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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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1 Abstract : words 243

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3 Proteases and Protease-Activated Receptors (PARs) are major mediators involved in irritable
4 bowel syndrome (IBS). Our objectives were to decipher the expression and functionality
5 (calcium signaling) of PARs in human dorsal root ganglia (DRG) neurons, and to define
6 mechanisms involved in human sensory neuron signaling by IBS patient mediators.
7 Human thoracic DRG were obtained from the national disease resource interchange.
8 Expression of PAR_1, PAR_2 and PAR_4 was assessed by immunohistochemistry and RT-qPCR in
9 whole DRG or in primary cultures of isolated neurons. Calcium signaling in response to PAR
10 agonist peptides (PAR-AP), their inactive peptides (PAR-IP), thrombin (10u/ml), supernatants
11 from colonic biopsies of IBS patients or healthy controls (HC), with or without PAR_1 or PAR_4
12 antagonist were studied in cultured human DRG neurons.
13 PAR_1, PAR_2 and PAR_4 were all expressed in human DRG, respectively in 20%, 40% and 40% of
14 the sensory neurons. PAR_1-AP increased intracellular calcium concentration in a dose-
15 dependent manner. This increase was inhibited by PAR_1 antagonism. In contrast, PAR_2-AP,
16 PAR_4-AP and PAR-IP did not cause calcium mobilization. PAR_1-AP-induced calcium flux was
17 significantly reduced by pre-incubation with PAR_4-AP, but not with PAR_2-AP. Thrombin
18 increased calcium flux, which was inhibited by a PAR_1 antagonist and increased by a PAR_4
19 antagonist. Supernatants from colonic biopsies of IBS patients induced calcium flux in human
20 sensory neurons compared to HC, this induction was reversed by a PAR_1 antagonist.
21 Taken together, our results highlight that PAR_1 antagonism should be investigated as a new
22 therapeutic target for IBS symptoms.

23

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

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27 **Introduction**

28 Irritable Bowel Syndrome (IBS) affects 11 to 20% of the Western population with a
29 higher prevalence in women [14; 31]. IBS associates abdominal pain, diarrhea (IBS-D),
30 constipation (IBS-C) or both (IBS-A, for alternate) [30]. Although IBS is a functional
31 gastrointestinal disorder, not associated with gross structural or biochemical abnormalities
32 [23], several recent studies indicate the presence of meaningful micro-organic changes [7].
33 One of the emerging ideas to explain the visceral pain associated with IBS is that sensory
34 neurons innervating the colon are hyperexcitable in these patients [10; 11; 13]. However,
35 because of the difficulties associated with human sensory neuron cultures, it has been
36 difficult to evaluate the relevance of identified mediators in the context of human pathology.

37 Among the molecular targets explored to decipher neuronal hyperexcitability in IBS,
38 several studies showed that proteases released by colonic biopsies of IBS patients were able
39 to activate mouse and rat intestinal neurons *in vitro* and to induce somatic and visceral
40 hypersensitivity *in vivo [9; 13; 17; 38; 47]*. Proteases are known to signal to mouse or rat
41 sensory neurons through the activation of Protease-Activated Receptors (PARs) [19; 46; 53;
42 55], a family of G protein-coupled receptors that includes 4 members: PAR_1, PAR_2, PAR_3 and
43 PAR_4 [40]. Only PAR_1, PAR_2 and PAR_4 seem to be able to signal through calcium mobilization
44 and to exert a role in nociception and pain (PAR_3 has been considered more as a co-factor
45 for other PAR activation[35] so far). PAR_1, $_2$ and $_4$ are activated by the proteolytic cleavage of
46 their N-terminal domain, which reveals a tethered ligand that binds and activates the
47 receptors. The role of PARs in visceral inflammation and pain has been well studied in
48 animal models[49; 50]. Both PAR_1 and PAR_2 agonists induce calcium mobilization in rodent
49 sensory neurons[17; 21]. PAR_2 agonists induce pain in somatic and visceral models [51; 53],
50 while PAR_1 and PAR_4 agonists attenuate nociception and pain symptoms in rodents [4-6; 32].
51 In mouse sensory neurons, the calcium mobilization induced by supernatants from colonic
52 biopsies of IBS patients is dependent on supernatant's proteolytic activity and on PAR_2
53 expression in mouse primary afferents [17]. In contrast, PAR_4 activation inhibits PAR_2-
54 induced calcium mobilization and intrinsic excitability of colonic dorsal root ganglia (DRG)
55 neurons, as well as overall pain [5; 6; 28]. However, in the context of human nociception and
56 pain, the effects of PAR agonists and PAR signaling mechanisms are largely unknown.

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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.

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87 Supernatants from colonic biopsies were obtained following a previously validated and
88 published method [8], with few modifications. Briefly, after removal, biopsies were
89 immersed in plastic tubes containing 1 ml of Hepes-Krebs solution. After weighing the
90 biopsies, supernatant volume was adjusted to incubate 15 mg of biopsies in 1 ml of buffer.
91 Incubation was carried out in oxygenation at 37°C for 25 min. Samples were centrifuged at
92 200g for 10 min and supernatant collected and stored at –20°C until the assay.
93

