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Protease-Activated Receptor 1 is implicated in irritable bowel syndrome mediators-induced signaling to thoracic human sensory neurons

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Proteases and Protease-Activated Receptors (PARs) are major mediators involved in irritable bowel syndrome (IBS). Our objectives were to decipher the expression and functionality (calcium signaling) of PARs in human dorsal root ganglia (DRG) neurons, and to define mechanisms involved in human sensory neuron signaling by IBS patient mediators.

Human thoracic DRG were obtained from the national disease resource interchange. Expression of PAR_1, PAR_2 and PAR_4 was assessed by immunohistochemistry and RT-qPCR in whole DRG or in primary cultures of isolated neurons. Calcium signaling in response to PAR agonist peptides (PAR-AP), their inactive peptides (PAR-IP), thrombin (10u/ml), supernatants from colonic biopsies of IBS patients or healthy controls (HC), with or without PAR_1 or PAR_4 antagonist were studied in cultured human DRG neurons.

PAR_1, PAR_2 and PAR_4 were all expressed in human DRG, respectively in 20%, 40% and 40% of the sensory neurons. PAR_1-AP increased intracellular calcium concentration in a dose-dependent manner. This increase was inhibited by PAR_1 antagonism. In contrast, PAR_2-AP, PAR_4-AP and PAR-IP did not cause calcium mobilization. PAR_1-AP-induced calcium flux was significantly reduced by pre-incubation with PAR_4-AP, but not with PAR_2-AP. Thrombin increased calcium flux, which was inhibited by a PAR_1 antagonist and increased by a PAR_4 antagonist. Supernatants from colonic biopsies of IBS patients induced calcium flux in human sensory neurons compared to HC, this induction was reversed by a PAR_1 antagonist.

Taken together, our results highlight that PAR_1 antagonism should be investigated as a new therapeutic target for IBS symptoms.

Keywords: Proteases; PARs; Protease-Activated Receptors; Visceral pain; Inflammation; Irritable Bowel Syndrome; Visceral hypersensivity; Thrombin; Human dorsal root ganglia neurons.

Introduction

Irritable Bowel Syndrome (IBS) affects 11 to 20% of the Western population with a higher prevalence in women [14; 31]. IBS associates abdominal pain, diarrhea (IBS-D), constipation (IBS-C) or both (IBS-A, for alternate) [30]. Although IBS is a functional gastrointestinal disorder, not associated with gross structural or biochemical abnormalities [23], several recent studies indicate the presence of meaningful micro-organic changes [7]. One of the emerging ideas to explain the visceral pain associated with IBS is that sensory neurons innervating the colon are hyperexcitable in these patients [10; 11; 13]. However, because of the difficulties associated with human sensory neuron cultures, it has been difficult to evaluate the relevance of identified mediators in the context of human pathology.

Among the molecular targets explored to decipher neuronal hyperexcitability in IBS, several studies showed that proteases released by colonic biopsies of IBS patients were able to activate mouse and rat intestinal neurons *in vitro* and to induce somatic and visceral hypersensitivity *in vivo [9; 13; 17; 38; 47]*. Proteases are known to signal to mouse or rat sensory neurons through the activation of Protease-Activated Receptors (PARs) [19; 46; 53; 55], a family of G protein-coupled receptors that includes 4 members: PAR_1, PAR_2, PAR_3 and PAR_4 [40]. Only PAR_1, PAR_2 and PAR_4 seem to be able to signal through calcium mobilization and to exert a role in nociception and pain (PAR_3 has been considered more as a co-factor for other PAR activation[35] so far). PAR_1, $_2$ and $_4$ are activated by the proteolytic cleavage of their N-terminal domain, which reveals a tethered ligand that binds and activates the receptors. The role of PARs in visceral inflammation and pain has been well studied in animal models[49; 50]. Both PAR_1 and PAR_2 agonists induce calcium mobilization in rodent sensory neurons[17; 21]. PAR_2 agonists induce pain in somatic and visceral models [51; 53], while PAR_1 and PAR_4 agonists attenuate nociception and pain symptoms in rodents [4-6; 32]. In mouse sensory neurons, the calcium mobilization induced by supernatants from colonic biopsies of IBS patients is dependent on supernatant's proteolytic activity and on PAR_2 expression in mouse primary afferents [17]. In contrast, PAR_4 activation inhibits PAR_2-induced calcium mobilization and intrinsic excitability of colonic dorsal root ganglia (DRG) neurons, as well as overall pain [5; 6; 28]. However, in the context of human nociception and pain, the effects of PAR agonists and PAR signaling mechanisms are largely unknown.

Proteases show different specificity for the different PARs[52]. For instance, thrombin can activate PAR_1, PAR_3, PAR_4 and to a lower extend PAR_2, trypsin can activate PAR_2, PAR_3 and PAR_4 and at high concentrations PAR_1 [25; 33; 42]. In the context of IBS, tryptase and trypsin-3 are up-regulated, and are considered as possible endogenous agonists for PARs[9; 43].

Our study aimed at deciphering PAR signaling in human sensory neurons, determining whether proteases and PARs could play a role in human sensory neuron biology and nociceptive mechanisms. Further, we investigated human sensory neuron responses to IBS mediators in cultured human DRG neurons. Although not only neurons projecting from the digestive tract are present in our cultures, the present study could give insights in the response of human primary afferents to mediators present in IBS patient tissues.

Methods

Chemicals

Each agonist and inactive peptides of PARs, respectively PAR-AP and PAR-IP, where purchased from Genscript (France): PAR_1-AP (TFFLR), PAR_2-AP (SLIGKV), PAR_4-AP (GYPGQV), PAR_1-IP (RLLFT), PAR_2-AP (LRGILS) and PAR_4-AP (YAPGQV). Thrombin, PAR_1-antagonist (SCH79797), PAR_2 antagonist (GB83) and PAR_4-antagonist (ML354) were obtained from Tocris (Denver, USA).

Patient biopsies and supernatant collection

Colon biopsy samples from 24 patients with IBS (8 IBS-D, 8 IBS-C, 8 IBS-A) and 5 healthy controls, HCs) undergoing colorectal cancer screening were collected during colonoscopy at the Department of Medical and Surgical Sciences of the University of Bologna, Italy (Supplementary Table 1, available online at http://links.lww.com/PAIN/A553). Rome III criteria were used for the diagnosis of IBS patients. Additional exclusion criteria were major abdominal surgery, celiac disease, asthma, allergic disorders, anti-inflammatory treatments, organic syndrome. Symptoms as bloating, pain and bowel habit changes in the last 12 months were also excluded from control group.

