ONLINE SUPPLEMENT

MATERIALS AND METHODS

Table S1: List of primary antibodies, their source and working dilutions

Antibody	Source	Host Species	Working Concentration
FSP	Abcam (UK)	Mouse	1:500
P75	Advanced Targetting Systems (USA)	Mouse	1:100
HNK1	Sigma (UK)	Mouse	1:100
AP2α	Novus Biologicals (USA)	Rabbit	1:50
PGP9.5	Cedarlane (Canada)	Mouse	1:250
Synaptophysin	Dako (UK)	Rabbit	1:50 (Pre-diluted)
SP	In house (Eedy et al., 91)	Rabbit	1:1000
CGRP	Peninsula (USA)	Rabbit	1:900
TRPA1	Abcam (UK)	Rabbit	1:100
TRPV1	Abnova (Taiwan)	Mouse	1:100

Harvesting total RNA

RNA was extracted from each sample (1 well) using the Picopure RNA Isolation kit as follows: culture medium was removed from PNE cultures and cells were washed with ice cold PBS for 5 minutes. Extraction buffer (100 µl) was then added and samples were incubated at 42°C for 30 minutes. Each sample was mixed gently using a pipette tip and collected in a microcentrifuge tube.

RNA purification columns were preconditioned by adding conditioning buffer (250 µl) to each column membrane. Membranes were incubated with the conditioning buffer for 5 minutes at room temperature. Columns were then centrifuged at high speed for 1 minute to remove buffer.

70% ethanol (100 µl) was added to each microcentrifuge containing the cell extract and samples were mixed gently by pipetting. The mixture was transferred to a preconditioned RNA purification column and RNA was bound to the column by centrifuging the column at low speed for 2 minutes. Flowthrough was removed by centrifuging the column at high speed for 30 seconds. Wash buffer 1 (100 µl) was added to each column and centrifuged for 1 minute at 8,000 x g. Wash buffer 2 (100 µl) was then added to each column and samples were centrifuged for 1 minute at 8,000 x g. A second aliquot of wash buffer 2 (100 µl) was added to each column and centrifuged at high speed for 2 minutes. The purification column was then transferred to a collection tube and elution buffer (11 µl) was applied directly to the column membrane. Membranes were incubated with elution buffer for 1 minute at room temperature before being centrifuged for 1 minute at 1000 x g to ensure complete coverage of the membrane with the buffer. RNA was then eluted by centrifuging for 1 minute at high speed.

RNA was quantified using 2 µl of cDNA solution on a Take3 plate. Absorbance readings at 260 nm and 280 nm were obtained along with a 260/280 ratio and the concentration of RNA per µl.

S2: TaqMan primer details

Gene	Reference No.	Chromosome No.	Probe sits on exon boundary	Base position	Amplicon length
TRPA1	HS00175798	8	2 - 3	441	124
TRPV1	HS00218912	17	8 - 9	1540	94
B2M	HS00984230	15	3 - 4	431	81
GUSB	HS00939627	7	8 - 9	1522	96

Table S3: Thermal profile for qPCR

Segment	Thermal profile	Cycles
1	2 minutes at 55°C	1
2	10 minutes at 95°C	1
3	15 seconds at 95°C 1 minute at 60°C	45

Microfluorimetric Calcium Imaging

$[Ca^{2+}]_i$ was measured by alternating excitation wavelengths of 340 and 380 nm light delivered from a dual monochromator (5 nm bandwidth) using a light chopper. Emitted fluorescence was measured from the side port of the microscope via an adjustable rectangular window, a filter (510 nm) and a photomultiplier tube (PMT) in the light path. Fluorescence equipment was controlled by Acquisition Engine software. Background fluorescence was measured at the end of each experiment by exposing the cell to 5 mM MnCl2 and quenching the fluorescent dye. Changes in the background-corrected fluorescence emitted at each excitation wavelength (R=340/380) was used as a measure of change in cytoplasmic Ca^{2+} concentrations.

Table S4: Cytokine details

Cytokine	Source
NGF	Peprotech (USA)
TNFα	Sigma (UK)
IL1β	Sigma (UK)
Poly I:C	Invivogen (UK)

RESULTS

Table S5: The presence of TRPA1 and TRPV1 mRNA in PNEs was confirmed by qPCR

Channel	Ct	Average Ct
TRPA1	25.785	25.547
	25.865	
	26.055	
	24.755	
	25.275	
TRPV1	34.94	34.644
	34.415	
	35.155	
	33.935	
	34.775	

FIGURE LEGENDS

Figure S1: hDPSCs express the neural crest stem cell marker P75 (A) and subpopulations of cells express HNK1 (B) and AP2α (C). Scale bars: 50 μm.