94 **Human Dorsal Root Ganglia Neurons Isolation**

95 Experiments were conducted under the Institutional Review Board numbers IRB00003888,
96 FWA00005831. Human DRG (thoracic position 11 and 12) were collected in Dulbecco's
97 Modified Eagle's medium by the National Disease Resource Interchange (NDRI). Twenty DRG
98 were obtained from 10 post-mortem donors (21-60 years old, 10 hours maximum post-
99 mortem) with the following exclusion criteria: chemotherapy, drug abuse, infectious disease,
100 neurodegenerative diseases and opioid medications. None of the donors had a reported
101 history of colitis or inflammatory bowel disease, but no information was provided about
102 possible IBS. DRG were cut in small pieces, rinsed in Hank's balanced salt solution (HBSS;
103 Thermo Fisher, Villebon-sur-Yvette, France) and digested in L-Cystein (pH 7.4, Sigma Aldrich,
104 Missouri, USA), Papain (27 µg/ml, Sigma Aldrich, Missouri, USA) for 20 minutes at 37°C,
105 rinsed 2 minutes in Leibovitz's L15 Medium (Thermo Fisher Scientific, Waltham,
106 Massachusetts, USA) containing 10 % of FBS. A second enzymatic dissociation was
107 performed in 4 mg/ml dispase II (Sigma Aldrich, Missouri, USA), and 1 mg/ml collagenase
108 (collagenase (type IV, Serlabo Technologies, France) for 15 minutes at 37°C, followed by
109 mechanical dissociation. This step was repeated until complete dissociation of the DRG up to
110 4 times. Finally, neurons were plated in 8-wells Nunc™ Lab-Tek™ II CC2™ chamber slide
111 system (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and cultured for 7 days in
112 complete Neurobasal-A medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
113 containing penicillin (100 µg/ml), streptomycin (100µg/ml), B27 (1X, Thermo Fisher
114 Scientific, Waltham, Massachusetts, USA), L-glutamine (1X, Thermo Fisher Scientific,
115 Waltham, Massachusetts, USA), and inhibitors of mitosis (Cytosine-B-arabinofuranoside
116 1.5µM, F-Uridine 10µM, Uridine, 10µM, Sigma Aldrich, Missouri, USA). Cytosine-B-
117 arabinofuranoside was removed after 3 days of culture and the medium was changed every
118 2 days.

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119 For RT-qPCR and single-cell PCR studies, human thoracolumbar DRG (T9-L1) were acquired
120 from five (three females, two males) human adult organ donors (22.2 ± 2.08 years of age)
121 during the removal of vital organs for transplantation. The harvested intact DRG were kept
122 for quantitative-reverse-transcription-PCR (RT-qPCR) mRNA expression studies, while
123 additional DRG were dissociated to allow individual DRG neurons to be isolated and studied
124 with single-cell-reverse-transcription-PCR (RT-PCR).
125
126 **Calcium imaging of human sensory neurons**
127 After washing with HBSS, cultured neurons were incubated for 1 hour (30 min at 37°C
128 followed by 30 min at room temperature) in solution containing fluo-4 acetoxymethyl (AM)
129 1mM (Thermo Fisher Scientific, Waltham, Massachusetts, USA) reconstituted in 0.01%
130 pluronic F-127 and 0.7% DMSO (Sigma Aldrich, Missouri, USA). The fluorescence was
131 measured at 460-490 nm excitation and 515 nm emission in each well. Neurons were
132 imaged using an inverted microscope (Zeiss, 10x-objective) and a CCD camera (Zeiss).
133 Acquisition parameters were kept constant within each experiment. A kinetic of 80
134 recordings (one per second) was performed. From 0 to 5-sec basal fluorescence was
135 determined, from 6 to 60-sec, neurons were exposed to the different molecules studied and
136 finally to a KCl solution (50 mM), in order to discriminate neurons from glial cells. Neurons
137 were identified by Image J software and variations in the fluorescence intensity of each
138 neuron was measured. Results were expressed as $\Delta F/F$, representing the fluorescence
139 intensity ratio between the highest measure during the 6 to 60 seconds period, and baseline
140 measure.
141
142 In a first set of experiments, neurons were treated with individual PAR agonist peptides:
143 PAR_1-AP (1, 10, 50 or 100 µM), PAR_2-AP (100 µM), PAR_4-AP (100 µM) or neurons were pre-
144 incubated for 5 minutes with PAR_2-AP (100 µM) or PAR_4-AP (100 µM), before being exposed
145 to PAR_1-AP (100 µM). For these experiments, the inactive peptides (PAR-IP, 100 µM) and
146 vehicle (HBSS) were used as control. In a second set of experiments, neurons were pre-
147 incubated for 5 minutes with antagonist of PAR_1 (SCH79797, 10 µM) or its vehicle (HBSS,
148 0.01% DMSO) and treated with PAR_1-AP (100 µM). In a third set of experiments, neurons
149 were pretreated 5 minutes with the PAR_1antagonist (SCH79797, 10 µM), the PAR_4 antagonist
150 (ML354, 10 µM) or vehicle (HBSS, 0.01% DMSO) and were then treated with thrombin (10

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

154

155 **Immunofluorescence in human Dorsal Root Ganglia**

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

176

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

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

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

184

185 **Single cell PCR:**

186 26 single dissociated DRG neurons were picked using a micromanipulator at 40x
187 magnification. Cells were under a continuous slow flow of sterile and RNA/DNAse free PBS to
188 reduce contamination. After a cell was picked, the glass capillary was broken into a tube
189 containing 10ul of Lysis buffer and DNAse (TaqMan® Gene Expression Cells-to-CT™ Kit;
190 Ambion). The whole content was used for cDNA synthesis (SuperScript® VILO™ cDNA
191 Synthesis Kit, Thermo Fisher) and PAR_1, PAR_2, PAR_4 expression was measured using
192 Taqman™ RT-qPCR for 50 cycles. For every coverslip, a bath control was taken and analyzed
193 together with samples. TUBB3 (Hs00964962_g1) expression served as positive control. One
194 cell was excluded because no TUBB3 expression was present. Twenty-six cells were used to
195 calculate frequency of presence of PAR_1, PAR_2 and PAR_4.