Supernatants from colonic biopsies were obtained following a previously validated and published method [8], with few modifications. Briefly, after removal, biopsies were immersed in plastic tubes containing 1 ml of Hepes-Krebs solution. After weighing the biopsies, supernatant volume was adjusted to incubate 15 mg of biopsies in 1 ml of buffer. Incubation was carried out in oxygenation at 37°C for 25 min. Samples were centrifuged at 200g for 10 min and supernatant collected and stored at –20°C until the assay.

Human Dorsal Root Ganglia Neurons Isolation

Experiments were conducted under the Institutional Review Board numbers IRB00003888, FWA00005831. Human DRG (thoracic position 11 and 12) were collected in Dulbecco's Modified Eagle's medium by the National Disease Resource Interchange (NDRI). Twenty DRG were obtained from 10 post-mortem donors (21-60 years old, 10 hours maximum post-mortem) with the following exclusion criteria: chemotherapy, drug abuse, infectious disease, neurodegenerative diseases and opioid medications. None of the donors had a reported history of colitis or inflammatory bowel disease, but no information was provided about possible IBS. DRG were cut in small pieces, rinsed in Hank's balanced salt solution (HBSS; Thermo Fisher, Villebon-sur-Yvette, France) and digested in L-Cystein (pH 7.4, Sigma Aldrich, Missouri, USA), Papain (27 µg/ml, Sigma Aldrich, Missouri, USA) for 20 minutes at 37°C, rinsed 2 minutes in Leibovitz's L15 Medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA) containing 10 % of FBS. A second enzymatic dissociation was performed in 4 mg/ml dispase II (Sigma Aldrich, Missouri, USA), and 1 mg/ml collagenase (collagenase (type IV, Serlabo Technologies, France) for 15 minutes at 37°C, followed by mechanical dissociation. This step was repeated until complete dissociation of the DRG up to 4 times. Finally, neurons were plated in 8-wells Nunc™ Lab-Tek™ II CC2™ chamber slide system (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and cultured for 7 days in complete Neurobasal-A medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA) containing penicillin (100 µg/ml), streptomycin (100µg/ml), B27 (1X, Thermo Fisher Scientific, Waltham, Massachusetts, USA), L-glutamine (1X, Thermo Fisher Scientific, Waltham, Massachusetts, USA), and inhibitors of mitosis (Cytosine-B-arabinofuranoside 1.5µM, F-Uridine 10µM, Uridine, 10µM, Sigma Aldrich, Missouri, USA). Cytosine-B-arabinofuranoside was removed after 3 days of culture and the medium was changed every 2 days.

For RT-qPCR and single-cell PCR studies, human thoracolumbar DRG (T9-L1) were acquired from five (three females, two males) human adult organ donors (22.2 ± 2.08 years of age) during the removal of vital organs for transplantation. The harvested intact DRG were kept for quantitative-reverse-transcription-PCR (RT-qPCR) mRNA expression studies, while additional DRG were dissociated to allow individual DRG neurons to be isolated and studied with single-cell-reverse-transcription-PCR (RT-PCR).

Calcium imaging of human sensory neurons

After washing with HBSS, cultured neurons were incubated for 1 hour (30 min at 37°C followed by 30 min at room temperature) in solution containing fluo-4 acetoxymethyl (AM) 1mM (Thermo Fisher Scientific, Waltham, Massachusetts, USA) reconstituted in 0.01% pluronic F-127 and 0.7% DMSO (Sigma Aldrich, Missouri, USA). The fluorescence was measured at 460-490 nm excitation and 515 nm emission in each well. Neurons were imaged using an inverted microscope (Zeiss, 10x-objective) and a CCD camera (Zeiss). Acquisition parameters were kept constant within each experiment. A kinetic of 80 recordings (one per second) was performed. From 0 to 5-sec basal fluorescence was determined, from 6 to 60-sec, neurons were exposed to the different molecules studied and finally to a KCl solution (50 mM), in order to discriminate neurons from glial cells. Neurons were identified by Image J software and variations in the fluorescence intensity of each neuron was measured. Results were expressed as $\Delta F/F$, representing the fluorescence intensity ratio between the highest measure during the 6 to 60 seconds period, and baseline measure.

In a first set of experiments, neurons were treated with individual PAR agonist peptides: PAR_1-AP (1, 10, 50 or 100 µM), PAR_2-AP (100 µM), PAR_4-AP (100 µM) or neurons were pre-incubated for 5 minutes with PAR_2-AP (100 µM) or PAR_4-AP (100 µM), before being exposed to PAR_1-AP (100 µM). For these experiments, the inactive peptides (PAR-IP, 100 µM) and vehicle (HBSS) were used as control. In a second set of experiments, neurons were pre-incubated for 5 minutes with antagonist of PAR_1 (SCH79797, 10 µM) or its vehicle (HBSS, 0.01% DMSO) and treated with PAR_1-AP (100 µM). In a third set of experiments, neurons were pretreated 5 minutes with the PAR_1antagonist (SCH79797, 10 µM), the PAR_4 antagonist (ML354, 10 µM) or vehicle (HBSS, 0.01% DMSO) and were then treated with thrombin (10

U/ml). In a last set of experiments, neurons were pretreated 5 minutes with the PAR_1 antagonist (SCH79797, 10µM) or vehicle, before being exposed to supernatants from colonic biopsies of IBS-D, IBS-C, IBS-A patients or HCs.

Immunofluorescence in human Dorsal Root Ganglia

Experiments were conducted under the Institutional Review Board numbers IRB00003888, and FWA00005831. Three Human DRG T11 and 3 DRG T12 (thoracic position 11 and 12) were cryoprotected in Tissue-Tek® optimum cutting temperature compound (Sakura Finetek, Netherlands) after their collection by the NDRI. Cryoprotected DRG were cut into 35 µm sections in a cryostat (Leica CM1950; Nanterre, France) and mounted on Superfrost slides (Thermo Fisher Scientific, Villebonne-sur-Yvette, France). Cultured DRG neurons were fixed with paraformaldehyde 4% during 20 min. Both slides and cultures were washed in Phosphate Buffered Saline (PBS), 0.5% Triton X-100, and 1% Bovine Serum Albumin solution (BSA, Sigma Aldrich, Missouri, USA) and were incubated overnight at 4°C with primary antibodies diluted at 1:100 for tissues and 1:500 for cultures and directed against PGP9.5, PAR_1, PAR_4 (respectively, AB86808, AB111976, AB70400, Abcam, Cambridge, England) and PAR_2 (LSB2321, LifeSpan, Seattle, USA). After washing in PBS, slices or cultures were incubated with the appropriate secondary antibody conjugated to Alexa Fluor 488 or Alexa Fluor 555, they were washed, and mounted with ProLong Gold reagent containing 40,6-diamidino-2-phenylindole (DAPI, Molecular Probes). Controls for the specificity of the antibodies include incubation in the absence of secondary antibody, incubation in the presence of immunizing peptides and the use of PAR-deficient tissues or cells (not shown). Images were acquired using Zeiss LSM-710 confocal microscopes (Carl Zeiss MicroImaging, Jena, Germany) with 10x objective in the inverted configuration [20]. Quantification of labelling was determined using Image J software.

