SRF siRNA-2 sense 5' ACAACAAGCUGCGGCGUUAUU, AS 5' UAACGCCGAGCUUGUUGUUU.

Transfection of primary sensory neuron cultures was performed with HiPerFect (Qiagen, Germantown, MD) following manufacturer's instructions.

2.4. Nuclear isolation

Dorsal root ganglia cultures were collected in Dulbecco's phosphate-buffered saline (PBS) by centrifugation and rinsed twice with cold PBS on ice. Supernatant was discarded and the cell pellet was resuspended in 150 μ L homogenization buffer (25 mM HEPES, 25 mM sucrose, 1.5 mM MgCl₂, 50 mM NaCl, pH to 7.2) with 1 μ g/mL pepstatin, 5 μ g/mL leupeptin, 100 nM PMSF, and 1 mM sodium orthovanadate added immediately before use. This resuspension was homogenized in a Potter Elvehjem homogenizer by 20 strokes and incubated on ice for 15 minutes. After incubation, NP-40 detergent was added to each sample to a final concentration of 0.5% NP-40, vortexed for 10 seconds, and centrifuged at 1,000g for 10 minutes at 4°C. The resulting supernatant represents the cytoplasmic fraction. The pellet is resuspended in general lysis buffer (1 mM sodium pyrophosphate, 50 mM HEPES [pH 7.5], 1% Triton X-100, 50 mM NaCl, 50 mM NaF, 5 mM EDTA [pH 8.0], pH to 7.4) and incubated on ice for 30 minutes with 10 seconds of vortexing in 10-minute intervals. Samples are then centrifuged for 30 minutes at 14,000g at 4°C, with the supernatant representing the nuclear fraction. Protein quantification is conducted following the Bradford method.⁵ Representative Western blot results of cytoplasmic and nuclear fractions resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and probed for nuclear pore complex protein 98 (NUP98) and β -actin (Supplementary Figure 1, available at <http://links.lww.com/PR9/A19>) demonstrate the high fidelity of this protocol.

2.5. Western blotting

Dorsal root ganglia cultures in 6-well plates were treated as described, rinsed twice with ice-cold PBS, and lysed in general lysis buffer, with 1 μ g/mL pepstatin, 5 μ g/mL leupeptin, 100 nM PMSF, and 1 mM sodium orthovanadate added immediately before use via 20 passes through a 24 g hypodermic needle. Lysates were incubated on ice for 15 minutes, then cleared by centrifuging at 4°C for 2 minutes at 1000g, before quantification of protein concentration by Bradford analysis.⁵ Equal 30 μ g samples across treatment parameters were resolved by 12.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA) using the semi-dry blotting method. Membranes were blocked in 5% nonfat milk in Tris-buffered saline with 0.1% Tween-20 nonionic detergent and incubated 18 hours with primary antibody anti-AKAP (Santa Cruz Biotechnology, Santa Cruz, CA),⁹ anti- β -Actin (Sigma-Aldrich, St. Louis, MO), anti-phospho-SRF Serine 162 (Cell Signaling Technology, Danvers, MA²⁰), and anti-SRF (Cell Signaling Technology).²² Anti-rabbit or anti-mouse secondary antibodies (GE Healthcare Life Sciences, Piscataway, NJ) were applied to rinsed blots, incubated at room temperature for 1 hour, and then blots were rinsed again. Membranes were incubated with enhanced chemiluminescence solution (GE Healthcare Life Sciences), exposed to X-ray film and developed for analysis. Membranes were subsequently rinsed with DPBS 4 \times 5 minutes at room temperature, and then chemically stripped for 40 minutes at 55°C in a hybridizing oven in stripping buffer (312.5 mM Tris HCl, 10% sodium dodecyl sulfate). Membranes were rinsed 4 \times 5 minutes at room temperature, and then reblocked and probed as before.

Membranes were scanned and immunoreactivities quantified using NIH Image-J shareware. Experiments were conducted with

minimum of 3 trials, and quantified results were analyzed for significance by 2-way analysis of variance with Bonferroni correction ad hoc.