196

197 **Statistical Analysis**

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

203

204 **Study approval**

205 The collection of biopsies from colonic patients was approved by the local Ethic Committee
206 (64/2004/O/Sper and EM14/2006/O) and conducted in accordance with the Declaration of
207 Helsinki. IBS patients and HCs gave their written and informed consent. Fixed and fresh
208 human DRG were collected with the NDRI (reference: DCEN1 001), all human DRG trials were
209 conducted following the opinion number 14-164 of the institutional review board
210 (IRB00003888) of French institute of medical research and health.

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213 **Results**

214 **-PAR expression in human DRG neurons-**

215 In whole human DRG, PAR mRNA expression was assessed by RT-qPCR analysis. The
216 relative mRNA abundance of PAR_2 was the highest followed by PAR_4 and PAR_1 (Figure 1a).
217 Then, to determine if PARs were expressed in neurons at the protein level, the percentage of
218 neurons, identified by Pgp9.5 immunostaining and expressing each PAR was studied (Figure
219 1b). PAR_1, PAR_2 and PAR_4 were respectively expressed in 20%, 40% and 40% of neurons
220 (Figure 1c). The percentage of PAR-positive neurons was expressed in function of the
221 diameter of neurons. PAR_1 was preferentially expressed in 30 to 50 µM diameter neurons,
222 PAR_2 in neurons with a diameter < 30 µM and PAR_4 in 30 to 50 µM diameter neurons (Figure
223 1d).

224 PAR expression was then assessed in cultured human DRG neurons. Cultures were
225 90% pure for Pgp9.5 neuronal marker staining (Supplementary Figure 1, available online at
226 http://links.lww.com/PAIN/A553). Single cell PCR experiments showed that PAR_1, PAR_2 and
227 PAR_4 mRNA were respectively expressed in 20%, 25% and 17% of neurons (Figure 2a left
228 panel). We found that 75% of PAR_1 expressing neurons also expressed PAR_2, 100% of the
229 PAR_4 expressing neurons also expressed PAR_2, whilst 25% of the PAR_1 expressing neurons
230 also expressed PAR_4 (Figure 2a right panel). PAR_1, PAR_2 and PAR_4 were respectively
231 expressed at the protein level in 35%, 36% and 27% of neurons (Figure 2b and 2c). These
232 results demonstrate that human DRG neurons in culture continue to express PAR_1, PAR_2 and
233 PAR_4 and no major or significant difference was observed between the level of expression of
234 PARs in whole DRGs and in cultured DRGs.

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

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

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

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

253 **- Thrombin signals to human DRG neurons**

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

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

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

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

283

284 **Discussion**

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

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

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303 mobilize calcium, but decreased pro-nociceptive mediator-induced calcium signaling [6; 28].
304 In human sensory neurons, we demonstrated that only PAR_1 agonist induced calcium
305 mobilization. Like in rodents, in human DRG neurons, PAR_4 was not able to mobilize calcium,
306 but decreased calcium mobilization induced by other agonists. Taken together, our results
307 clearly demonstrated that both PAR_1 and PAR_4 were present and functional in human
308 primary afferents, where they exerted opposing effects. The PAR peptide agonists used in
309 the present study have been well characterized for their selectivity [26; 27]. The doses that
310 were used for these peptide agonists in the present study are considered to be highly
311 selective for their targeted receptors in cell culture assays. Therefore, we can reasonably
312 think that the results obtained are truly representative of PAR selective activation. Indeed,
313 this was confirmed for PAR_1 activation by incubation in the presence of the PAR_1 antagonist.
314 At the concentrations used, both SCH79797 and ML354 antagonists were considered
315 selective for inhibition of PAR_1 and PAR_4 respectively, compared to the inhibition of other
316 PARs [1; 57]. However, PAR_1-independent effects have also been described for the
317 SCH79797 compound [22], and one cannot rule out that the effects of this antagonist alone
318 cannot be due to MAPK inhibition as it has been shown in cell lines at similar concentrations
319 [22]. The PAR_4 antagonist ML354 is potent only at micro-molar ranges, showing a reasonable
320 selectivity for PAR_4, but one cannot rule out at this concentration off-target binding for this
321 antagonist [57].

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

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

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

354 In agreement with the results previously generated in mouse primary afferents [6;
355 28], we observed in human neurons as well, that PAR_4-AP did not mobilize calcium, but
356 significantly reduced calcium mobilization of stimulated sensory neurons. Here, we
357 demonstrated for the first time an inhibitory effect for PAR_4 activation on human sensory
358 pathways, suggesting that like in rodents [5; 6], PAR_4 activation could contribute at reducing
359 pain and hypersensitivity. Taken together, our results highlight opposite effects for PAR_1 and
360 PAR_4 activation in human primary sensory afferents. Considering that both receptors are
361 activated by thrombin, although at different concentrations, it was important to investigate
362 the overall effect of thrombin on human primary neuron calcium signaling. Thrombin is
363 attracted to PAR_1 by a Hirudin-like site located at the N-terminal end of the receptor [56].
364 Thrombin binding facilitates the cleavage of PAR_1, which thus does not require high

