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Protease activated receptor 2 (PAR₂) activation is sufficient to induce the transition to a chronic pain state

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Abstract

Protease Activated Receptor Type 2 (PAR₂) is known to play an important role in inflammatory, visceral and cancer-evoked pain based on studies using PAR₂ knockout (PAR₂^{-/-}) mice. Here we have tested the hypothesis that specific activation of PAR₂ is sufficient to induce a chronic pain state via extracellular signal-regulated kinase (ERK) signaling to protein synthesis machinery. We have further tested whether the maintenance of this chronic pain state involves a brain-derived neurotrophic factor (BDNF) / tropomyosin related kinase B (trkB) / atypical protein kinase C (aPKC) signaling axis. We observed that intraplantar injection of the novel, highly specific PAR₂ agonist, 2-aminothiazol-4-yl-LIGRL-NH₂ (2-at), evokes a long-lasting acute mechanical hypersensitivity (ED₅₀ ~ 12 pmoles), facial grimacing and causes robust hyperalgesic priming as revealed by a subsequent mechanical hypersensitivity and facial grimacing to prostaglandin E₂ (PGE₂) injection. The pro-mechanical hypersensitivity effect of 2-at is completely absent in PAR₂^{-/-} mice as is hyperalgesic priming. Intraplantar injection of the upstream ERK inhibitor, U0126 and the eukaryotic initiation factor (eIF) 4F complex inhibitor, 4EGI-1, prevented the development of acute mechanical hypersensitivity and hyperalgesic priming following 2-at injection. Systemic injection of the trkB antagonist ANA-12 likewise inhibited PAR₂-mediated mechanical hypersensitivity, grimacing and hyperalgesic priming. Inhibition of aPKC (intrathecal delivery of ZIP) or trkB (systemic administration of ANA-12) after the resolution of 2-at-induced mechanical hypersensitivity reversed the maintenance of hyperalgesic priming. Hence, PAR₂ activation is sufficient to induce neuronal plasticity leading to a chronic pain state, the maintenance of which is dependent on a BDNF/trkB/aPKC signaling axis.

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Keywords

Hyperalgesic priming; translation control; proteinase activated receptor; MAPK; atypical PKC; BDNF; PAR₂

INTRODUCTION

Protease Activated Receptor Type 2 (PAR₂) is a G-protein coupled receptor (GPCR) implicated in disease conditions including allergic asthma [43], cancer [53], arthritis [24], and chronic pain [42]. PAR₂ can be activated in response to various exogenous and endogenous proteases [47]. Proteolytic cleavage of the N terminus results in exposure of a tethered ligand which activates the receptor to induce signaling [42]. The primary method to study PAR₂ has been small peptides or peptidomimetics that mimic the naturally cleaved tethered ligand thus bypassing proteolytic cleavage of the N-terminal domain. This approach can be problematic, however, because this peptide sequence also activates mas-related G protein-coupled receptors (Mrgpr, GPCRs) that are specifically expressed in the sensory system and are involved in pain and itch signaling [35]. While PAR₂^{-/-} mice have been indispensable for elucidating the role of this receptor in normal physiology and pathology [42], a lack of suitable pharmacological tools have hindered full exploration of the role of this receptor in disease conditions, including chronic pain [1]. We have developed highly potent, efficacious and specific agonists [7; 21; 22] and used them here to explore the role of PAR₂ in the development of a chronic pain state.

PAR₂ is thought to play an important role in inflammatory [9; 42; 57], visceral [10; 12; 26; 50; 60] and cancer-evoked [5; 31; 32; 36] pain based on studies using PAR₂^{-/-} mice and/or antagonists suggesting an important role of PAR₂ in pathological pain. Hyperalgesic priming models have emerged as an important paradigm for probing plasticity associated with chronic pain in the nociceptive system [48]. We have previously demonstrated that a single injection of interleukin-6 (IL-6) induces hyperalgesic priming and that this priming is dependent on plasticity in the peripheral and central nervous system [4; 38–40]. This is consistent with similar experiments in rats using inflammatory stimuli [48]. Importantly, PAR₂^{-/-} mice fail to show nociceptive sensitization in many inflammatory pain models [9] and PAR₂ mediates alterations in dorsal root ganglion BDNF levels [5], a critical factor for hyperalgesic priming [39; 40]. Based on this, we hypothesized that specific PAR₂ activation should be sufficient to lead to the development of a chronic pain state as reflected by induction of hyperalgesic priming.