2.6. Immunohistochemistry

Dorsal root ganglia from male Sprague-Dawley rats were obtained by surgical resection from adults 225 to 250 g in weight and processed/prepared as we have described previously.⁸ Individual sections from 3 different rats were stained with antibodies specific to SRF (1:400; Cell Signaling Technologies²²), AKAP79/150 (1:100; Santa Cruz Biotechnology⁹), and TRPV1 (1:2000; NeuroMics [GP14103]¹⁷). Species-appropriate Alexa Fluor secondary antibodies (Molecular Probes at 1:200 dilution) were used to visualize TRPV1 (Alexa Fluor 488), AKAP79/150 (Alexa Fluor 633), and SRF (Alexa Fluor 568). Immunofluorescence was evaluated, and images were obtained with the use of a Nikon 90i microscope equipped with a C1si laser scanning confocal imaging system (Nikon, Melville, NY). Images were processed for illustration purposes with Adobe Photoshop CS2 (Adobe, San Jose, CA) and are representative of 3 individual animals.

For quantification, 8 DRG images were chosen randomly, and an unbiased observer quantified positively stained cells. Image J (NIH) was used for identifying positive expression against a background-quantified low threshold.

2.7. Hyperalgesic priming behavior

Antisense oligonucleotides were designed against rat SRF DNA, using custom-design shareware available on invitrogen.com. The AS sequence created and used was as follows: 5'—GCG TGA AGA TCA AGA TGG. The mismatch (MM) sequence created and used was as follows: 5'—GGC TCT TCT AGT TCC AGC. Antisense and MM oligonucleotides (ODNs) were dissolved in sterile-filtered PBS and stored at -20°C . Rats were briefly anesthetized with 2.5% isoflurane before a 28 1/2-gauge needle insulin syringe was inserted intrathecally on the midline between vertebrae L5 and L6. Injection location at the level of the cauda equine was confirmed by tail flick.¹⁴ Thirty microgram of ODN in 20 μL sterile PBS was administered once daily for 3 consecutive days. Behavioral testing was completed the following day within 24 hours of the final injection. After behavioral testing and within 24 hours of the final ODN administration, L4 to L6 DRG were bilaterally harvested. Dorsal root ganglia were homogenized in homogenization buffer containing 1% Triton X-100, cleared by centrifugation at 1000g for 1 minute, quantified using the Bradford method, with 50 μg aliquots resolved by SDS-PAGE.

All injections were given intraplantarly in 50 μL volumes using a 28-gauge needle inserted through the lateral footpad just under the skin to minimize tissue damage. Drug doses were taken from previous work by Aley et al.¹ Drug stocks were dissolved in PBS. For rat priming experiments, rats were tested for baseline responses before injections, and then injected with carrageenan (50 μL of a 0.1% solution in PBS) or vehicle (PBS) on day 1. On days 2, 3, and 4, rats were injected intrathecally with AS and MM oligonucleotides. Rats were tested again on day 5 for baseline responses, and then injected with PGE₂ (100 ng) followed by repeated mechanical threshold testing at 15 minutes, 30 minutes, 2 hours, and 4 hours post-PGE₂ injection.

2.8. Serum response element-GFP reporter assay

Rat DRG were cultured in 48-well plates, grown for 5 days, and transfected with cDNA for the SRE-green fluorescent protein

reporter, negative control (no promoter-GFP cDNA) or positive control (CMV-GFP cDNA), following manufacturer's instructions (Qiagen), serum starved for 18 hours, and then treated with serum-free media, fetal calf serum (serum media, SM), (RS)-3,5-dihydroxyphenylglycine (group 1 mGluR agonist, DHPG), DHPG + 2-Methyl-6-(phenylethynyl) pyridine hydrochloride (noncompetitive antagonist of mGluR5, MPEP), DHPG + GF 109203X (PKC α , β 1, δ , ϵ , and ζ isoform inhibitor, GFX), or Phorbol 12-myristate 13-acetate (PKC activator, PMA) for 24 hours. The assay was then performed following manufacturer's instructions, and GFP fluorescence was quantified on a Tecan SpectraFluor Plus (488 nm excitation and 530 nm emission). Negative control emission values were subtracted from each experimental condition, and reported values were quantified as a percentage of the positive control (CMV-GFP). Results represent mean \pm SEM from 6 individual samples per treatment, with significance determined by 1-way analysis of variance with the Tukey multiple comparisons correction.

Representative images were generated from similar transfection and treatment conditions using an EVOS FL Color Imaging Systems transmitted light microscope (Life Technologies Corp, Bothell, WA). Combined DIC and GFP-fluorescence images were collected with a 10 \times objective to provide optimal analysis of cell types expressing GFP. Images are representative of 3 individual trials per transfection/treatment condition.