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

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

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395 One crucial step in studying the relevance of PARs as therapeutic targets is the
396 definition of protease profile and activities associated with pathological states. Indeed, the
397 opposite roles for PAR_1 and PAR_4 we have defined here in human primary afferent signaling,
398 suggest that depending on the proteases present and their concentration, pro- or anti-
399 nociceptive signals could be prominent. Thrombin can activate both PAR_1 and PAR_4, but as
400 mentioned above, at different concentrations. Therefore, the concentration of active
401 thrombin detected in colonic tissues from IBS patients could give an important indication on
402 the activation status of PARs and the overall nociceptive signals. Cathepsin G activates PAR_4
403 [44], and disarms PAR_1 [41]. Here again, the presence of cathepsin G in tissues could modify
404 nociceptive status and accordingly participate to pain relief. Thus, it is clear that protease
405 profiling will be an important step towards our comprehension of nociceptive signaling to
406 primary afferents. However, the results presented here clearly defined that PAR_1 activation
407 on sensory neurons is involved in sensory response to mediators associated with IBS in
408 humans, even though the proteases responsible for PAR_1 activation are not yet defined.
409 Whether PAR_1 activation can potentiate transient receptor potential channels as it was
410 demonstrated in rodent sensory neurons both for PAR_1 [48] and PAR_2 [2; 3; 15; 16; 18; 24]
411 would still have to be confirmed in human primary afferents.

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

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

424

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425 **Authors participation**

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

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439

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610 **Figure legends**

611 **Figure 1: Expression of PARs in whole human DRG.** (**a**) Relative expression of PAR_1
612 (white bar), PAR_2 (gray bar) and PAR_4 (black bar) mRNA in human DRG normalized with
613 GAPDH expression; n=5 human DRG from 5 different donors. (**b**) Representative pictures of
614 PAR_1 (blue), PAR_2 (red), PAR_4 (green) and PGP9.5 (white, arrows = neurons, arrow heads =
615 nerves fibers) immunodetection on slices of human DRG; scale bar = 50 µm. (**c**) Percentage
616 of PAR expression on PGP9.5 positive cells in slices of T11 and T12 DRG from 3 donors.
617 For PAR_1 expression 154 neurons were counted, 162 for PAR_2 and 89 for PAR_4. (**d**)
618 Percentage of PAR–immunoreactive neurons according to the diameter of neuronal perikarya.
619 (c), (d), n = 3 x (T11 + T12). For each condition, an average of the percentage obtained on 3
620 pictures was performed.
621

622 **Figure 2: Expression of PARs in cultured human DRG neurons.**
623 (**a**) Expression of PAR_1 (in white), PAR_2 (in gray) and PAR_4 (in black) mRNA transcripts by
624 single-cell RT-qPCR (left panel) of human neurons (TUBB3 positive cells). Pie charts
625 representation of the expression (dark color) or not (light color) of PAR_1 (in blue), PAR_2 (in
626 red) and PAR_4 (in green) mRNA in human neurons (right panel). Each segment represents a
627 single neuron. n = 26 neurons. (**b**) Representative pictures of PARs (in red) and PGP9.5 (in
628 green) immunodetection in cultures of human DRG; scale bar = 50 µm. (**c**) Percentage of
629 PAR expression on PGP9.5-positive cells in human DRG neuron cultures from 5 different
630 donors. Two wells for each condition were counted:112 neurons for PAR_1 labelling, 134 for
631 PAR_2 and 88 for PAR_4 were counted.
632

633 **Figure 3: Effects of PAR-AP in human sensory neurons.**
634 (**a**) Representative trace of calcium flux experiment obtained in one well of human sensory
635 neuron culture exposed to PAR_1-AP, PAR_2-AP or PAR_4-AP (100 µM each). Percentage of
636 responding neurons (**b**) and amplitude of intracellular calcium mobilization ($\Delta F/F$; **c**) in
637 human sensory neurons exposed to PAR agonist peptides (PAR-AP, 100 µM, black bar) or
638 inactive peptides (PAR-IP, 100 µM, white bar). n=6 to 8 independent experiments of 3 wells
639 per condition and 20-53 neurons per well. (**d**) Calcium flux amplitude of responding neurons
640 exposed to PAR_1-IP (100 µM, white bar) or to increasing doses of PAR_1-AP (1, 10, 50 and
641 100 µM, black bar) pretreated or not with a PAR_1 antagonist (SCH79797, 10µM). n=4 to 5
642 independent experiments of 3 wells per condition and 36-62 neurons per well. (**e**)

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Representative trace of calcium flux experiment obtained in one well of human sensory neurons culture exposed to PAR_1-AP (100 µM) and pre-incubated with PAR_2-AP or PAR_4-AP (100 µM each). Percentage of responding neurons (**f**) and amplitude of intracellular calcium mobilization (ΔF/F; **g**) in human sensory neurons exposed to PAR_1-AP (100 µM, all bars) and pretreated with PAR_2-IP or PAR_4-IP (100 µM, white bar), PAR_2-AP (100 µM, gray bar) or PAR_4-AP (100µM, black bar). n=3 to 4 independent experiments of 3 wells per condition and 30-58 neurons per well. Statistical analysis was performed using Kruskal-Wallis analysis of variance and subsequent Dunn's post hoc test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, significantly different from the corresponding inactive-peptide groups; £££ $p < 0.001$, significantly different from PAR_1-AP (100µM).

Figure 4: Effects of thrombin in human DRG neurons.