Figure S2: PNEs express the neuropeptides SP (A) and CGRP (B), a characteristic of sensory neurons. Scale bar 100 μm.

Figure S3: PNEs displayed spontaneous fluctuations in $[Ca^{2+}]_i$. Viability of PNEs was determined through the observation of increases in $[Ca^{2+}]_i$ in the absence of agonist challenges. Figures A and B are representative traces of the varying degrees of spontaneous activity observed in PNEs.

Figure S4: Changes in intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) in response to capsaicin were investigated in PNEs demonstrating spontaneous activity. Stimulation with capsaicin evoked an instantaneous increase in $[Ca^{2+}]_i$ levels (A). $[Ca^{2+}]_i$ fell back to basal levels immediately after peaking. In the presence of the TRPV1 antagonist capsazepine PNEs exhibited an attenuated response to capsaicin compared with cells that had not been exposed to the antagonist (B). The change in absorbance ratio (ΔRatio) was graphed for statistical analysis to show specific TRPV1 activation in PNEs (C). Bars represent SEM, * $P < 0.05$, ** $P < 0.01$.

Figure S5: PNEs demonstrating spontaneous activity were stimulated with cinnamaldehyde and an instantaneous increase in $[Ca^{2+}]_i$ levels was observed (A). $[Ca^{2+}]_i$ fell back to basal levels immediately after peaking. In the presence of the TRPA1 antagonist HC030031 PNEs exhibited an attenuated response to cinnamaldehyde compared to cells that had not been exposed to the antagonist (B). The change in absorbance ratio (ΔRatio) was graphed for statistical analysis to show specific TRPA1 activation in PNEs (C). Bars represent SEM, * $P < 0.05$, ** $P < 0.01$.

Figure S6: PNEs were treated with pro-inflammatory cytokines TNFα and IL1β to determine whether the lack of change in TRPA1 gene expression was specific to NGF treated cells. No significant changes in TRPA1 gene expression was observed in PNEs treated with either TNFα or IL1β. Bars represent SEM.

Figure S7: PNE TRPV1 channels did not become hyper-responsive following 20 minutes incubation with the pro-inflammatory mediator NGF (100 ng/ml) (A). Similarly there were no significant changes in TRPV1 gene expression following NGF treatment for 6 or 24 hours (B). Bars represent SEM, * $P < 0.05$.

Figure S8: Vehicle controls. PNE currents were not altered in response to 0.001% DMSO alone. Currents generated by cinnamaldehyde and capsaicin were included for comparison. Bars represent SEM.

Figure S9: PNEs were treated with a range of Poly I:C concentrations (2 µg/ml – 20 µg/ml) in order to determine the optimal working concentration for experiments. IL8 release was used a measure of response. PNEs treated with 2 µg/ml PolyI:C generated the largest response in terms of IL8 release compared to 10 µg/ml and 20 µg/ml and thus was selected for use in further experiments. Bars represent SEM, *** $P < 0.001$.