3. Results

Serum response factor controls the developmental phenotypes of multiple tissues and cell types, including embryonic DRG neurons.²⁹ Immunohistochemical analysis of intact rat DRG identifies coexpression with AKAP150 in both TRPV1(+) and TRPV1(-) neurons (**Fig. 1A-D**). When quantified, SRF was expressed in $37 \pm 5.9\%$ of AKAP(+) positive neurons, and in 13.5% of TRPV1(+) neurons (**Fig. 1E, F**). Importantly, SRF was expressed in both neuronal and nonneuronal cells, indicating a potential role in pain processes transduced by primary afferents as well as glial support cells.

Serum response factor phosphorylation by PKC simulates translocation of the transcription factor to the nucleus to associate with DNA SREs.²⁵ Cultured sensory neurons from DRG were treated with phorbol 12-myristate 13-acetate (PMA), a PKC activator, and lysed for Western blot measurement of SRF phosphorylation at Ser162. In both intact and cultured DRG, SRF phosphorylation at Ser162 increased after PKC activation by PMA (1 μM , **Fig. 2A**). Metabotropic glutamate receptors, including group 1 mGluR1/5 are expressed in sensory neurons and signal through G α q-coupled mechanisms to increase PKC activity in DRG cultures.¹² Therefore, we set out to determine whether receptor-mediated PKC activation could also drive SRF phosphorylation. In **Figure 2B**, treatment of DRG cultures with mGluR5-specific agonist (S)-3,5-dihydroxyphenylglycine (DHPG) resulted in a 2-fold increase in SRF phosphorylation at Ser162, in a manner sensitive to PKC inhibition by GF 109203X (GFX). As a control, nuclear preparations of cultured DRG neurons were also collected and monitored for SRF phosphorylation at Ser162, showing an increase in nuclear localization of the phosphorylated protein in nuclei after DHPG treatment, when normalized to nuclear pore complex protein 98 (NUP98) expression (**Fig. 2C**).

To confirm that mGluR5 activation was driving PKC-mediated SRF activation of SRE-containing genetic material, we used an SRE-driven Green Fluorescent Protein reporter construct transfected into DRG cultures. After transfection, cultures were maintained in serum-free media for 18 hours to baseline GFP

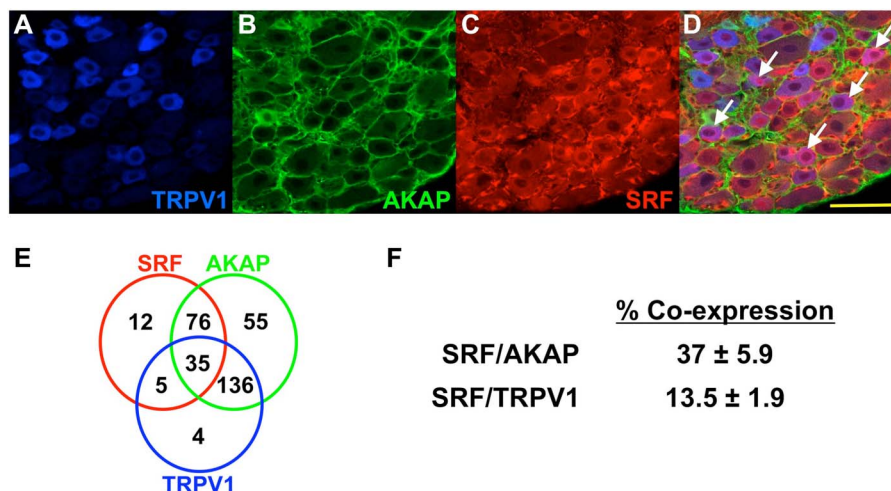


Figure 1. Immunohistochemical expression of serum response factor in rat dorsal root ganglia. Dorsal root ganglia dissected from male Sprague-Dawley rat and imaged by confocal microscopy for (A) TRPV1 (blue), (B) AKAP (AKAP150, green), and (C) SRF (red), with colocalization represented in panel (D). White arrows indicate cells expressing all 3 proteins. Yellow line represents 50 μ m in length; images are representative of 4 individual sections taken from 6 L4 to L6 DRG. (E) Venn diagram of coexpression between SRF (red circle), AKAP (AKAP150, green circle), and TRPV1 (blue circle). (F) Percentages of SRF coexpression with AKAP (AKAP150) or TRPV1 calculated from 8 randomly selected coverslips. DRG, dorsal root ganglia; SRF, serum response factor.

expression, then treated with DHPG vehicle (DMSO, 0.01%), DHPG, DHPG + mGluR5-specific antagonist 2-Methyl-6-(phenylethynyl) pyridine (MPEP), DHPG + GFX, PMA, or placed in serum-containing media (10% fetal calf serum), and incubated for 24 hours. GFP expression was quantified by the spectrofluorometric measurement, revealing induction of GFP fluorescence in cultures treated with DHPG (**Fig. 3**). The increase in GFP-reporter expression appears to have occurred predominantly in neurons, although several glial also demonstrate GFP-fluorescence (Panel H). This increase was blocked by cotreatment with either MPEP or GFX, whereas both PMA and serum-media exposure produced increased GFP fluorescence, as expected (positive controls). Taken together, these data suggest that mGluR5 activation drives PKC-dependent SRF-transcription in cultured sensory neurons.