Our findings demonstrate a clear role for PAR₂ in induction of pain plasticity suggestive of a chronic pain state. Interestingly, while most previous studies in dorsal root ganglion (DRG) neurons have focused on PAR₂-mediated signaling via protein kinases A and C and/or phospholipase C and its role in regulation of ion channels [2; 3; 17; 23; 36; 44], we find that ERK signaling to translational machinery downstream of PAR₂ activation is required for mechanical hypersensitivity and hyperalgesic priming. Moreover, PAR₂-mediated induction and maintenance of hyperalgesic priming is dependent on BDNF/trkB/aPKC signaling, as we have previously shown for other mediators that induce this form of

nociceptive plasticity [39; 45]. We have elucidated a novel role for PAR₂ in pathological pain plasticity and identified the signaling mechanisms leading to this effect.

MATERIALS AND METHODS

Experimental animals

Male ICR mice (Harlan, 20–25 g) or C57Bl/6 (Jackson Labs, data in Fig 2) mice were used for the study. Approval for animal studies was obtained from the Institutional Animal Care and Use Committee of The University of Arizona, The University of Alabama at Birmingham and The University of Texas at Dallas. All animal procedures were in accordance with International Association for the Study of Pain guidelines. PAR₂^{-/-} mice were obtained from Jackson Labs and bred at University of Arizona. Littermates were used in all experiments. Male mice from both genotypes were used.

Drugs and primary antibodies

2-aminothiazol-4-yl-LIGRL-NH₂ (2-at, [7]) was prepared by semimanual solid-phase peptide synthesis performed in fritted syringes using a Domino manual synthesizer obtained from Torviq (Niles, MI). Crude peptides were purified by HPLC and size exclusion chromatography. Purity of the peptides was ensured using analytical HPLC (Waters Alliance 2695 separation model with a dual wavelength detector, Waters 2487) with a reverse-phase column (Waters Symmetry, 4.6 × 75 mm, 3.5 μm; flow rate, 0.3 ml/min). Structure was characterized by electrospray ionization on a Thermoelectron (Finnigan) LCQ ion trap instrument (low resolution), a Bruker Ultraflex III MALDI-TOF/TOF (low resolution), or a Bruker 9.4 T Fourier transform ion-cyclotron resonance (high resolution accurate mass) mass spectrometer.

For the Western blot experiments, rabbit polyclonal antibodies were obtained from Cell Signaling: phospho-ERK (Thr202/Tyr204, cat# 9101), total-ERK (cat# 9102). U0126 and U0124 were from Tocris; Prostaglandin E₂ (PGE₂) was from Cayman Chemical; 4EGI-1 [41] was from Axxora; ANA-12 [11] was from Maybridge and ZIP and Scrambled ZIP were from Anaspec. Stock solutions of U0126, U0124, 4EGI1 and ANA-12 were made in 100% DMSO. ZIP and Scrambled ZIP stock solutions were made in distilled H₂O. All compounds except U0126, U0124 and ANA-12 were diluted to final concentrations in saline for injection. U0126 was diluted to final concentration in 45% β-cyclodextran. ANA-12 was diluted to final concentration in 10% polyethylene glycol 300.

Behavioral testing

Animals were placed in acrylic boxes with wire mesh floors and allowed to habituate for approximately 1 h on all testing days. Paw withdrawal thresholds were measured using calibrated von Frey filaments (Stoelting, Wood Dale, IL) by stimulating the plantar aspect of left hind paw using the up-down method [13]. A mouse model based on 'hyperalgesic priming model' originally developed by Levine and colleagues [48] and adapted for mice was used for the study. Baseline mechanical thresholds of the left hind paw were measured prior to 2-at injection. For the dose-response experiment, 2-at was injected at escalating doses into the plantar surface of the left hind paw in a volume of 25 μL (diluted in saline).

The approximate ED₅₀ dose (30 pmoles) of 2-at was used for all subsequent experiments. For acute mechanical hypersensitivity experiments with U0126, 4EGI-1 or ANA-12, 2-at (30 pmoles) was injected into the plantar surface of the left hind paw in a volume of 25 μ L (diluted in saline) and paw withdrawal thresholds were measured at 1 h, 3 h, Day 1, Day 2, Day 5 and Day 14 post injection. U0126 (10 μ g, [38]) or U0124 (control) (10 μ g) was co-injected into the plantar surface of the left hind paw with 2-at. 4EGI-1 (25 μ g, [38]) or vehicle was co-injected into the plantar surface of the left hind paw with 2-at. ANA-12 (1 mg/kg, [39]) was injected intra-peritoneally (IP) for 3 days starting on the day of 2-at injection. For all hyperalgesic priming experiments, mice received an injection of PGE₂ (100 ng, [4; 38]) in the plantar surface of left hind paw in a volume of 25 μ L 14 days following initial intraplantar injection of 2-at. Following PGE₂ injection, paw withdrawal thresholds were again measured at 3 h and 24 h following the PGE₂ injection. For maintenance of hyperalgesic priming experiments with ANA-12, ANA-12 (1 mg/kg) was injected IP for 3 days starting on day 11 following 2-at administrations. For maintenance of hyperalgesic priming experiments with ZIP, ZIP (10 μ g, Anaspec, [4; 39]) or scrambled ZIP (10 μ g, Anaspec) was injected intrathecally (IT) on day 12 following 2-at injections.