Quantitative-reverse-transcription-PCR (RT-qPCR):

RNA was extracted from either whole human DRG or single human DRG neurons using RNA-isolation kits (PureLink™ and Cells-to-CT™; Ambion). RT-qPCR was performed using human-specific Taqman primers for PAR_1, PAR_2, PAR_4 and GAPDH (Hs00169258_m1, Hs00608346_m1, Hs00765740_m1, Hs01006385_g1, Hs99999905_m1). The comparative

cycle threshold method was used to quantify the abundance of target transcripts to reference genes.

Single cell PCR:

26 single dissociated DRG neurons were picked using a micromanipulator at 40x magnification. Cells were under a continuous slow flow of sterile and RNA/DNAse free PBS to reduce contamination. After a cell was picked, the glass capillary was broken into a tube containing 10ul of Lysis buffer and DNAse (TaqMan® Gene Expression Cells-to-CT™ Kit; Ambion). The whole content was used for cDNA synthesis (SuperScript® VILO™ cDNA Synthesis Kit, Thermo Fisher) and PAR_1, PAR_2, PAR_4 expression was measured using Taqman™ RT-qPCR for 50 cycles. For every coverslip, a bath control was taken and analyzed together with samples. TUBB3 (Hs00964962_g1) expression served as positive control. One cell was excluded because no TUBB3 expression was present. Twenty-six cells were used to calculate frequency of presence of PAR_1, PAR_2 and PAR_4.

Statistical Analysis

Data are presented as means ± standard error of the mean (SEM). Analyses were performed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA). Comparisons between-groups were performed by Mann-Whitney test or Wilcoxon matched pairs test. Multiple comparisons within groups were performed by Kruskal-Wallis test, followed by Dunn's post-test. Statistical significance was accepted at $P < 0.05$.

Study approval

The collection of biopsies from colonic patients was approved by the local Ethic Committee (64/2004/O/Sper and EM14/2006/O) and conducted in accordance with the Declaration of Helsinki. IBS patients and HCs gave their written and informed consent. Fixed and fresh human DRG were collected with the NDRI (reference: DCEN1 001), all human DRG trials were conducted following the opinion number 14-164 of the institutional review board (IRB00003888) of French institute of medical research and health.

Results

-PAR expression in human DRG neurons-

In whole human DRG, PAR mRNA expression was assessed by RT-qPCR analysis. The relative mRNA abundance of PAR_2 was the highest followed by PAR_4 and PAR_1 (Figure 1a). Then, to determine if PARs were expressed in neurons at the protein level, the percentage of neurons, identified by Pgp9.5 immunostaining and expressing each PAR was studied (Figure 1b). PAR_1, PAR_2 and PAR_4 were respectively expressed in 20%, 40% and 40% of neurons (Figure 1c). The percentage of PAR-positive neurons was expressed in function of the diameter of neurons. PAR_1 was preferentially expressed in 30 to 50 µM diameter neurons, PAR_2 in neurons with a diameter < 30 µM and PAR_4 in 30 to 50 µM diameter neurons (Figure 1d).

PAR expression was then assessed in cultured human DRG neurons. Cultures were 90% pure for Pgp9.5 neuronal marker staining (Supplementary Figure 1, available online at http://links.lww.com/PAIN/A553). Single cell PCR experiments showed that PAR_1, PAR_2 and PAR_4 mRNA were respectively expressed in 20%, 25% and 17% of neurons (Figure 2a left panel). We found that 75% of PAR_1 expressing neurons also expressed PAR_2, 100% of the PAR_4 expressing neurons also expressed PAR_2, whilst 25% of the PAR_1 expressing neurons also expressed PAR_4 (Figure 2a right panel). PAR_1, PAR_2 and PAR_4 were respectively expressed at the protein level in 35%, 36% and 27% of neurons (Figure 2b and 2c). These results demonstrate that human DRG neurons in culture continue to express PAR_1, PAR_2 and PAR_4 and no major or significant difference was observed between the level of expression of PARs in whole DRGs and in cultured DRGs.

-Effects of PAR agonists on calcium mobilization in human DRG neurons-

Calcium flux in response to specific synthetic agonist peptides of each PAR was quantified in human sensory neuron cultures. Only PAR1-activating peptide (PAR_1-AP, 100-µM) evoked a transient increase in [Ca2+]i that was maximal after 20 seconds and declined to baseline afterwards, compared to PAR_2-AP and PAR_4-AP (Figure 3a). This activation was characterized by an increased percentage of responding neurons (Figure 3b) and increased amplitude ($\Delta F/F$) of response (Figure 3c), compared to its inactive peptide (PAR_1-IP, 100-µM). PAR_1-AP-induced calcium mobilization was dose-dependent (Figure 3d) and abolished

by a PAR_1 antagonist (SCH79797, 10-µM) (Figure 3d). Agonists and inactive peptides of PAR_2 and PAR_4 (all at 100-µM) had no significant effect on calcium signaling in human cultured DRG (Figure 3a, 3b and 3c).

As PAR_2 and PAR_4 agonist peptides did not induce calcium mobilization, we tested their potential inhibitory role on PAR_1-AP-induced calcium flux. Human DRG were pretreated with PAR_2-AP or PAR_4-AP and, 5 min after later, PAR_1-AP was added (all agonists at 100 µM). The transient increase in [Ca2+]i induced by PAR_1-AP was decreased by PAR_4-AP but not by PAR_2-AP pretreatment (Figure 3e). Both the percentage of responding neurons and the amplitude of response induced by a PAR_1 agonist were significantly reduced by PAR_4-AP, but not by PAR_2-AP pretreatment (Figure 3f and 3g).

- Thrombin signals to human DRG neurons

Considering the calcium mobilization responses of human sensory DRG neurons to PAR_1 and PAR_4 peptidic agonists, we investigated the effects of thrombin (10U/ml), a known endogenous activator of both PAR_1 and PAR_4. The average amplitude of thrombin-induced calcium response in human DRG neurons was significantly increased compared to vehicle (Figure 4a). Pre-treatment with a PAR_1 antagonist (SCH79797, 10 µM) significantly reduced the effects of thrombin on the percentage of responding neurons. Pre-treatment with a PAR_4 antagonist (ML354, 10 µM) had no effect on this parameter (Figure 4b). Considering only neurons that responded to thrombin by mobilizing calcium (23% or all neurons), the amplitude of their response to thrombin was significantly increased in the presence of PAR_4 antagonist, but was not modified by a PAR_1 antagonist (Figure 4c). These results demonstrated that in human DRG neurons, thrombin activates both PAR_1 and PAR_4, exerting opposite effects in terms of calcium mobilization.