Serum response factor and AKAP150 are coexpressed in DRG neurons (**Fig. 1**), prompting us to investigate whether the promoter region 5' to the AKAP150 start site on chromosome 14 in rats contains SREs for SRF-stimulated transcription. As depicted in **Figure 4A**, 2 SREs exist 5' of the AKAP150 start codon, although one is closer than the other. Unfortunately, we

were unable to delineate any exon/intron inclusion in the sequence analysis, so we next investigated the hypothesis that SRF positively upregulates AKAP150 expression. First, we used 2 custom-designed, SRF-specific siRNA sequences, identified as SRF siRNA-1 and siRNA-2. In **Figure 4B**, SRF siRNA-2 inhibited SRF expression in transfected DRG cultures. We next investigated whether mGluR5 activation, which was previously demonstrated to stimulate SRF-dependent DNA transcription, could stimulate an increase in AKAP150 expression in DRG neurons. Indeed, 24-hour treatment of DRG cultures with mGluR5-specific agonist DHPG increased AKAP150 protein expression in a manner sensitive to PKC-inhibition by GFX (**Fig. 4C**), suggesting a functional transcriptional link between SRF and AKAP150. To confirm this deduction, we repeated the experiment in the presence of SRF-siRNA-2, or MM (negative control) siRNA. As shown in **Figure 4D**, DHPG-stimulated AKAP150 upregulation was blocked in cultures pretreated with siRNA specific for SRF. Collectively, these results intimate that mGluR5 activation can stimulate SRF-dependent upregulation of AKAP150 protein expression in DRG cultures.

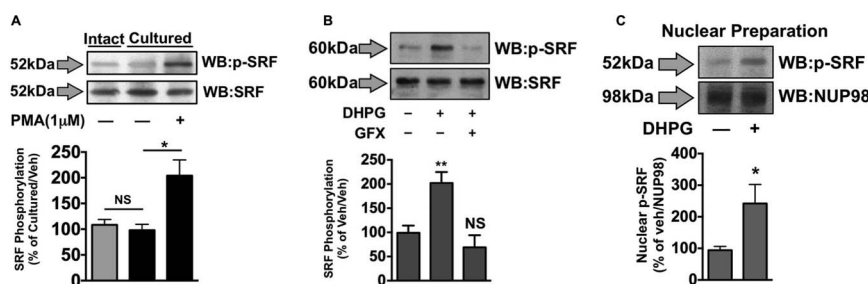


Figure 2. Serum response factor is expressed and activated in cultured dorsal root ganglia. L4 to L6 DRG dissected and cultured from male, adult Sprague-Dawley rats and prepared for in vitro biochemical analyses. (A) Intact and cultured DRG were homogenized and lysed, with equal aliquots resolved by SDS-PAGE for Western blot (WB) analysis. Cultured DRG were serum-starved for 18 hours and then treated with either vehicle (95% ethanol) or PMA (1 μ M, 10 minutes, 37°C) and probed for phospho-SRF at Ser162. NS = no significance, ** P < 0.01, the Student t test, n = 4. (B) Cultured DRG were serum-starved for 18 hours and then treated with vehicle (ddH₂O) or mGluR5 agonist DHPG (10 μ M, 10 minutes) in the presence or absence of PKC inhibitor GFX (10 μ M) and probed for phospho-SRF at Ser162. NS = no significance, ** P < 0.01, compared with DHPG(-), GFX(-) group, 1-way ANOVA with Bonferroni correction, n = 4. (C) Cultured DRG were serum-starved for 18 hours and then treated with vehicle (ddH₂O) or mGluR5 agonist DHPG (10 μ M, 10 minutes) and then homogenized for nuclear isolation. Nuclear preparations were probed for phospho-SRF and nuclear pore complex protein 98 (NUP98). * P < 0.05, the Student t test, n = 3. ANOVA, analysis of variance; DRG, dorsal root ganglia; PKC, protein kinase C; SRF, serum response factor.