The protocol originally developed by Langford and colleagues for testing facial grimacing in mice was utilized for this study [33]. Mice were placed individually on a tabletop in cubicles (9 $\times$ 5 $\times$ 5 cm high) with 2 walls of transparent acrylic glass and 2 walls of removable stainless steel. Two high-resolution (1920 $\times$ 1080) digital video cameras (High-definition Handycam Camcorder, model HDR-CX100, Sony, San Jose, CA) were placed immediately outside both acrylic glass walls to maximize the opportunity for clear head shots. For acute experiments, video was taken immediately before 2-at injections, and at the indicated time points following 2-at injections. For the hyperalgesic priming experiment, video was taken immediately before PGE₂ injections, and 3 and 24 hrs following PGE₂ injections.

Primary neuronal cultures

Mouse dorsal root ganglia (DRG) were excised aseptically and placed in Hank's Buffered Salt Solution (HBSS, Invitrogen) on ice. The ganglia were dissociated enzymatically with collagenase A (1 mg/ml, 25 min, Roche) and collagenase D (1 mg/ml, Roche) with papain (30 U/ml, Roche) for 20 min at 37°C. To eliminate debris 70 μ m (BD) cell strainers were used. The dissociated cells were suspended in DMEM/F12 (Invitrogen) containing 1 $\times$ pen-strep (Invitrogen), 1 $\times$ GlutaMax (Invitrogen) and 10% fetal bovine serum (Hyclone). The cells were plated in 6-well plates (BD Falcon) and incubated at 37°C in a humidified 95% air/5% CO₂ incubator. Cultures were maintained in resuspension media until time of treatment. On day 5 the cells were washed in DMEM/F12 media for 30 mins and subsequently were treated as described in results.

Western blotting

Protein was extracted from cells in lysis buffer (50 mM Tris HCl, 1% Triton X-100, 150 mM NaCl, and 1 mM EDTA at pH 7.4) containing protease and phosphatase inhibitor mixtures (Sigma) with an ultrasonicator on ice, and cleared of cellular debris and nuclei by centrifugation at 14,000 RCF for 15 min at 4°C. 15 μ g of protein per well were loaded and separated by standard 7.5% SDS-PAGE. Proteins were transferred to Immobilon-P

membranes (Millipore) and then blocked with 5% dry milk for 3 h at room temperature. The blots were incubated with phospho-ERK (p-ERK) or total-ERK (t-ERK) primary antibody (Cell Signaling Technologies) overnight at 4°C and detected the following day with donkey anti-rabbit antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch). Signal was detected by ECL on chemiluminescent films. Phosphoprotein was normalized to the expression of the total protein on the same membrane. Densitometric analysis was performed using Image J software (NIH).

Statistical Analysis and Data Presentation

Data are shown as means and the standard error of the mean ($\pm$ SEM) of 8 independent cell culture wells or 6 mice per group (for behavioral studies). Graph plotting and statistical analysis used Graphpad Prism Version 5.03 (Graph Pad Software, Inc. San Diego, CA, USA). Statistical evaluation was performed by two-way analysis of variance (ANOVA), followed by Bonferroni's post-hoc tests for multiple comparisons or by unpaired t-test for pair-wise comparisons. Dose-response calculations were done using non-linear regression with variable slope (four parameters) with area under the curve calculations from cumulative mechanical hypersensitivity data. The *a priori* level of significance at 95% confidence level was considered at $p < 0.05$.