-IBS patient mediators mobilize calcium in human DRG neurons through a PAR_1-dependent mechanism-

IBS supernatants, but not supernatants from healthy control, evoked a transient increase in [Ca2+]i that was maximal after 30 seconds and declined to baseline afterwards (Figure 5a). Supernatants from colonic biopsies of IBS patients significantly increased the amplitude of the calcium flux response and the percentage of responding neurons compared to healthy control supernatants (Figure 5b and 5c). Neither the percentage of responding

neurons, nor the amplitude of the response of human neurons to IBS patient tissue biopsy supernatants correlated with abdominal pain scores or abdominal frequency scores (supplementary Figure 2, available online at http://links.lww.com/PAIN/A553). When considered by subgroups, only supernatants from IBS-A patients induced a significant increase in the number of responding neurons (Supplementary Figure 3, available online at http://links.lww.com/PAIN/A553). PAR_1 antagonist (SCH79797, 10 µM) pretreatment of human sensory neurons decreased the transient increase in [Ca2+]i induced by IBS supernatants (Figure 5d). The antagonist significantly reduced both the percentage of responding neurons to IBS supernatants (all IBS subgroups together) and the amplitude of their response (Figure 5e and 5f).

Discussion

Since their discovery in rodent neurons [46; 53], PARs have been considered as new important therapeutic targets for the treatment of pain. A number of *in vivo* and *in vitro* studies have confirmed this potential role for PARs, particularly in the context of visceral pain and hypersensitivity [19; 49; 51; 52]. However, the relevance of considering PAR signaling as an important pathway for human pain has not been thoughtfully addressed thus far. Indeed, only one study performed in human subjects refers to a possible role for PAR_2 in pruritus[45], but no study has investigated the expression and functionality of PARs in human DRG neurons. Here, we provide evidences that PAR_1, PAR_2 and PAR_4 are all expressed in human sensory neurons. Furthermore, we showed that in culture, the expression of PARs is generally conserved and that the culture conditions we have defined for human DRG neurons can be used to investigate the functionality of human sensory neurons. Although our results provide new insights on human primary afferent signaling, the link to activation of pain pathways would clearly require further experiments.

Previous studies performed in rodent primary afferents have demonstrated the expression of the three functional PARs: PAR_1, PAR_2 and PAR_4 [21; 46]. We confirmed here that those three receptors are also expressed in human primary afferent neurons both in cell bodies of whole DRG neurons and in cultures. In rodent sensory neurons, both PAR_1 and PAR_2 agonists induced calcium mobilization [15-17; 21]. In contrast, PAR_4 agonists did not

mobilize calcium, but decreased pro-nociceptive mediator-induced calcium signaling [6; 28]. In human sensory neurons, we demonstrated that only PAR_1 agonist induced calcium mobilization. Like in rodents, in human DRG neurons, PAR_4 was not able to mobilize calcium, but decreased calcium mobilization induced by other agonists. Taken together, our results clearly demonstrated that both PAR_1 and PAR_4 were present and functional in human primary afferents, where they exerted opposing effects. The PAR peptide agonists used in the present study have been well characterized for their selectivity [26; 27]. The doses that were used for these peptide agonists in the present study are considered to be highly selective for their targeted receptors in cell culture assays. Therefore, we can reasonably think that the results obtained are truly representative of PAR selective activation. Indeed, this was confirmed for PAR_1 activation by incubation in the presence of the PAR_1 antagonist. At the concentrations used, both SCH79797 and ML354 antagonists were considered selective for inhibition of PAR_1 and PAR_4 respectively, compared to the inhibition of other PARs [1; 57]. However, PAR_1-independent effects have also been described for the SCH79797 compound [22], and one cannot rule out that the effects of this antagonist alone cannot be due to MAPK inhibition as it has been shown in cell lines at similar concentrations [22]. The PAR_4 antagonist ML354 is potent only at micro-molar ranges, showing a reasonable selectivity for PAR_4, but one cannot rule out at this concentration off-target binding for this antagonist [57].

Although DRG cultures were 90% pure for neurons, some glial cells or other supporting cells might still be present in our culture conditions. PARs are known to be expressed and functional in glial cells and to some extent, glial PAR activation might account for some of the calcium responses observed in our cultures.

Translating cellular signaling of sensory neurons into nociceptive response *in vivo* is complex, and the study of calcium signaling in primary afferents cannot fully reflect pain pathway activation. We found no correlation between pain severity scores and pain frequency scores with the percentage of responding neurons or with the amplitude of the response to IBS tissue supernatants. This could be due to the low number of samples we included in this study considering the precious nature of human DRG neuron cultures. Indeed, other animal studies suggest that calcium signals in sensory neurons are often associated with pro-nociceptive signals. Calcium mobilization and increasing excitability of

neurons reflected the sensitization of neurons associated to visceral hypersensitivity [12]. However, in rodents, while PAR_1 agonists mobilized calcium in primary afferents [21], they also increased nociceptive threshold and reduced inflammatory hyperalgesia [4; 32]. In the present study, we showed that PAR_1 specific activation mobilized calcium in human sensory neurons. The pro-nociceptive nature of this PAR_1 signal is supported by our subsequent observations demonstrating the involvement of PAR_1 activation in IBS patient biopsy supernatants-induced activation of human sensory neurons. Interestingly, in human submucosal and myenteric plexi, PAR_1-AP (TFLLR), thrombin or supernatants of colonic biopsies from IBS patients were also able to induce spike discharges and calcium signaling. This reflected enteric neuronal excitability [29; 34]. This suggests that in humans, PAR_1 might be functional both in submucosal and myenteric neurons, as well as in primary afferents (per our results). In the context of IBS, this means that PAR_1 could contribute as our results suggested, to hyperexcitability of extrinsic sensory neurons leading to visceral hypersensitivity symptoms, but could also contribute to motor and secretory dysfunctions controlled by intrinsic enteric neurons. However, one has to be careful establishing a link between primary afferent response and activation of pain pathways, as in rodents, PAR_1 can be activated in primary afferents, but was shown to be analgesic. Therefore, the pro-nociceptive effects of PAR_1 agonists remain to be demonstrated in humans. Our study paves the way for studying in human clinical trials the potential that PAR_1 antagonists already developed for use in human could have at reducing pain and hyperalgesia.