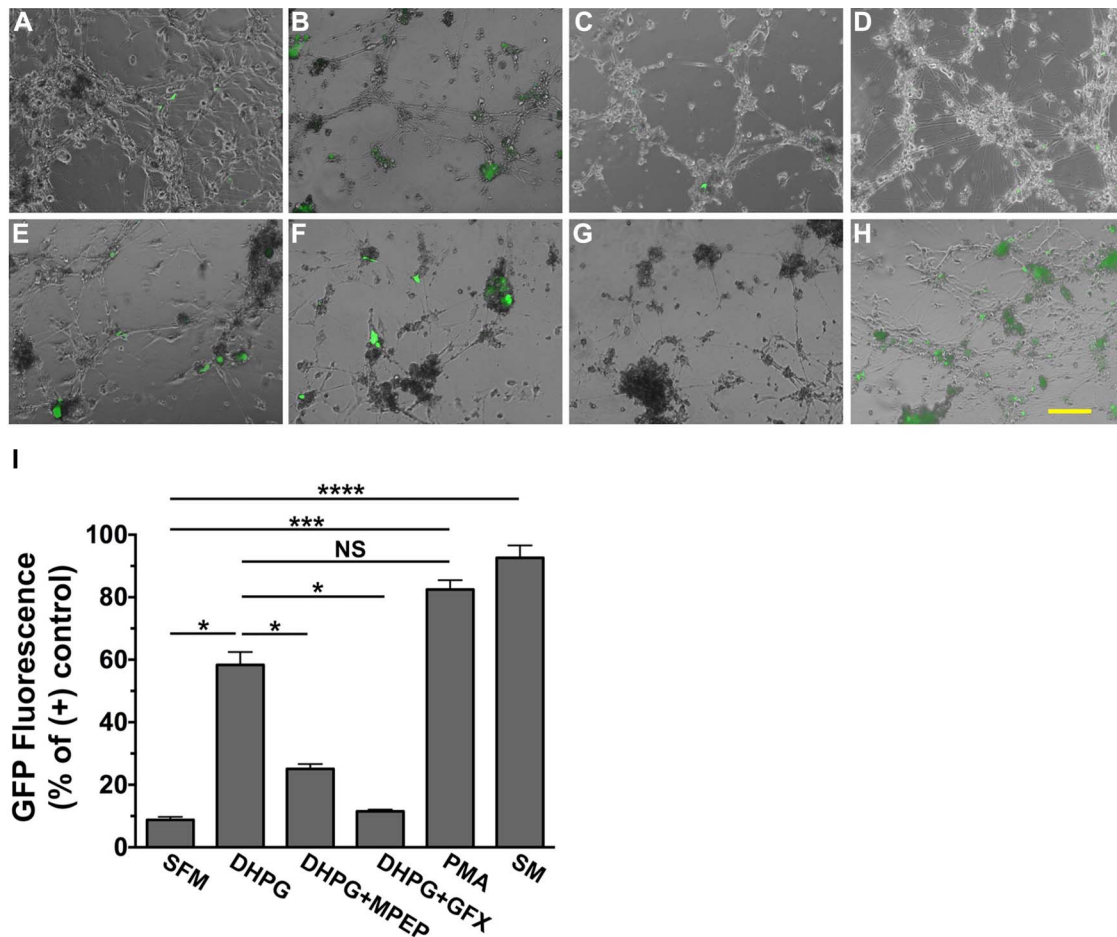


Figure 3. Serum response element gfp-promoter upregulation by mglur5 activation. GFP-reporter containing 5'-serum response element was transfected into cultured DRG and quantified for GFP-fluorescence after 18-hour serum starvation, and then 24-hour exposure to serum-free media (SFM), DHPG (10 μ M), DHPG + MPEP (1 μ M, mGluR5 antagonist), DHPG + GFX (10 μ M), PMA (PKC activator, 1 μ M), or normal 10% serum media (SM). Representative images taken at 10 \times with merged GFP-immunofluorescence and differential interference contrast (DIC) microscopy with the following treatments: (A) SFM, (B) DHPG, (C) DHPG + MPEP, (D) DHPG + GFX, (E) PMA, (F) SM, (G) negative control (SRE-cDNA without GFP reporter), and (H) positive control (CMV-promoter with GFP-reporter), yellow line represents 100 μ m. (I) Quantification of GFP fluorescent intensity, NS = no significance, **P*, 0.05, ****P* < 0.001, *****P* < 0.0001, as indicated, 1-way ANOVA with the Tukey multiple comparisons test, *F* value of 16.75, *n* = 6/treatment. ANOVA, analysis of variance; DRG, dorsal root ganglia; PKC, protein kinase C; SFM, serum-free media; SRE, serum response element.