RESULTS

The specific PAR₂ agonist 2-at induces long-lasting mechanical hypersensitivity, facial grimacing and hyperalgesic priming revealed by subsequent exposure to a sub-threshold dose of PGE₂

We first investigated if a single injection of PAR₂ agonist is sufficient to induce acute mechanical hypersensitivity and hyperalgesic priming. Mice were injected with increasing doses of the PAR₂ agonist 2-at (3, 8, 30, 300 or 3,000 pmoles) into the left hind paw and mechanical thresholds were measured over the ensuing 14 days. 2-at induced long-lasting acute mechanical hypersensitivity in a dose dependent manner (Fig 1A) with a calculated ED₅₀ of 12.3 pmoles (Fig 1B, 95% confidence interval = 2.57 – 58.5 pmoles). The 30, 300 and 3000 pmole doses were not statistically different from each other except at the 24 hr time point where the 30 and 3000 doses were significantly different. We assessed for hyperalgesic priming with an intraplantar injection, into the same hindpaw, of the inflammatory mediator PGE₂ (100 ng) after the resolution of 2-at-mediated mechanical hypersensitivity. Mice previously receiving vehicle displayed only a transient hypersensitivity following PGE₂ injection. In contrast, mice receiving 2-at injection all developed long-lasting mechanical hypersensitivity lasting at least 24 h (Fig 1C). The most robust response was observed in the mice previously treated with 30 pmoles 2-at. We used this dose in all subsequent experiments.

Pain induces affective changes in behavior that may not readily be captured by withdrawal reflex-mediated behavioral assessments [8]. This affective pain component can be measured by facial expressions [33] as has been well characterized in humans [16; 30]. To measure an affective pain response to 2-at, mice were injected with 2-at in the left hind paw and grimacing was measured utilizing the mouse grimace scale (MGS). 2-at induced an increase

in MGS score (Fig 1D) following 2-at injection compared to the mice treated with vehicle. Following injection of 100 ng PGE₂ in the left hind paw of previously primed mice, we also observed an increase in MGS score (Fig 1E). Hence, PAR₂ agonism acutely induces mechanical hypersensitivity and grimacing and a transition to a chronic state of pain plasticity where a subthreshold dose of PGE₂ is capable of inducing mechanical hypersensitivity and an affective pain response.

Next, we investigated if the 2-at-mediated effects are PAR₂ specific. Wild-type and PAR₂^{-/-} C57Bl/6 mice were injected with 2-at in the left hind paw and mechanical thresholds were assessed. 2-at induced long-lasting acute mechanical hypersensitivity in wild type mice but not in PAR₂^{-/-} mice (Fig 2A). Similarly, injection of PGE₂ induced precipitation of hyperalgesic priming in wild-type mice but not in PAR₂^{-/-} mice. Hence, 2-at acts in a PAR₂-dependent fashion to induce a chronic pain state.

PAR₂-mediated mechanical hypersensitivity and hyperalgesic priming depends on ERK- and eIF4F complex signaling

Next, we sought to understand the downstream signaling mechanisms underlying PAR₂ induced mechanical hypersensitivity and hyperalgesic priming. Following activation, PAR₂ is quickly phosphorylated via a G-protein receptor kinase pathway that leads to β-arrestin binding and activation of ERK signaling [1]. While extensive investigations have examined the role of PAR₂-mediated PKC, PKA, phospholipase C and Ca²⁺ signaling in nociceptive plasticity [3; 17; 23; 44], possible connections to ERK signaling have not been explored in behavioral experiments. To investigate if ERK signaling is required for PAR₂-induced mechanical hypersensitivity and/or hyperalgesic priming, mice were co-injected with 30 pmoles 2-at and the MEK inhibitor U0126 (10 μg) or an inactive control compound U0124 (10 μg) in the left hind paw and paw withdrawal thresholds were measured. U0126 inhibited the development of mechanical hypersensitivity induced by 2-at injection (Fig 3A) and attenuated PGE₂ precipitated hyperalgesic priming (Fig 3A). To verify that PAR₂ signaling induced activation of ERK, DRG neurons in culture were treated with PAR₂ agonist, 2-at (10 μM), for 5 min. Treatment of DRG neurons induced a significant increase in ERK phosphorylation (Fig 3B).

We have demonstrated that mechanical hypersensitivity and hyperalgesic priming induced by IL-6 depends on protein translation regulated by the eIF4F complex [4; 38; 40]. Hence we hypothesized that eIF4F complex formation may be required for PAR₂-induced mechanical hypersensitivity and hyperalgesic priming. To test this we used the eIF4F complex formation inhibitor 4EGI-1 [41]. Mice were co-injected with 2-at and 4EGI-1 (25 μg) or vehicle in the left hind paw. 4EGI-1 inhibited the development of mechanical hypersensitivity induced by 2-at injection (Fig 4) and strongly attenuated PGE₂-precipitated hyperalgesic priming (Fig 4). Consistent with previous results with IL-6 and nerve growth factor, ERK signaling to translational machinery is required for a full mechanical hypersensitivity response and for the establishment of hyperalgesic priming.