Percentage of responding neurons (**a**) and amplitude of intracellular calcium mobilization (ΔF/F; **b**) in human DRG neurons exposed to thrombin (10 U/mL, black bar) or its vehicle (HBSS, white bar). n=3 to 4 independent experiments of 3 wells per condition and 31-45 neurons per well. Statistical analysis was performed using Mann-Whitney test. * $p<0.05$, ** $p<0.01$, significantly different from HBSS group. Percentage of responding neurons (**c**) and amplitude of intracellular calcium mobilization (ΔF/F; **d**) in human sensory neurons exposed to thrombin (10 U/mL, all bars) and pretreated with PAR_1 antagonist (SCH79797, 10 µM, gray bar), PAR_4 antagonist (ML354, 10 µM, black bar) or their vehicle (HBSS, white bar). n=3 independent experiments of 3 wells per condition and 39-68 neurons per well. Statistical analysis was performed using Kruskal-Wallis analysis of variance and subsequent Dunn's post hoc test. * $p<0.05$, *** $p<0.001$, significantly different from the corresponding inactive-peptide groups.

Figure 5: Effects of supernatants from colonic biopsies of IBS patients or healthy controls on calcium mobilization in human DRG neurons.

(**a**) Representative trace of calcium flux experiment obtained in one well of human sensory neuron culture exposed to supernatant of diarrhea-predominant IBS patient (hexagon) or to healthy control (HC, circle). Amplitude of intracellular calcium mobilization (ΔF/F; **b**) in human sensory neurons and percentage of responding neurons (**c**) exposed to supernatants from colonic biopsies of IBS patients: constipation-predominant (-C, triangle), diarrhea-predominant (-D, hexagon), alternate (-A, square) or to supernatants from colonic biopsies of healthy control (HC, circle). (**d**) Representative trace of calcium flux experiment obtained in