FIGURES

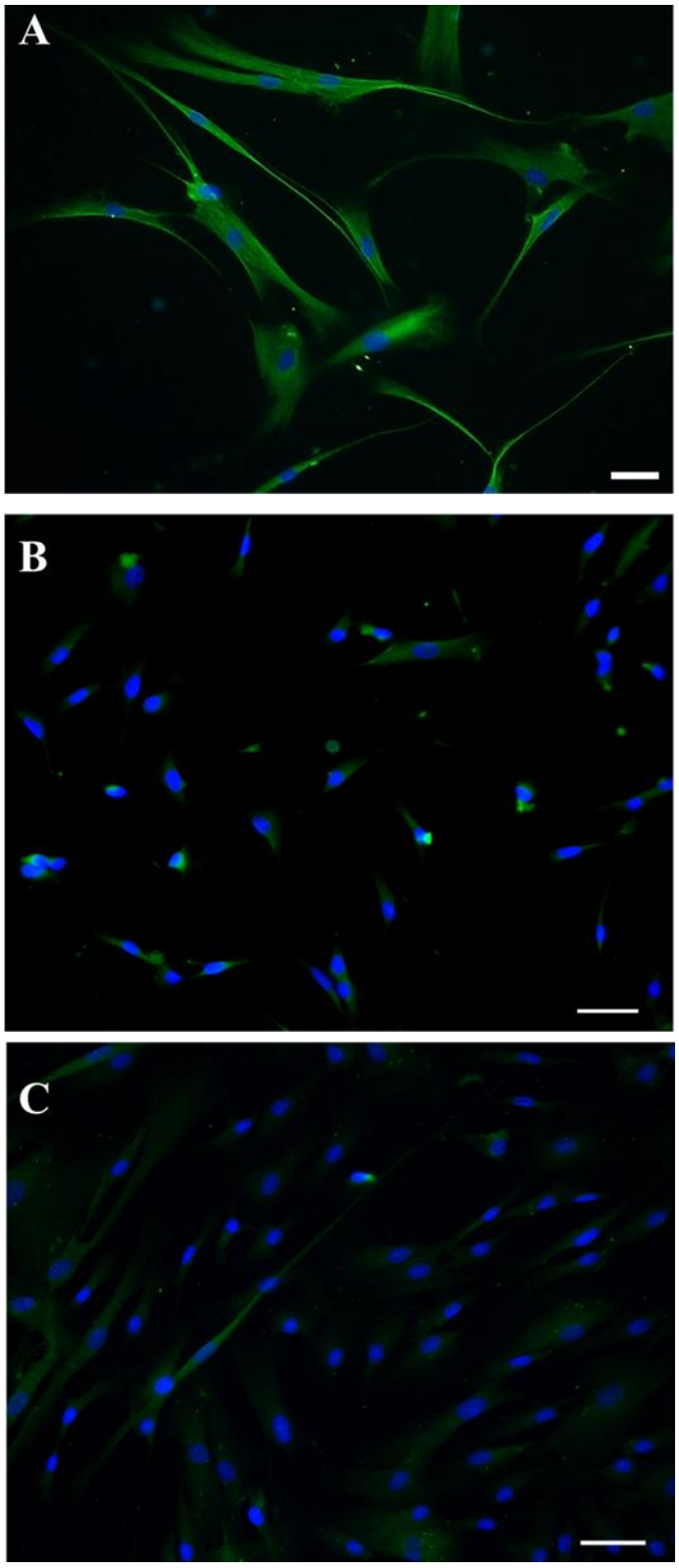


Figure S1

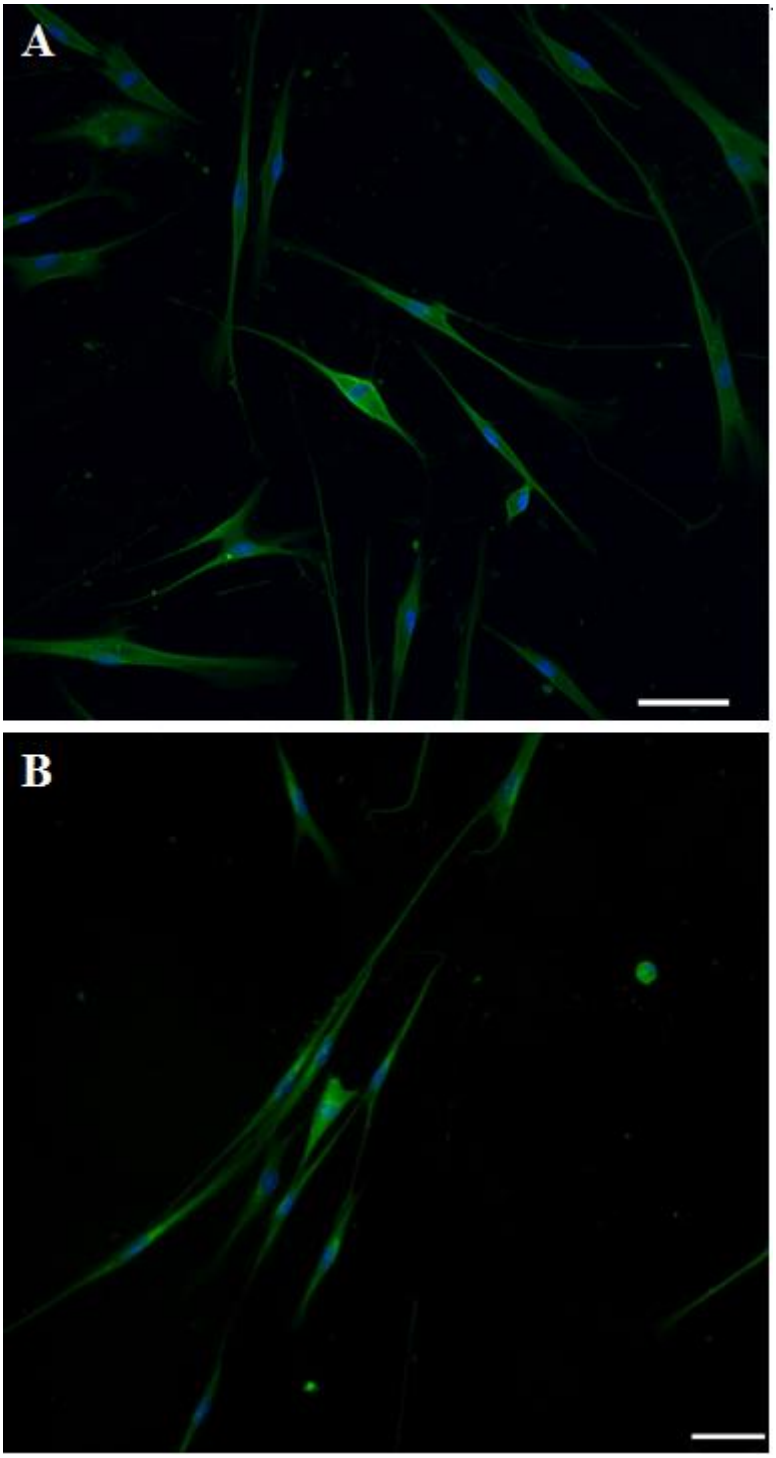


Figure S2