In agreement with the results previously generated in mouse primary afferents [6; 28], we observed in human neurons as well, that PAR_4-AP did not mobilize calcium, but significantly reduced calcium mobilization of stimulated sensory neurons. Here, we demonstrated for the first time an inhibitory effect for PAR_4 activation on human sensory pathways, suggesting that like in rodents [5; 6], PAR_4 activation could contribute at reducing pain and hypersensitivity. Taken together, our results highlight opposite effects for PAR_1 and PAR_4 activation in human primary sensory afferents. Considering that both receptors are activated by thrombin, although at different concentrations, it was important to investigate the overall effect of thrombin on human primary neuron calcium signaling. Thrombin is attracted to PAR_1 by a Hirudin-like site located at the N-terminal end of the receptor [56]. Thrombin binding facilitates the cleavage of PAR_1, which thus does not require high

concentrations of thrombin for its activation (0.1 to 1u/ml). In contrast, PAR_4 has no hirudin-like site and higher concentrations of thrombin are requested to activate PAR_4 (10u/ml) [36]. We deliberately used a concentration of thrombin that would activate both PAR_1 and PAR_4 and observed that thrombin induced a PAR_1-dependent calcium mobilization in human primary afferents (Figure 4 a,b). We also observed that the amplitude of the thrombin response in human sensory neurons was reduced by concomitant PAR_4 activation. Indeed, PAR_4 blockade enhanced the amplitude of thrombin-induced calcium response (Figure 4c). In keeping with these functional results, our single cell PCR data show that a population of human DRG neurons expressing PAR_1 also expresses PAR_4. Interestingly, PAR_4 was strongly expressed in human DRG neurons. The magnitude of PAR_4 effect on PAR_1- or thrombin-induced calcium signals in primary afferents was not as strong as it could have been expected considering the strong PAR_4 expression. In primary afferents, PAR_4 might have other function than counteracting the PAR_1-induced signals.

In contrast to studies reporting calcium mobilization in rodent sensory neurons after stimulation with PAR_2-APs, or in other human cell lines [37; 54], we observed that human sensory neurons did not mobilize calcium after exposure to PAR_2-APs. Interestingly, PAR_2 expression in human sensory DRG neurons was clearly demonstrated both at the mRNA and protein levels (Figures 1 and 2). This result was quite surprising since numerous studies have demonstrated that human cells expressing PAR_2, mobilize calcium after exposure to PAR_2 tethered ligand synthetic peptide [39]. The lack of calcium mobilization in human sensory neurons exposed to PAR_2 synthetic agonist does not mean that the receptor is silent or non-functional. Indeed, other signaling pathways that have been previously described for PAR_2, such as pERK or cAMP signaling might be implicated in human sensory neurons. Such pathways would have to be investigated in future studies. Furthermore, in human submucosal neurons, PAR_2-AP induces very weak calcium mobilization [34], while PAR_2 has been shown to be functional and potently activated by the Trypsin-3 protease in the same neurons [43]. This suggests that depending on the type of agonists that are tested (synthetic peptides or proteases), the cell response might be diverse and more or less potent. Similar findings have been reported for PAR pharmacology in a number of cells and tissues [39].

One crucial step in studying the relevance of PARs as therapeutic targets is the definition of protease profile and activities associated with pathological states. Indeed, the opposite roles for PAR_1 and PAR_4 we have defined here in human primary afferent signaling, suggest that depending on the proteases present and their concentration, pro- or anti-nociceptive signals could be prominent. Thrombin can activate both PAR_1 and PAR_4, but as mentioned above, at different concentrations. Therefore, the concentration of active thrombin detected in colonic tissues from IBS patients could give an important indication on the activation status of PARs and the overall nociceptive signals. Cathepsin G activates PAR_4 [44], and disarms PAR_1 [41]. Here again, the presence of cathepsin G in tissues could modify nociceptive status and accordingly participate to pain relief. Thus, it is clear that protease profiling will be an important step towards our comprehension of nociceptive signaling to primary afferents. However, the results presented here clearly defined that PAR_1 activation on sensory neurons is involved in sensory response to mediators associated with IBS in humans, even though the proteases responsible for PAR_1 activation are not yet defined. Whether PAR_1 activation can potentiate transient receptor potential channels as it was demonstrated in rodent sensory neurons both for PAR_1 [48] and PAR_2 [2; 3; 15; 16; 18; 24] would still have to be confirmed in human primary afferents.

One major limitation of the present study though is that even if we have harvested thoracic DRGs containing neurons projecting from the colon, not all neurons present in the culture dishes are colonic projections. We cannot define whether colonic projections are responding to supernatants of IBS patients, or even whether colonic projections are indeed expressing the different PARs. In animal studies, retrograde labeling of projecting neurons is used to identify the origin of the neurons, but this is hardly applicable to human studies.

In conclusion, this study describes in human sensory neurons, the expression of PARs and their ability to generate calcium signaling. The results highlight the functionality of PAR_1 as an activator of calcium-dependent signaling and PAR_4 as an inhibitor of such signaling. Most importantly, mediators from IBS patient tissues signal to human primary afferent in a PAR_1-dependent mechanism, illustrating the potential of PAR_1 antagonism as a new therapeutic option to treat symptoms associated with IBS.

Authors participation

CD, TB, SG-C, SMB, and CR performed experiments and statistical analysis. MRG and GB provided technical and material supports. CD, NC, NV have drafted the manuscript. NC and NV designed and supervised the study and obtained funding.

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Figure legends

Figure 1: Expression of PARs in whole human DRG. (**a**) Relative expression of PAR_1 (white bar), PAR_2 (gray bar) and PAR_4 (black bar) mRNA in human DRG normalized with GAPDH expression; n=5 human DRG from 5 different donors. (**b**) Representative pictures of PAR_1 (blue), PAR_2 (red), PAR_4 (green) and PGP9.5 (white, arrows = neurons, arrow heads = nerves fibers) immunodetection on slices of human DRG; scale bar = 50 µm. (**c**) Percentage of PAR expression on PGP9.5 positive cells in slices of T11 and T12 DRG from 3 donors. For PAR_1 expression 154 neurons were counted, 162 for PAR_2 and 89 for PAR_4. (**d**) Percentage of PAR–immunoreactive neurons according to the diameter of neuronal perikarya. (c), (d), n = 3 x (T11 + T12). For each condition, an average of the percentage obtained on 3 pictures was performed.

Figure 2: Expression of PARs in cultured human DRG neurons.

(**a**) Expression of PAR_1 (in white), PAR_2 (in gray) and PAR_4 (in black) mRNA transcripts by single-cell RT-qPCR (left panel) of human neurons (TUBB3 positive cells). Pie charts representation of the expression (dark color) or not (light color) of PAR_1 (in blue), PAR_2 (in red) and PAR_4 (in green) mRNA in human neurons (right panel). Each segment represents a single neuron. n = 26 neurons. (**b**) Representative pictures of PARs (in red) and PGP9.5 (in green) immunodetection in cultures of human DRG; scale bar = 50 µm. (**c**) Percentage of PAR expression on PGP9.5-positive cells in human DRG neuron cultures from 5 different donors. Two wells for each condition were counted:112 neurons for PAR_1 labelling, 134 for PAR_2 and 88 for PAR_4 were counted.