A BDNF/trkB/aPKC signaling axis is required for establishment and maintenance of PAR₂-mediated acute mechanical hypersensitivity and hyperalgesic priming

BDNF is expressed by nociceptors [28; 61] and its release in the dorsal horn sensitizes postsynaptic responses [5; 28; 39; 49; 61; 62]. BDNF displays considerable plasticity in expression following injury including de novo expression in a subpopulation of sensory neurons [37] and increased expression in microglia [15]. We have demonstrated that the initiation and maintenance of hyperalgesic priming evoked by IL-6 depends on BDNF signaling via trkB in the spinal dorsal horn [39]. Hence, we hypothesized that trkB activation is required for establishment and maintenance of PAR₂ agonist induced hyperalgesic priming. Mice were injected with 2-at and the small molecule trkB antagonist, ANA-12 (1 mg/kg, [11]), or vehicle was injected IP for 3 days starting the day of 2-at injection. ANA-12 inhibited the development of mechanical hypersensitivity induced by 2-at injection (Fig 5) and blocked the development of PGE₂-precipitated hyperalgesic priming (Fig 5).

Atypical PKCs participate in synaptic plasticity linked to learning and memory [51; 52] and chronic pain [45]. We have demonstrated that inhibition of aPKCs with a peptide inhibitor, ZIP, leads to a reversal of the maintenance of hyperalgesic priming [4; 39]. Moreover, we have shown that BDNF signals via aPKCs in spinal synaptosomes [39]. Hence we hypothesized that aPKCs and trkB are required for maintenance of PAR₂ agonist induced hyperalgesic priming. To test this, mice were injected with 2-at or IL-6 and following resolution of the initial mechanical hypersensitivity, mice were injected with the small molecule TrkB antagonist, ANA-12 (1 mg/kg) or vehicle IP for 2 days (Fig 6A). ANA-12 treatment led to a significant attenuation of PGE₂-precipitated hyperalgesic priming in mice previously exposed to 2-at and to IL-6, consistent with our previous results with IL-6. To test the role of aPKCs, following resolution of initial mechanical hypersensitivity, mice were injected with ZIP or scrambled ZIP IT on day 12 after 2-at. ZIP effectively reversed the maintenance of hyperalgesic priming (Fig 6B). Hence, a BDNF/trkB/aPKC signaling axis is implicated in PAR₂-mediated hyperalgesic priming.

PAR₂-mediated grimace behavior is mediated by local translation regulation signaling to the eIF4F complex and trkB receptors

Finally we sought to address signaling events regulating affective pain induced by PAR₂ activation. We assessed two unique signaling pathways: PAR₂ signaling to translation machinery in the periphery and BDNF/trkB signaling. When 2-at was injected with the eIF4F complex inhibitor 4EGI-1 at the same dose we previously showed attenuated mechanical hypersensitivity we also observed an inhibition of grimacing at 3 and 24 hrs after injection (Fig 7A). While 2-at-induced mechanical hypersensitivity lasts for ~ 7 days, grimacing induced by 2-at completely resolved by 2 days after injection showing that the duration of the affective component of PAR₂ signaling is shorter lived than mechanical hypersensitivity. Also paralleling mechanical hypersensitivity findings, 4EGI-1 given at the time of 2-at injection blocked the development of grimacing induced by subsequent PGE₂ injection in primed animals (Fig 7A). With the trkB antagonist, ANA-12, given systemically, we also observed similar findings to our mechanical hypersensitivity experiment. ANA-12 completely blocked grimacing at 1 and 3 hrs after injection and also

attenuated the subsequent response to PGE₂ (Fig 7B). We conclude that local translation and BDNF/trkB signaling regulate affective pain behaviors induced by PAR₂ activation.

DISCUSSION

We have shown that a single intraplantar injection of a highly specific PAR₂ agonist evokes a long-lasting acute mechanical hypersensitivity, facial grimacing and causes robust hyperalgesic priming as revealed by precipitation of mechanical hypersensitivity and facial grimacing in response to a normally subthreshold dose of PGE₂. Furthermore, we elucidate signaling mechanisms responsible for these effects (summarized in Fig 8A and B) providing evidence for a role of translation control in PAR₂-mediated pain plasticity and downstream dorsal horn signaling via a BDNF/trkB/aPKC signaling axis. The present findings provide compelling evidence that specific PAR₂ activation is sufficient to induce neuronal plasticity leading to a chronic pain state and further substantiate PAR₂ as an important therapeutic target for pain therapeutics.