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

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Figure 1

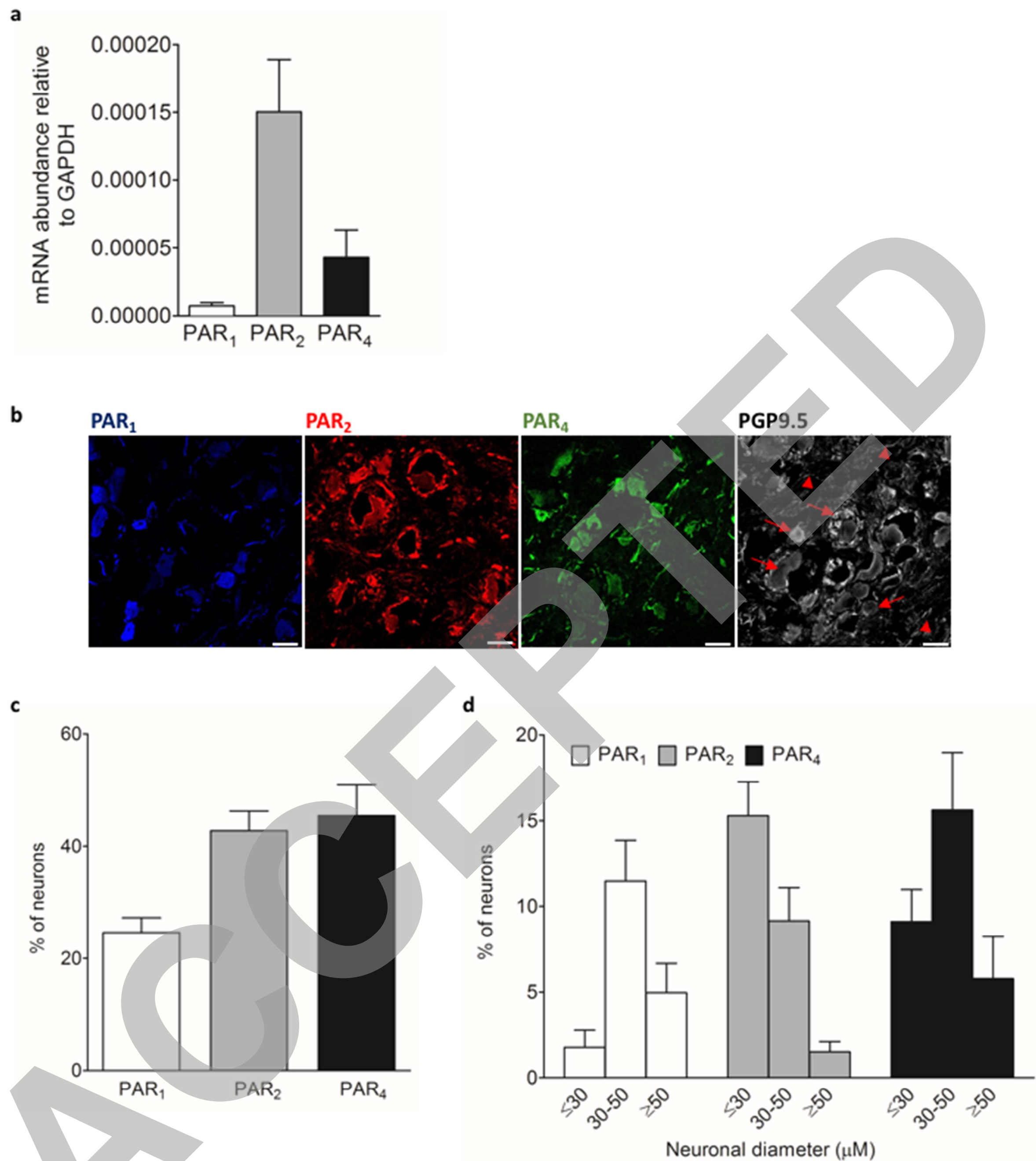

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Figure 2

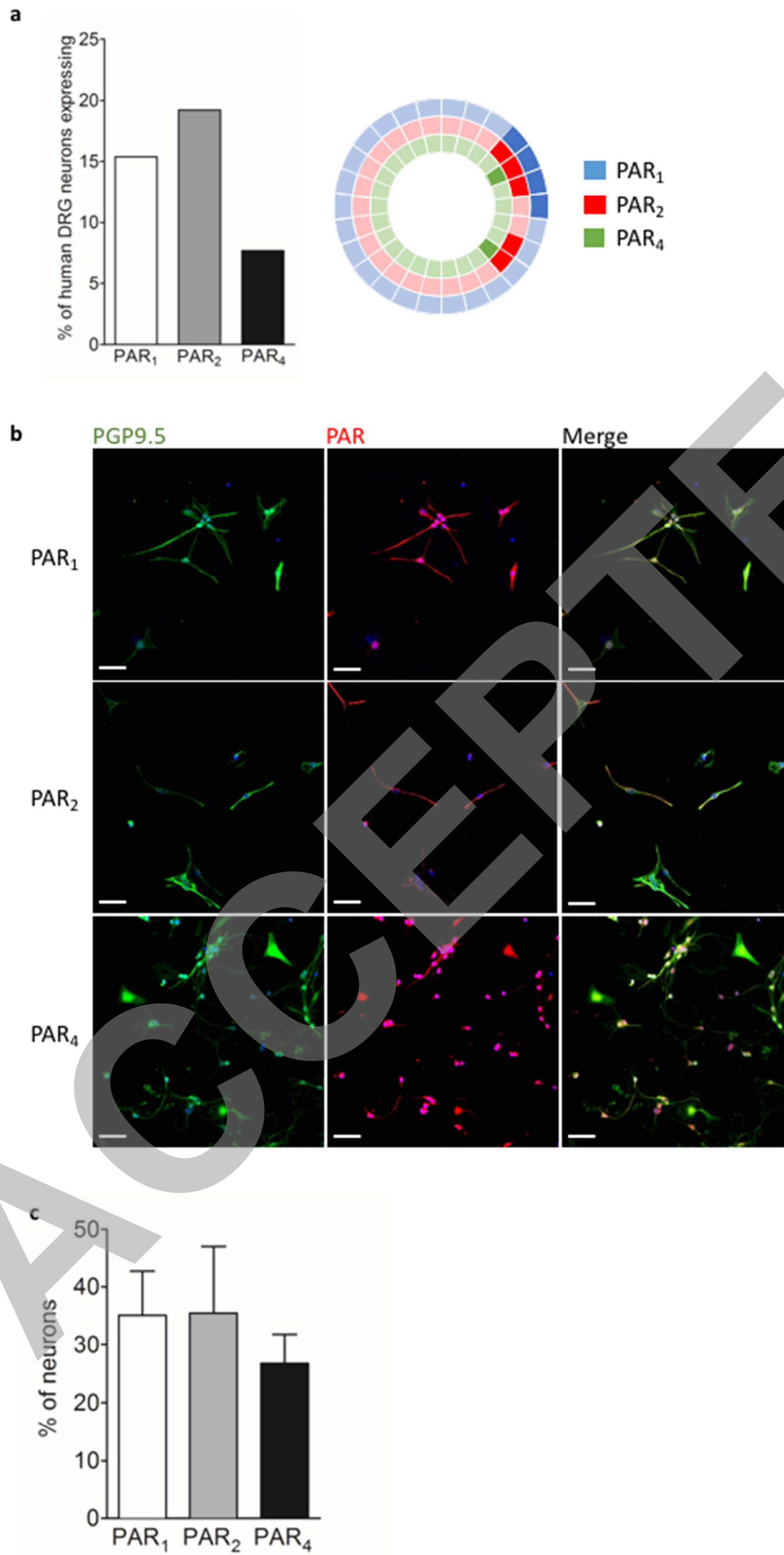

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Figure 3