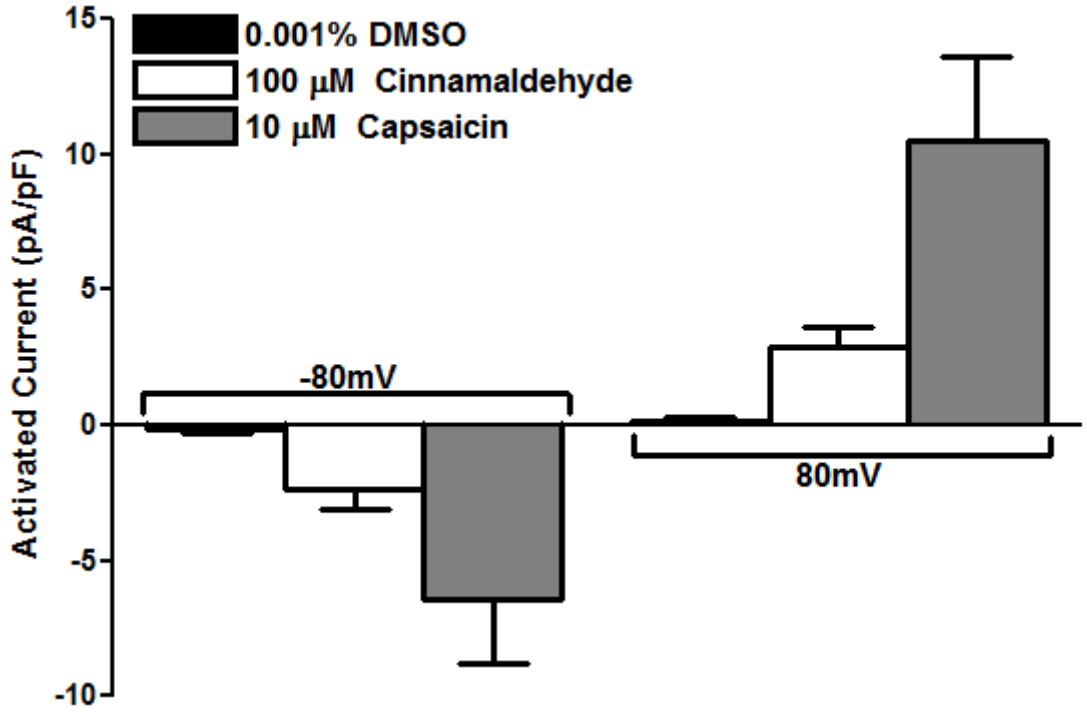

0.001% DMSO
100 μM Cinnamaldehyde
10 μM Capsaicin
Activated Current (pA/pF)
15
10
5
0
-5
-10
-80mV
80mV