Hyperalgesic priming models have emerged as an important experimental paradigm for probing plasticity leading to chronic pain in the nociceptive system [48]. While multiple studies have explored the role of PAR₂ in a variety of pain states, our study is the first to demonstrate the importance of PAR₂ in pain plasticity suggestive of a chronic pain state. Importantly, we have done this using traditional reflex withdrawal-evoked pain measures and using new behavioral assays that capture affective components of pain. We find that acute injection of a PAR₂ agonist causes an increase in facial grimace score demonstrating that activation of PAR₂ is sufficient to induce an affective pain state. Interestingly this affective pain state does not last for the same duration as mechanical hypersensitivity (2 versus 7 days). This differential duration may reflect ongoing afferent input (in the case of grimace) versus longer lasting plasticity mechanisms that promote increased mechanical sensitivity. Alternatively it may reflect suppression of facial expression changes over a longer time course as has been proposed previously [33]. To our knowledge only one other previous study has examined a role for PAR₂ in non-evoked pain measures [31]. Lam and colleagues developed the dolognawmeter assay, a behavioral paradigm that challenges mice to gnaw through obstacles to reach a chamber and demonstrated that while head and neck cancers negatively influence performance on the dolognawmeter task, PAR₂^{-/-} mice were unaffected despite the normal progression of cancer [31]. Importantly head and neck cancers secrete proteases that act on PAR₂ to induce this functional impairment [31; 32]. Our study complements these findings providing strong evidence that PAR₂ activation is sufficient to induce an affective pain state in mice. Furthermore we show that this affective pain state requires peripheral translation and BDNF/trkB signaling. It is therefore likely that diseases associated with protease secretion (e.g., inflammatory bowel disease [12]) are strongly influenced by the actions of PAR₂ in terms of the presence of spontaneous ongoing pain. Since this is a primary complaint of these disorders, the present findings place new prominence on PAR₂ as a therapeutic target for this most salient clinical symptom.

While hyperalgesic priming models are emerging as a compelling preclinical model of the transition to a chronic pain state it is as yet unclear if hyperalgesic priming can be observed in humans [56]. There is evidence that chronic pain states in humans are often discontinuous

[58] or can be brought about by priming from a previous injury [29]. Two excellent examples are chronic post-surgical pain where the chronic phase is often preceded by pain-free periods [58] and migraine and other headache disorders where pain is often brought on by triggers but is clearly not continuous [19]. A potential explanation for these features is underlying plasticity mechanisms that change susceptibility to pain when subthreshold insults are encountered. While it remains to be seen if mechanisms of hyperalgesic priming modeled in preclinical studies have broad application to clinical features of chronic pain, these models do offer new mechanistic insights into the maintenance of plasticity in the nociceptive system and how they may set the stage for pain susceptibility. In agreement with the viewpoint of others [48; 56], we posit that hyperalgesic priming models the transition to a chronic pain that is plausibly linked to chronic pain in humans.