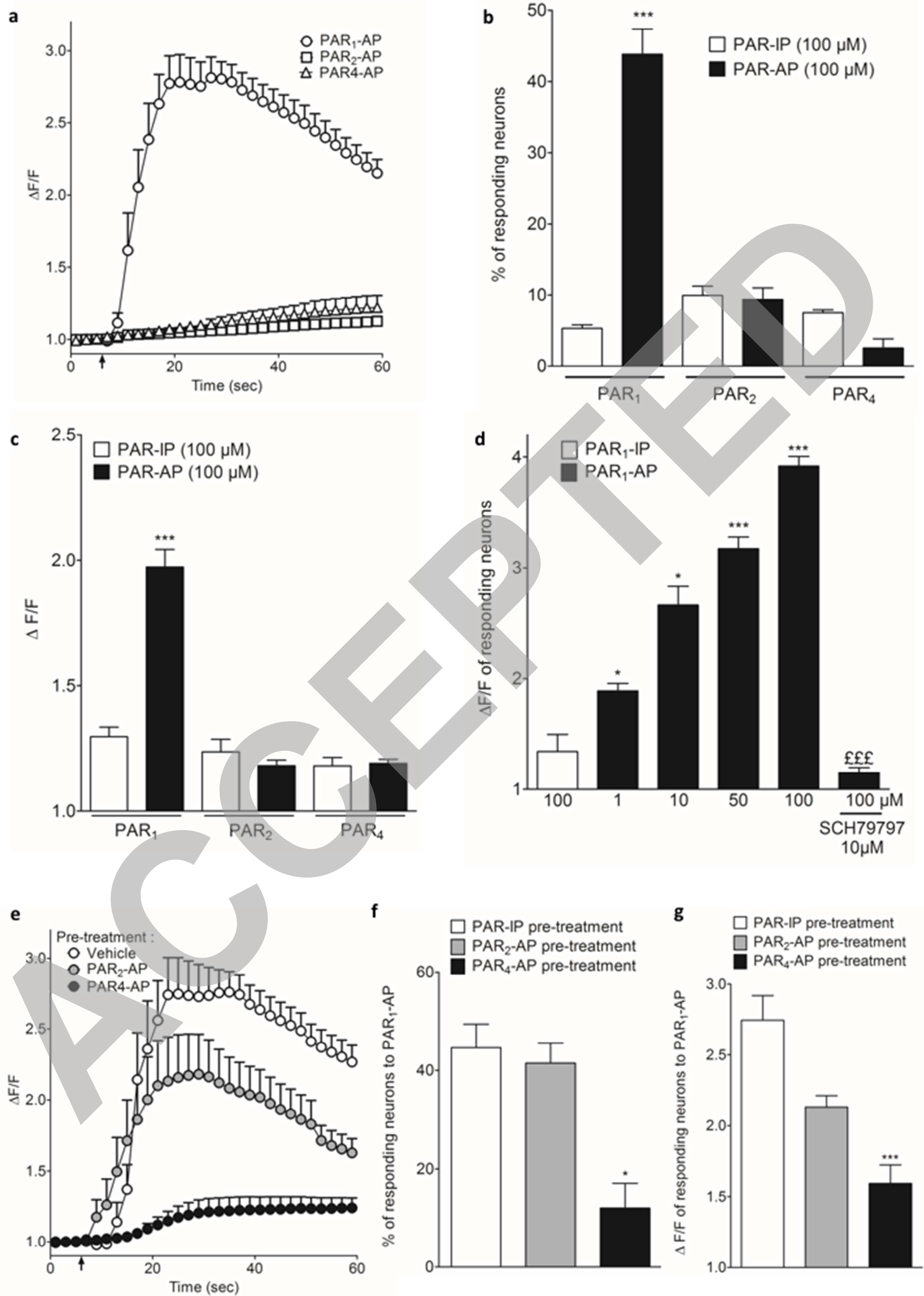

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Figure 4

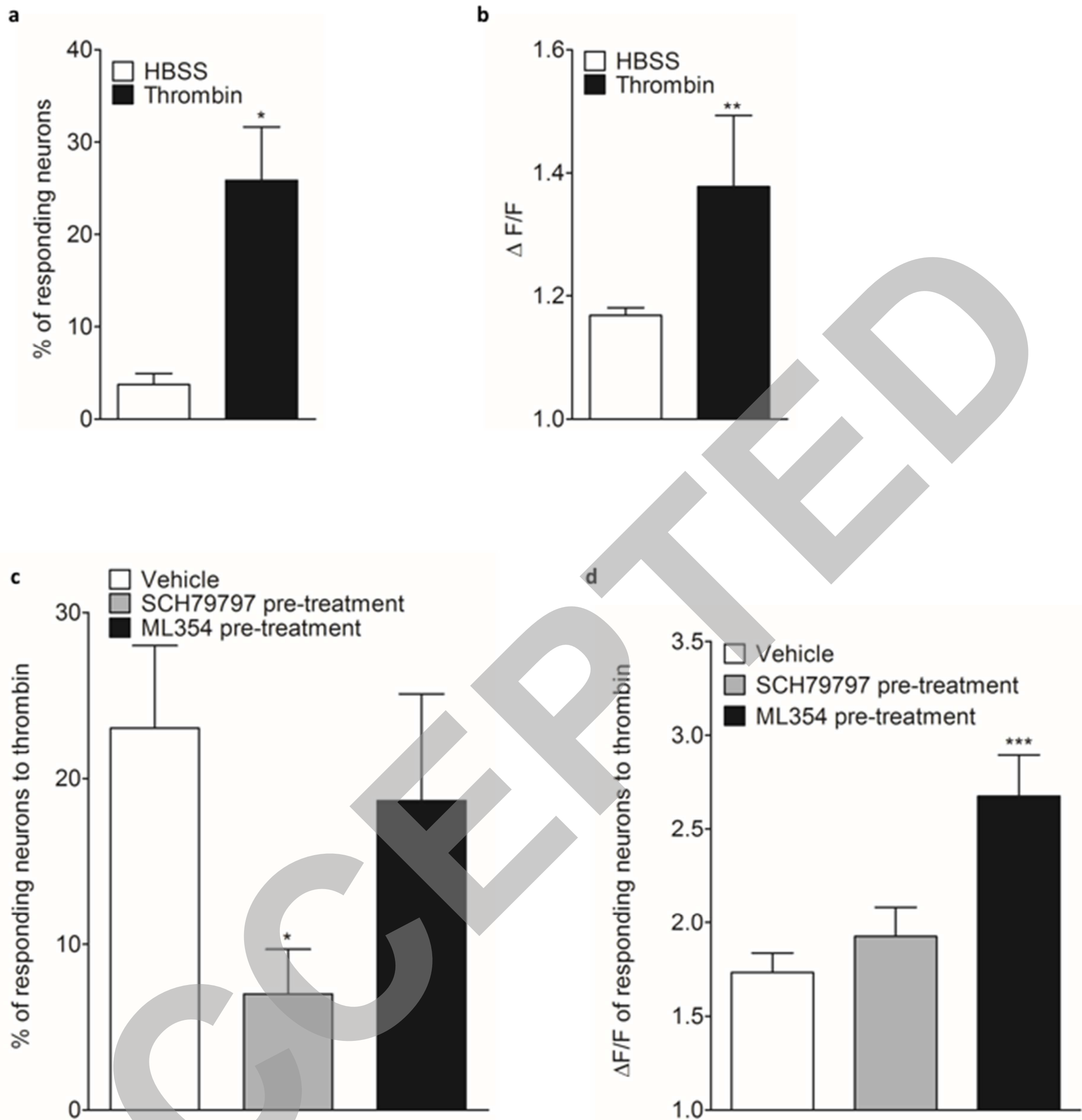

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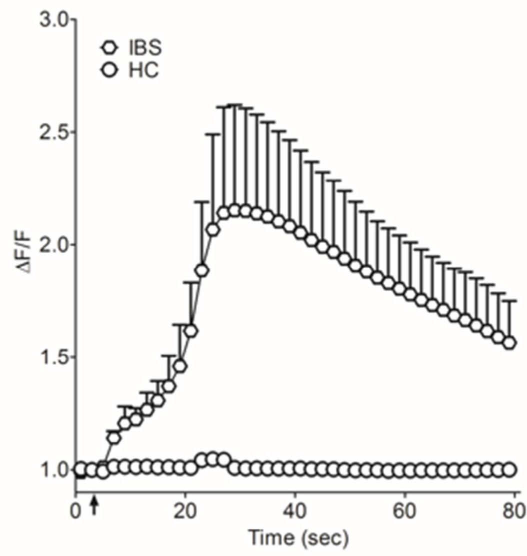
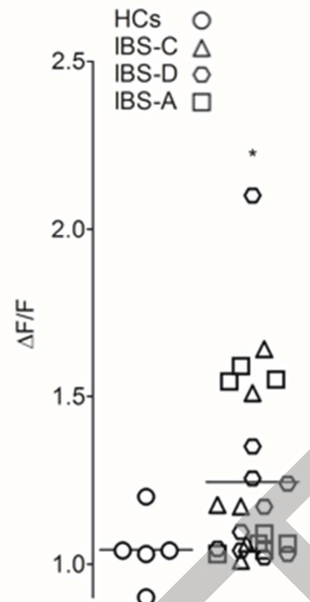
Figure 5

a