Figure 3: Effects of PAR-AP in human sensory neurons.

(**a**) Representative trace of calcium flux experiment obtained in one well of human sensory neuron culture exposed to PAR_1-AP, PAR_2-AP or PAR_4-AP (100 µM each). Percentage of responding neurons **(b**) and amplitude of intracellular calcium mobilization (ΔF/F; **c**) in human sensory neurons exposed to PAR agonist peptides (PAR-AP, 100 µM, black bar) or inactive peptides (PAR-IP, 100 µM, white bar). n=6 to 8 independent experiments of 3 wells per condition and 20-53 neurons per well. (**d**) Calcium flux amplitude of responding neurons exposed to PAR_1-IP (100 µM, white bar) or to increasing doses of PAR_1-AP (1, 10, 50 and 100 µM, black bar) pretreated or not with a PAR_1 antagonist (SCH79797, 10µM). n=4 to 5 independent experiments of 3 wells per condition and 36-62 neurons per well. (**e**)

643 Representative trace of calcium flux experiment obtained in one well of human sensory
644 neurons culture exposed to PAR_1-AP (100 µM) and pre-incubated with PAR_2-AP or PAR_4-
645 AP (100 µM each). Percentage of responding neurons **(f)** and amplitude of intracellular
646 calcium mobilization (ΔF/F; **g**) in human sensory neurons exposed to PAR_1-AP (100 µM, all
647 bars) and pretreated with PAR_2-IP or PAR_4-IP (100 µM, white bar), PAR_2-AP (100 µM, gray
648 bar) or PAR_4-AP (100µM, black bar). n=3 to 4 independent experiments of 3 wells per
649 condition and 30-58 neurons per well. Statistical analysis was performed using Kruskal-
650 Wallis analysis of variance and subsequent Dunn's post hoc test. * $p<0.05$, ** $p<0.01$, ***
651 $p<0.001$, significantly different from the corresponding inactive-peptide groups; £££ $p <$
652 0.001, significantly different from PAR_1-AP (100µM).
653
654 **Figure 4: Effects of thrombin in human DRG neurons.**
655 Percentage of responding neurons **(a)** and amplitude of intracellular calcium mobilization
656 (ΔF/F; **b**) in human DRG neurons exposed to thrombin (10 U/mL, black bar) or its vehicle
657 (HBSS, white bar). n=3 to 4 independent experiments of 3 wells per condition and 31-45
658 neurons per well. Statistical analysis was performed using Mann-Whitney test. * $p<0.05$, **
659 $p<0.01$, significantly different from HBSS group. Percentage of responding neurons **(c)** and
660 amplitude of intracellular calcium mobilization (ΔF/F; **d**) in human sensory neurons exposed
661 to thrombin (10 U/mL, all bars) and pretreated with PAR_1 antagonist (SCH79797, 10 µM,
662 gray bar), PAR_4 antagonist (ML354, 10 µM, black bar) or their vehicle (HBSS, white bar).
663 n=3 independent experiments of 3 wells per condition and 39-68 neurons per well. Statistical
664 analysis was performed using Kruskal-Wallis analysis of variance and subsequent Dunn's
665 post hoc test. * $p<0.05$, *** $p<0.001$, significantly different from the corresponding inactive-
666 peptide groups.
667 **Figure 5: Effects of supernatants from colonic biopsies of IBS patients or healthy**
668 **controls on calcium mobilization in human DRG neurons.**
669 (**a**) Representative trace of calcium flux experiment obtained in one well of human sensory
670 neuron culture exposed to supernatant of diarrhea-predominant IBS patient (hexagon) or to
671 healthy control (HC, circle). Amplitude of intracellular calcium mobilization (ΔF/F; **b**) in
672 human sensory neurons and percentage of responding neurons (**c**) exposed to supernatants
673 from colonic biopsies of IBS patients: constipation-predominant (-C, triangle), diarrhea-
674 predominant (-D, hexagon), alternate (-A, square) or to supernatants from colonic biopsies of
675 healthy control (HC, circle). (**d**) Representative trace of calcium flux experiment obtained in

one well of human sensory neurons culture exposed to supernatant of alternate-predominant IBS patient (square) and pretreated with PAR_1 antagonist (SCH79797, 10 µM, gray square) or its vehicle (white square). Amplitude of intracellular calcium mobilization (ΔF/F; **e**) in human sensory neurons and percentage of responding neurons (**f**) exposed to supernatants from colonic biopsies of IBS patients: constipation-predominant (-C, triangle), diarrhea-predominant (-D, hexagon) or alternate (-A, square) and pretreated with PAR_1 antagonist (SCH79797, 10 µM), or its vehicle. Data are represented as scattered dot plot with line at mean. Each symbol represents one patient. n=6 independent experiments of 3 wells per condition and 42-53 neurons per well. Statistical analysis was performed using Mann-Whitney test (**b** and **c**) or Wilcoxon matched pairs test (**e** and **f**). * $p<0.05$, ** $p<0.01$, significantly different from HC (**b** and **c**) or from IBS group (**e** and **f**).