Following the validation of PAR₂ in inducing affective signs of pain and in hyperalgesic priming, we sought to identify the downstream signaling mechanisms underlying PAR₂ induced mechanical hypersensitivity and hyperalgesic priming. Proteolytic cleavage of the N terminus of PAR₂ exposes a tethered ligand that activates the receptor to induce intracellular Ca²⁺ signaling following activation of G_q G-protein (Fig 8A). PAR₂ is quickly phosphorylated leading to β -arrestin binding and activation of ERK signaling [18]. The β -arrestin portion of this signaling event has been widely studied in terms of desensitization of the receptor, especially at high agonist concentrations, and may explain why a 30 pmole dose was more efficacious than higher doses *in vivo*. ERK signaling plays an important role in DRG neuron excitability [54; 59] and previous studies have linked PAR₂-mediated ERK signaling to alterations in DRG neuron function *in vitro* [27] (Fig 8B). Many previous studies have focused on PAR₂-mediated G_q signaling and its role in regulation of ion channels, including transient receptor potential (TRP) family members in induction of pain *in vivo* [3; 17; 23; 44; 60] (Fig 8A). We demonstrate that PAR₂-induced acute mechanical hypersensitivity and hyperalgesic priming is dependent on ERK signaling and that 2-ATP activates ERK phosphorylation in DRG neurons *in vitro*. This is important because previous studies demonstrating activation of ERK by PAR₂ *in vitro* have relied on SLIGRL at concentrations that are not PAR₂ specific [27] and these activities could be attributed to MrgprC11. ERK regulates DRG excitability via direct phosphorylation of channels, mainly voltage gated sodium channels [54], and through changes in gene expression via translation regulation [4; 38; 40]. Translation regulation downstream of ERK activation is mediated by MAPK interacting kinase (MNK) signaling to eukaryotic initiation factor (eIF) proteins that bind the m⁷GTP cap structure of mRNAs to regulate the initiation step of translation [46] (Fig 8B). This signaling event is occluded by the eIF4F complex inhibitor, 4EGI-1 [41]. We have previously shown that 4EGI-1 inhibits nerve growth factor (NGF) and interleukin 6 (IL-6) induced mechanical hypersensitivity and hyperalgesic priming. Here we show that 4EGI-1 likewise inhibits PAR₂-mediated mechanical hypersensitivity, hyperalgesic priming and facial grimacing [38]. This finding, combined with other studies that have highlighted the crucial role of translation regulation in nociceptor plasticity and a transition to chronic pain [6; 20], further substantiates the importance of local translation in sensory neurons for the induction of pain plasticity. Moreover, it demonstrates a novel signaling mechanism for PAR₂ that has not been explored extensively and represents the first evidence for a GPCR signaling to the translation machinery in DRG neurons to induce a state of pain plasticity.

Finally, these are the first findings to show that peripheral translation mechanisms are required to induce an affective pain state via receptors that engage this signaling pathway.

Multiple lines of evidence suggest that BDNF, originating either from DRG neurons [61] or spinal microglia [15] plays an important role in synaptic plasticity in the dorsal horn and maintenance of chronic pain states. Expression of BDNF is upregulated in DRG after injury [37]. Although there are likely different sources of BDNF in different preclinical pain models, there is consensus that it is released in an activity-dependent fashion in the spinal dorsal horn [34] and activates trkB receptors found on dorsal horn neurons [15; 39; 61; 62] or the presynaptic endings of DRG neurons [14]. Activation of trkB receptors can lead to multiple downstream effects such as phosphorylation of NMDA receptors [14], increased eIF4F complex formation and local protein synthesis [55], stimulation of aPKC phosphorylation [39] and/or degradation of the K⁺, Cl⁻ cotransporter KCC2 [25]. Herein we demonstrate that BDNF is required for initiation as well as maintenance of PAR₂ induced hyperalgesic priming and for PAR₂-mediated grimacing. This finding is in line with the demonstration that PAR₂ plays an important role in changes in BDNF expression in the dorsal horn in a model of bone cancer pain [5]. This finding is also in line with our previous demonstration that disruption of BDNF/trkB signaling with the trkB antagonist ANA-12 relieves acute mechanical hypersensitivity induced by IL-6 and blocks the initiation and maintenance of hyperalgesic priming [39]. They are also consistent with our finding that BDNF activates aPKCs in the spinal dorsal horn where aPKC inhibition with the peptide inhibitor ZIP leads to a resolution of plasticity associated with hyperalgesic priming [39]. It is important to note that while we favor the hypothesis that BDNF has a spinal mechanism of action in the initiation and maintenance of hyperalgesic priming and in the grimacing effect, ANA-12 was administered systemically and we cannot definitively conclude a site of action based on these studies.

In conclusion, we have elucidated a novel role for PAR₂ in pathological pain plasticity and identified the novel signaling mechanisms leading to this effect. The present findings provide a better understanding of the mechanisms through which PAR₂ activation may lead to the development of chronic pain states. They also highlight the importance of this target for the development of future pain therapeutics.

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Figure 1. The potent PAR₂ agonist 2-at induces mechanical hypersensitivity and hyperalgesic priming in mice

A) Mice were injected with increasing doses of 2-at in the left hind paw and mechanical thresholds were measured at indicated time points. 2-at induced long-lasting acute mechanical hypersensitivity in a dose dependent manner. B) Dose-response data for cumulative area under the curve responses for each dose. C) Mice were injected with 100ng PGE₂ in the left hind paw following resolution of initial hypersensitivity. PGE₂ injection induced a long-lasting mechanical hypersensitivity in mice previously exposed to effective doses of 2-at. D) Mice were injected with 30 pmole dose of 2-at in the left hind paw and

facial grimacing was measured using the mouse grimace scale (MGS) at indicated time points. 2-at induced facial grimacing. E) Mice were injected with 100 ng PGE₂ in the left hind paw following resolution of initial hypersensitivity. PGE₂ injection induced an increase in MGS only in mice previously treated with 2-at. ** $p < 0.01$, *** $p < 0.001$; two way anova with Bonferroni's multiple comparisons test.