The *in vitro* analyses of SRF transcriptional control of AKAP150 expression prompted us to turn our investigation towards functional *in vivo* analyses. Given the important role of AKAP150 to mechanical hyperalgesic priming,^{4,24} we questioned whether SRF is also involved. To develop this experiment, we used an oligonucleotide approach to knock down *in vivo* SRF through intrathecal injections, as we have described previously.³ The SRF-siRNA-2 sequence previously used in **Figure 3** was used to create an SRF oligonucleotide for *in vivo* injection. As depicted in **Figure 5A**, intrathecal injection of the SRF oligonucleotide reduces SRF protein expression in extracted DRG lysates over those animals treated with an MM oligonucleotide. With this verification of specificity, we proceeded to divide adult male rats into 4 groups to analyze mechanical hyperalgesic priming, as we and others have performed.^{1,24} Importantly, we used a chronic mechanical rodent behavior model that uses carrageenan priming because carrageenan-induced hyperalgesia is inhibited by mGluR5 antagonism.²⁷ The 4 groups included animals with no treatment, carrageenan (Cg) priming, Cg-primed animals that received MM oligonucleotide injections, and Cg-primed rats that received SRF-oligonucleotide (AS) injections. Rats were randomly selected to receive intrathecal injections during the 3 days

between vehicle/Cg and PGE₂ intraplantar injections into the same hind paw. As we have demonstrated previously, Cg-priming extends mechanical hypersensitivity after PGE₂ in a manner that mimics persistent pain (**Fig. 5B**). However, oligonucleotide knock down of SRF expression reversed the persistent mechanical hypersensitivity phenotype, indicating a functional, *in vivo* importance for the transcription factor.

4. Discussion

Chronic pain is considered a multifactorial phenomenon. Numerous publications have identified not only specific signaling pathways that contribute to the generation and maintenance of chronic pain, but also anatomical structures that support pain persistence. The difficulty in treating chronic pain effectively and without contributing to the opioid-use epidemic is identifying singular targets for pharmaceutical development that can address both peripheral and central afferent sensitization. However, the understanding that peripheral sensitization predicates central would suggest that addressing the mechanisms of initial peripheral sensitization could prevent eventual central sensitization. In this article, we identify a transcriptional process