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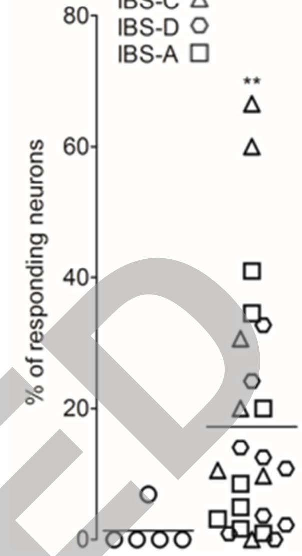

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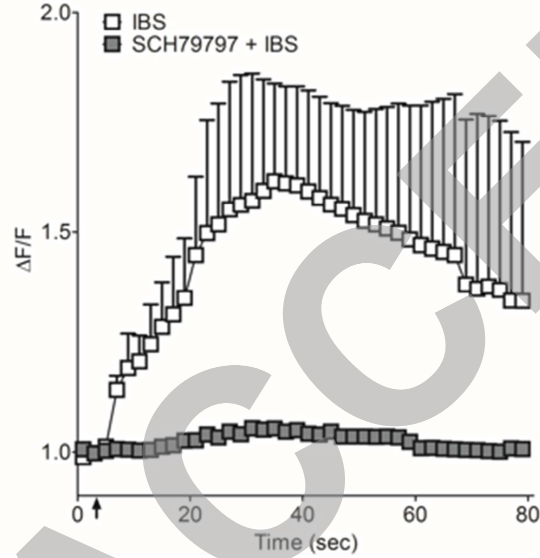

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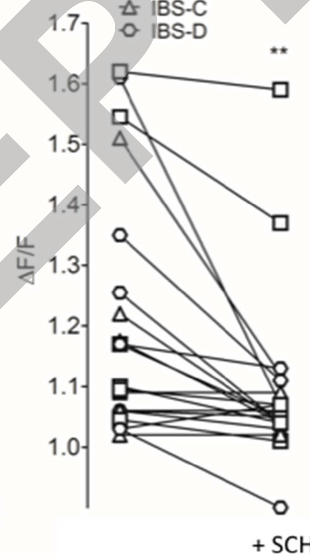

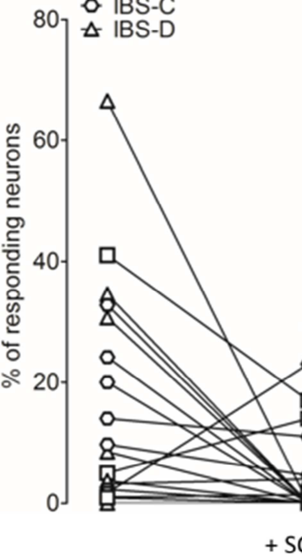

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