Figure 1

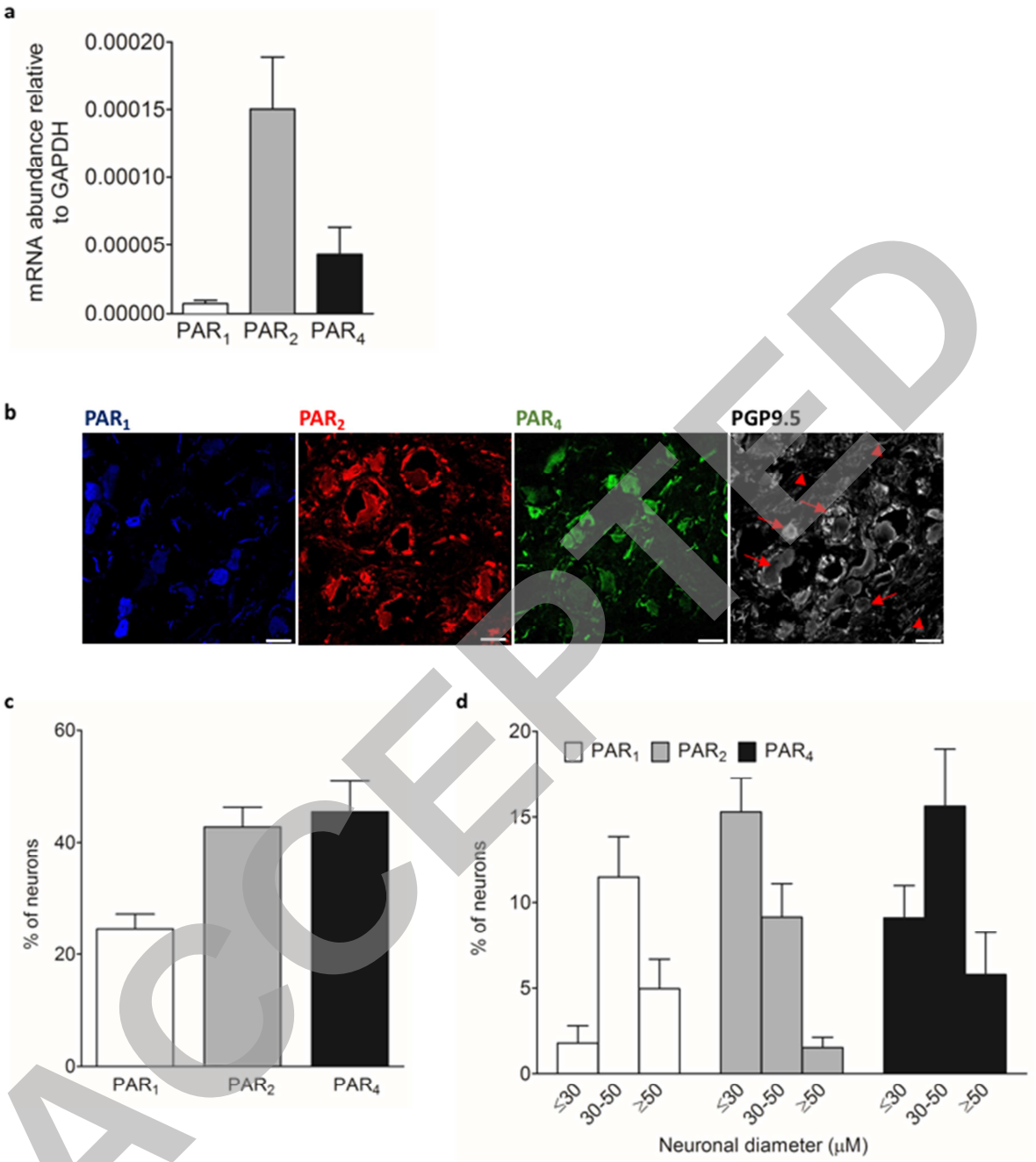

Figure 2

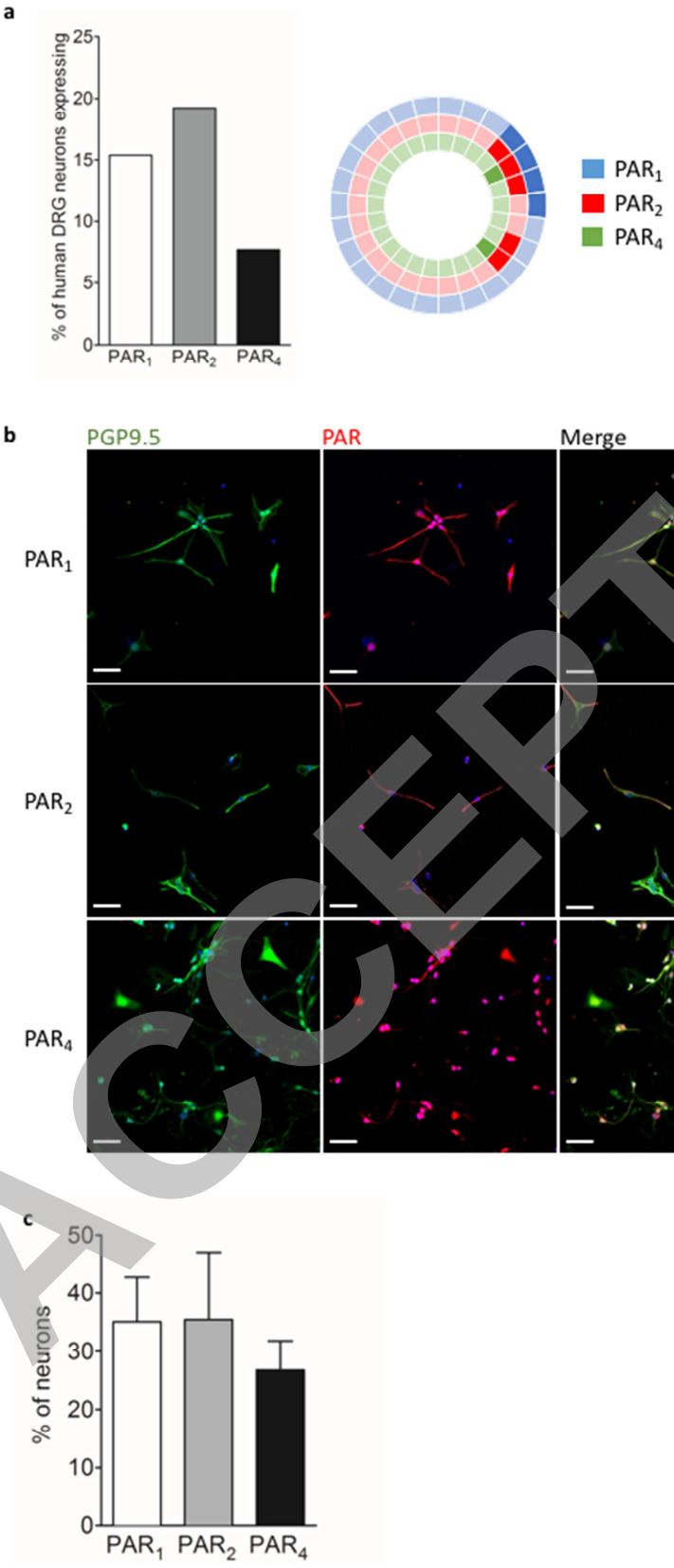