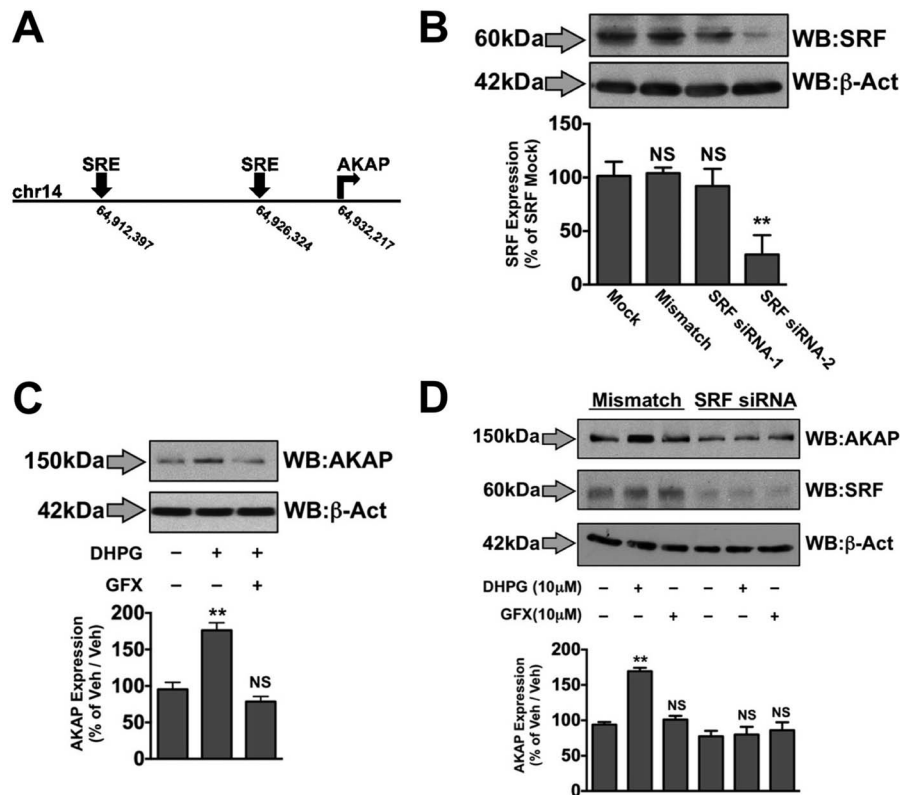


Figure 4. Serum response factor mediates AKAP upregulation. (A) Representation of AKAP150 gene with 2 individual serum response elements located 5' from start site. (B) Dorsal root ganglia cultures transfected in a mock setting, with mismatch siRNA, SRF siRNA-1, or SRF siRNA-2. Cell lysates were resolved by SDS-PAGE and probed for SRF and β -actin expression by WB to determine SRF siRNA efficacy. (C) Dorsal root ganglia cultures serum-starved for 18 hours and then treated for 24 hours with vehicle (ddH₂O) or DHPG (10 μ M) in the presence/absence of GFX (10 μ M, PKC inhibitor). Cell lysates were resolved by SDS-PAGE and probed for AKAP and β -actin expression by WB. (D) Dorsal root ganglia cultures treated with DHPG and GFX as indicated after transfection with either mismatch siRNA or SRF-siRNA-2 and an 18-hour serum starvation. For panels (B–D), WB results are representative of 4 individual trials. ** $P < 0.01$, NS = no significance, 2-way ANOVA with Bonferroni correction. ANOVA, analysis of variance; DRG, dorsal root ganglia; PKC, protein kinase C; SRE, serum response element; SRF, serum response factor; WB, Western blot.

that could explain how a number of signaling pathways contribute to the “chronification” of pain.

AKAP150 scaffolding protein has been identified as important in the efficient organization of signaling kinases and their targets within nociceptors, contributing to neuronal sensitization and

reduced TRP receptor thresholds.^{4,9,10,18,31} Here, we have identified how extended glutamate exposure may upregulate AKAP150 expression through the transcription factor SRF. In this mechanism, activation of mGluR5/PKC signaling pathway stimulates SRF transcription and the upregulation of AKAP150

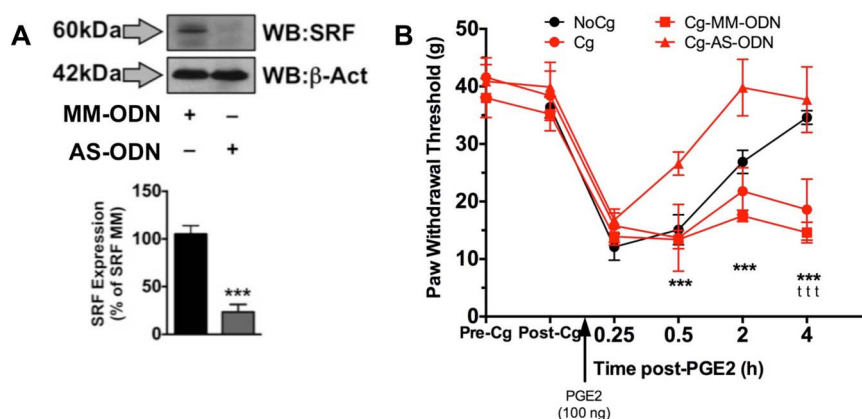


Figure 5. Serum response factor supports chronic hyperalgesia. (A) Serum response factor–specific antisense oligonucleotide (AS-ODN) knocks down in vivo DRG SRF expression 80% compared with control mismatch oligonucleotide (MM-ODN). *** $P < 0.005$, the Student t test. (B) Chronic hyperalgesic responses to a mechanical stimulus are reduced in rats intrathecally injected with MM-ODN or AS-ODN. ttt $P < 0.005$ for NoCg vs Cg, *** $P < 0.005$ for Cg-treated (red circle) vs Cg-AS-ODN (red triangle), 2-way ANOVA with Bonferroni post hoc. ANOVA, analysis of variance; DRG, dorsal root ganglia; SRF, serum response factor; WB, Western blot.

expression. Furthermore, oligonucleotide-mediated knock down of SRF in rat DRG in vivo reduced persistent mechanical hypersensitivity in a model of carrageenan-induced hyperalgesic priming. A signal transduction model in **Figure 6** illustrates these findings, and provides a mechanism by which extended glutamate exposure could stimulate an accumulation of AKAP150 scaffolding protein, thereby enabling the potential for increased TRP channel responsivity and peripheral afferent depolarization.