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Figure 2. 2-at effects are PAR₂-dependent

A) Wild type and PAR₂^{-/-} C57Bl/6 mice were injected with 30 pmoles 2-at in the left hind paw and mechanical thresholds were measured at indicated time points. 2-at induced long-lasting acute mechanical hypersensitivity in wild type but not PAR₂^{-/-} mice. B) 2-at induced hyperalgesic priming is absent in PAR₂^{-/-} mice. *** p < 0.001; two way anova with Bonferroni's multiple comparisons test.

Figure 3. PAR₂-mediated acute mechanical hypersensitivity and hyperalgesic priming initiation is ERK dependent

A) Mice were co-injected with 2-at and U0126 (10 µg) or U0124 (10 µg) in the left hind paw. U0126 inhibited the development of mechanical hypersensitivity induced by 2-at and attenuated PGE₂-precipitated hyperalgesic priming. (B) Treatment of DRG neurons with 2-at or 2-at-PEG₃-PALM for 5 min induced an increase in phosphorylation of ERK. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two way anova with Bonferroni's multiple comparisons test for (A) and (B) and unpaired t-test for (B).

Figure 4. PAR₂-mediated acute mechanical hypersensitivity and hyperalgesic priming initiation is eIF4F complex dependent

Mice were co-injected 2-at and the eIF4F complex formation inhibitor 4EGI-1 (25 µg) or vehicle in the left hind paw. 4EGI-1 inhibited the development of mechanical hypersensitivity induced by 2-at injection and attenuated PGE₂-precipitated hyperalgesic priming. * $p < 0.05$, *** $p < 0.001$; two way anova with Bonferroni's multiple comparisons test.

Figure 5. PAR₂-mediated acute mechanical hypersensitivity and hyperalgesic priming initiation is dependent on BDNF/trkB signaling

Mice were injected with 2-at in the left hind paw. ANA-12 (1 mg/kg) or vehicle was injected IP for 3 days starting at the time of 2-at injection. ANA-12 inhibited the development of mechanical hypersensitivity induced by 2-at and attenuated PGE₂-precipitated hyperalgesic priming. ** $p < 0.01$, *** $p < 0.001$; two way anova with Bonferroni's multiple comparisons test.

Figure 6. Maintenance of PAR₂-mediated hyperalgesic priming is dependent on trkB and aPKC signaling

Mice were injected with 2-at or IL-6 in the left hind paw on Day 1. Following resolution of initial mechanical hypersensitivity, mice were injected with the small molecule trkB antagonist, ANA-12 (1 mg/kg) or vehicle IP for 2 days (A) or with a single IT injection of ZIP or scrambled ZIP (B). ANA-12 and ZIP attenuated PGE₂-precipitation of hyperalgesic priming. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two way anova with Bonferroni's multiple comparisons test.

Figure 7. Inhibition of eIF4F complex formation or trkB antagonism attenuates PAR₂-induced grimacing

A) Mice were injected with 2-at and vehicle or 2-at and 4EGI-1 (25 μ g) into the left hindpaw and facial scoring was done at the indicated time points. 4EGI-1 blocked 2-at-induced grimacing and attenuated the subsequent response to PGE₂ injection into the left hindpaw.

B) ANA-12, given systemically also attenuated 2-at induced grimacing 1 and 3 hrs after injection and blocked grimacing in response to PGE₂ injection 14 days later. * p < 0.05, ** p < 0.01, *** p < 0.001; two way anova with Bonferroni's multiple comparisons test.

Figure 8. PAR₂ signaling via ERK to the translational machinery as a novel mechanism of PAR₂-mediated pain plasticity

Agonist activation of PAR₂ is known to stimulate two major signaling events, Ca²⁺ mobilization (A) and ERK activation (B). While a role for Ca²⁺ mobilization has been well described in terms of PAR₂-mediated thermal hypersensitivity the role of ERK activation is not understood. The current work indicates that PAR₂-mediated stimulation of ERK leads to a translation-dependent mechanical hypersensitivity and hyperalgesic priming (B). MNK1/2 kinases are implicated in this effect because they signal downstream of ERK to eIF4 proteins that regulate protein synthesis. These eIF4 proteins are targets for 4EGI-1, which abrogates PAR₂-mediated mechanical hypersensitivity and hyperalgesic priming.