Figure 3

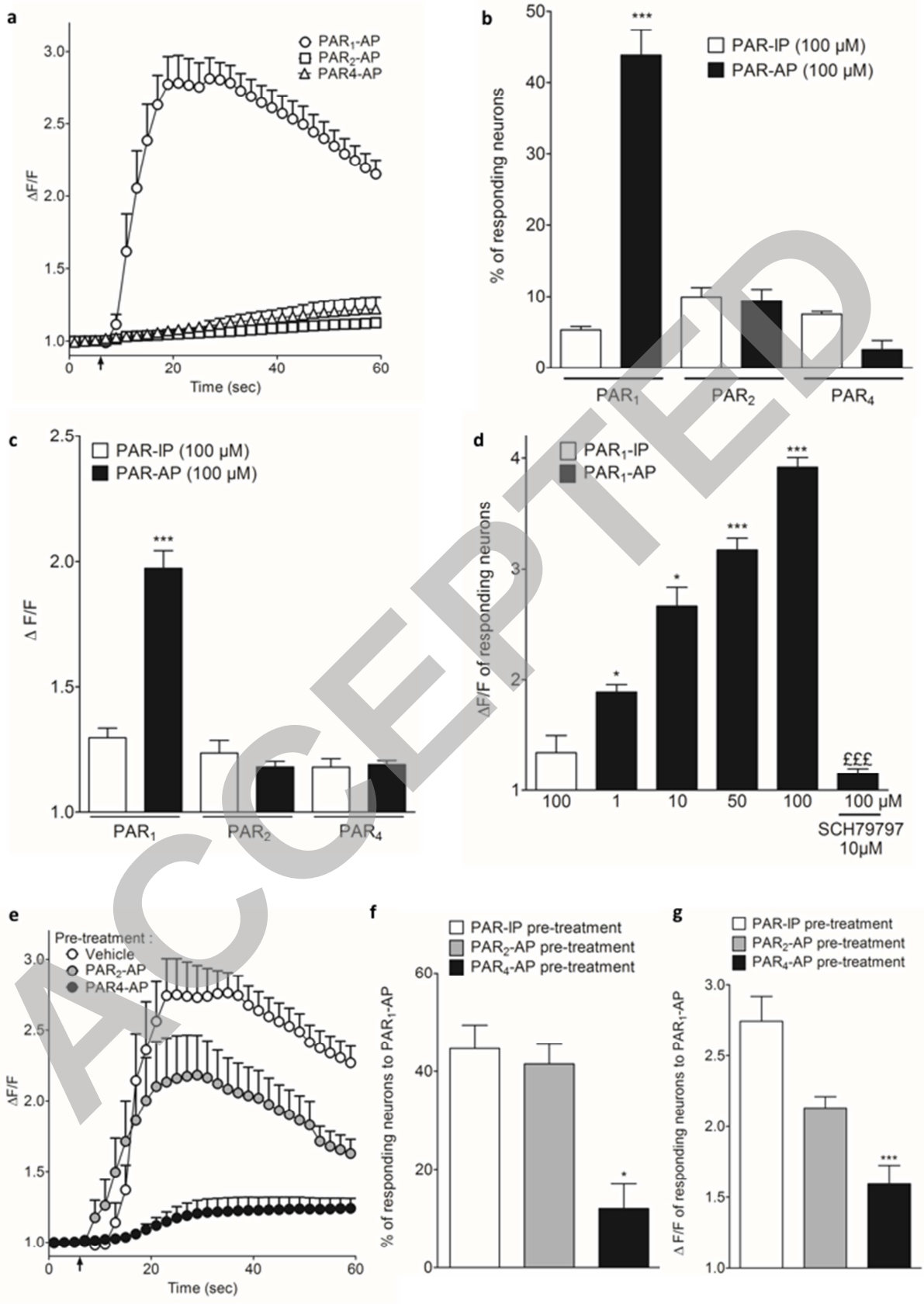

Figure 4

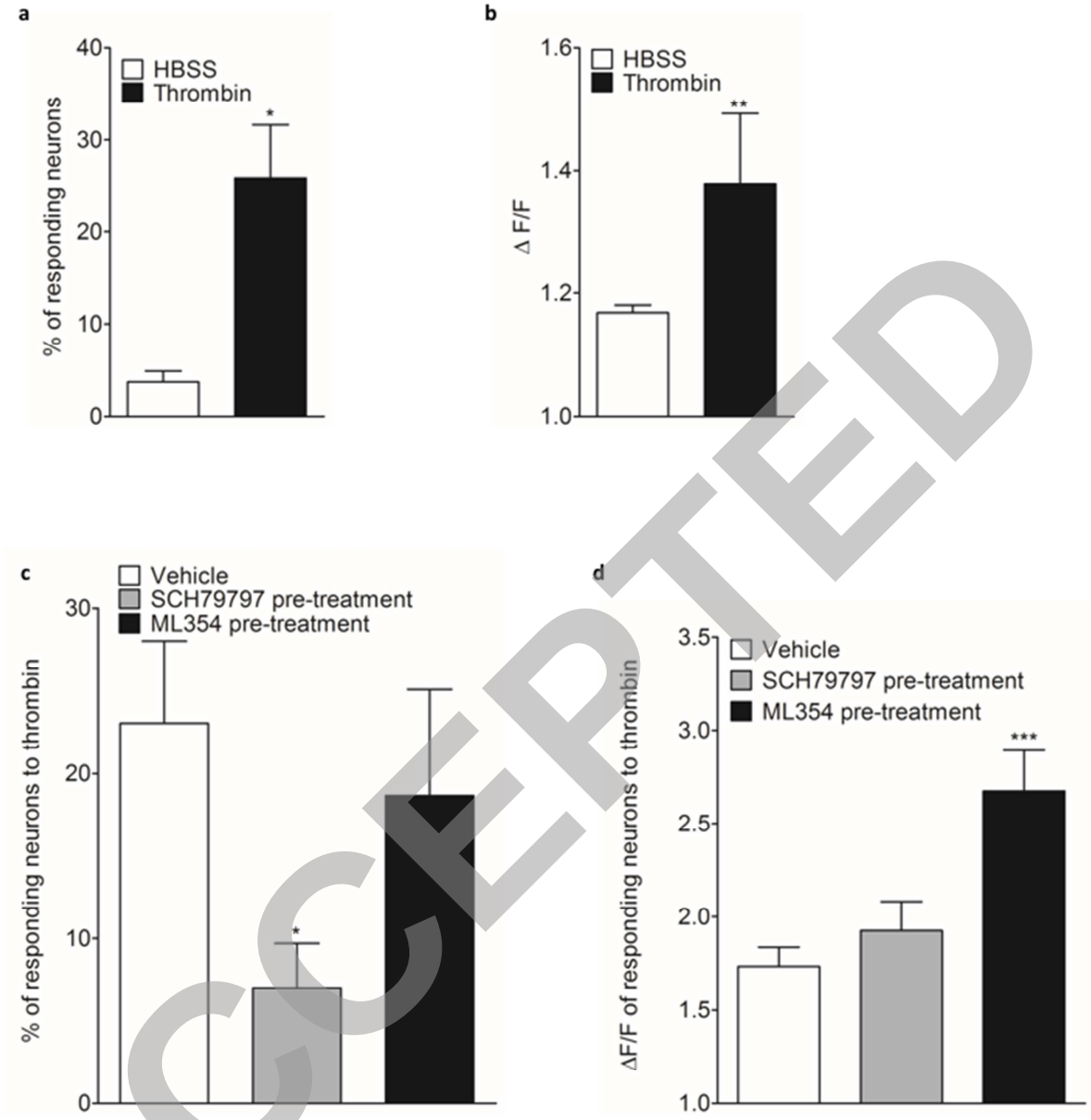

Figure 5