Previous work by our group has identified AKAP150 as an important component of hyperalgesic priming.⁴ Here, experimental results indicate that SRF also plays a role (**Fig. 4**). Although it may appear that our main hypothesis would be supported more so by performing the same experiment in AKAP150 WT and KO mice, or vice versa (AKAP150 oligonucleotide knock down in SRF WT and KO mice), both of these avenues would likely produce confounding results. First, AKAP150 modulates multiple receptors and channels that are likely involved in the persistent hypersensitivity associated with mechanical hyperalgesic priming, including TRPA1,⁴ TRPV1,²⁸ and potentially other glutamatergic ionotropic channels.⁷ Second, SRF likely mediates the transcription of other molecules such as *c-fos* that are possibly associated with persistent pain.^{13,19} Of course, applying AKAP150 oligonucleotide knock down in this behavioral model, although possible, would not provide much information in an SRF knock out mouse, given that the genomic ablation of SRF is embryonic lethal.² Taken together, our approach falls short in identifying that SRF-mediated upregulation of AKAP150 contributes to hyperalgesic priming, but does provide evidence that the transcription factor contributes to persistent mechanical hypersensitivity. Indeed, GFP-fluorescence in Panel H of **Figure 3** could indicate that expression of mGluR5 in peripheral glia could open up new roles for accumulated glutamate in the periphery in respects to persistent inflammatory hypersensitivity. Furthermore, the tenet between these behavioral results and our biochemical data agrees that SRF-mediated upregulation of AKAP150 could contribute to mechanical hypersensitivity associated with persistent pain.

The involvement of SRF-dependent transcription in pain behaviors is a potentially important phenomenon that bears additional investigation. The phosphorylation of SRF at Ser162 by PKC is a mechanism that could be considered downstream of

many inflammatory signaling systems. For this study, we used the broad-spectrum PKC inhibitor GF 109203X (GFX), given that multiple isoforms are believed to stimulate SRF transcription,²¹ and that even more isoforms are expressed in sensory neuronal systems and involved in pain. Although a drawback with using broad-spectrum PKC inhibitors are the nonspecific effects on other kinases at high concentrations, the data presented here support the direct signaling mechanism stimulated from mGluR5-Gαq-PKC-SRF.

Transcriptional regulation of pain behavior is a field of research that has been studied previously. From Runx1-mediated upregulation of TRPV1 expression in DRG neurons,²⁶ to transcription factor EB-mediated lysosomal activation in nerve injury pain,¹¹ many transcription factors have been localized and studied individually. Here, our studies focused on SRF, as a positive modulator of AKAP150 gene transcription in rat DRG neurons. As results presented herein identify a relationship between SRF and pain, it is important to remember that SRF likely promotes transcription of multiple genes in sensory neurons. These other proteins likely contribute to the variation between hyperalgesic priming behavior in control vs SRF oligonucleotide-treated animals. However, the findings from this work will stimulate future studies into the role of SRF, as it pertains to not only persistent pain but other models that lack definitive treatment targets for analgesia.

Disclosures

The authors have no conflict of interest to declare.

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Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at <http://links.lww.com/PR9/A19>.

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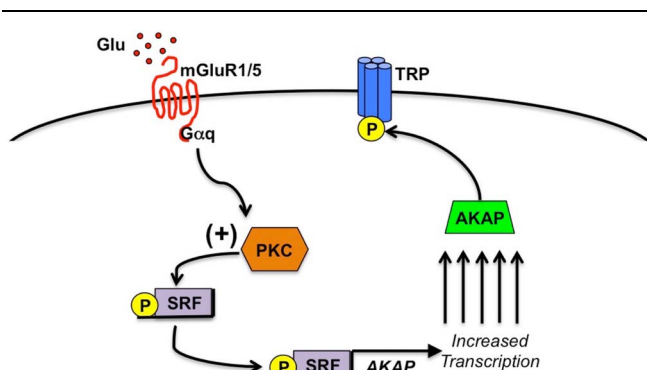


Figure 6. Schematic of mGluR5-mediated transcriptional upregulation of AKAP150. Activation of mGluR5 stimulates PKC-phosphorylation of serum response factor (SRF). When phosphorylated, SRF binds to serum response elements 5' of specific genes and drives transcription. Continued mGluR5 activity, driving constant SRF phosphorylation increases AKAP150 transcription and expression, further sensitizing nociceptors to continued activation and pain. PKC, protein kinase C.

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