Figure S3

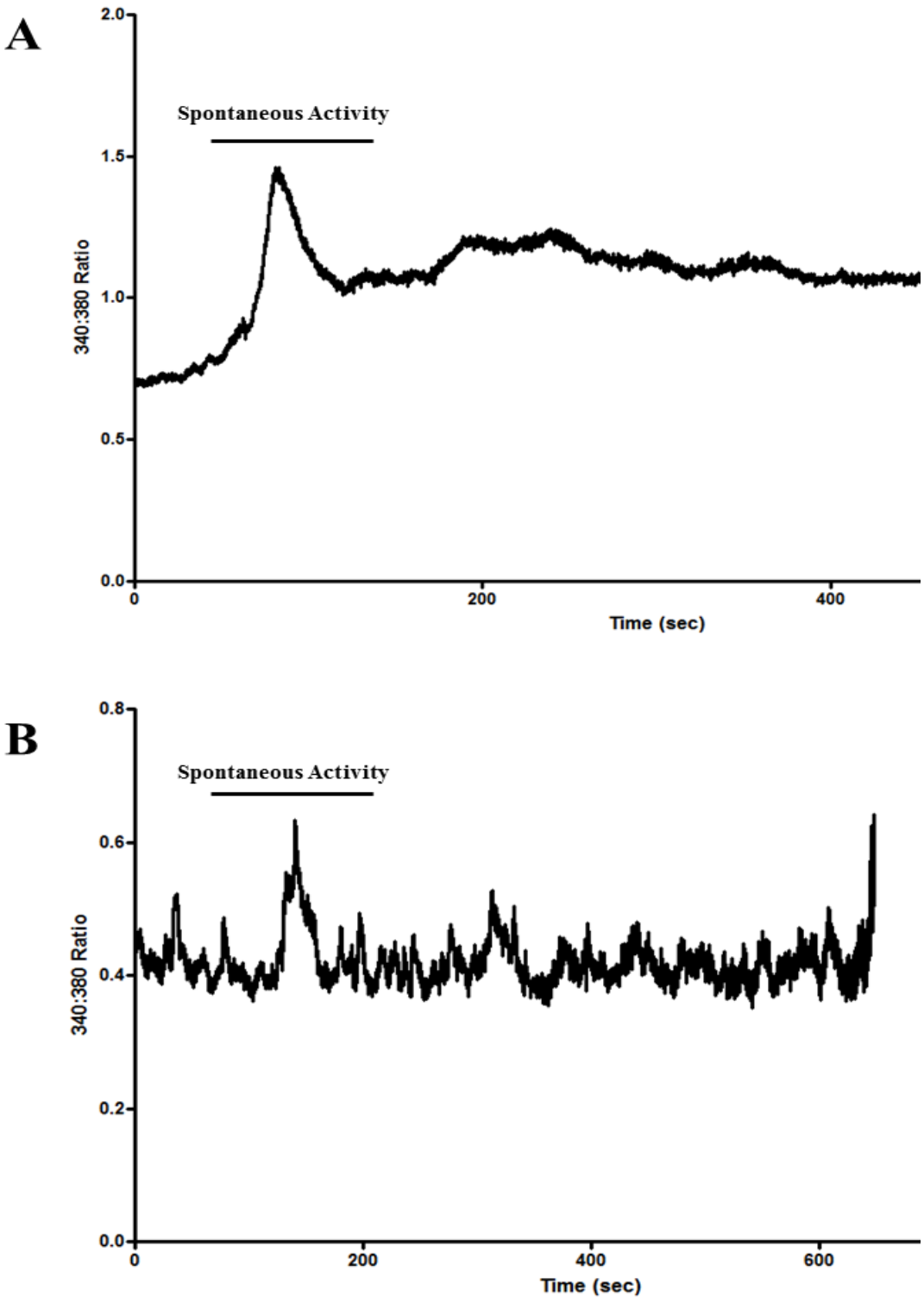

A
Spontaneous Activity
340:380 Ratio
2.0
1.5
1.0
0.5
0.0
0
200
400
Time (sec)
B
Spontaneous Activity
340:380 Ratio
0.8
0.6
0.4
0.2
0.0
0
200
400
600
Time (sec)

Figure S4

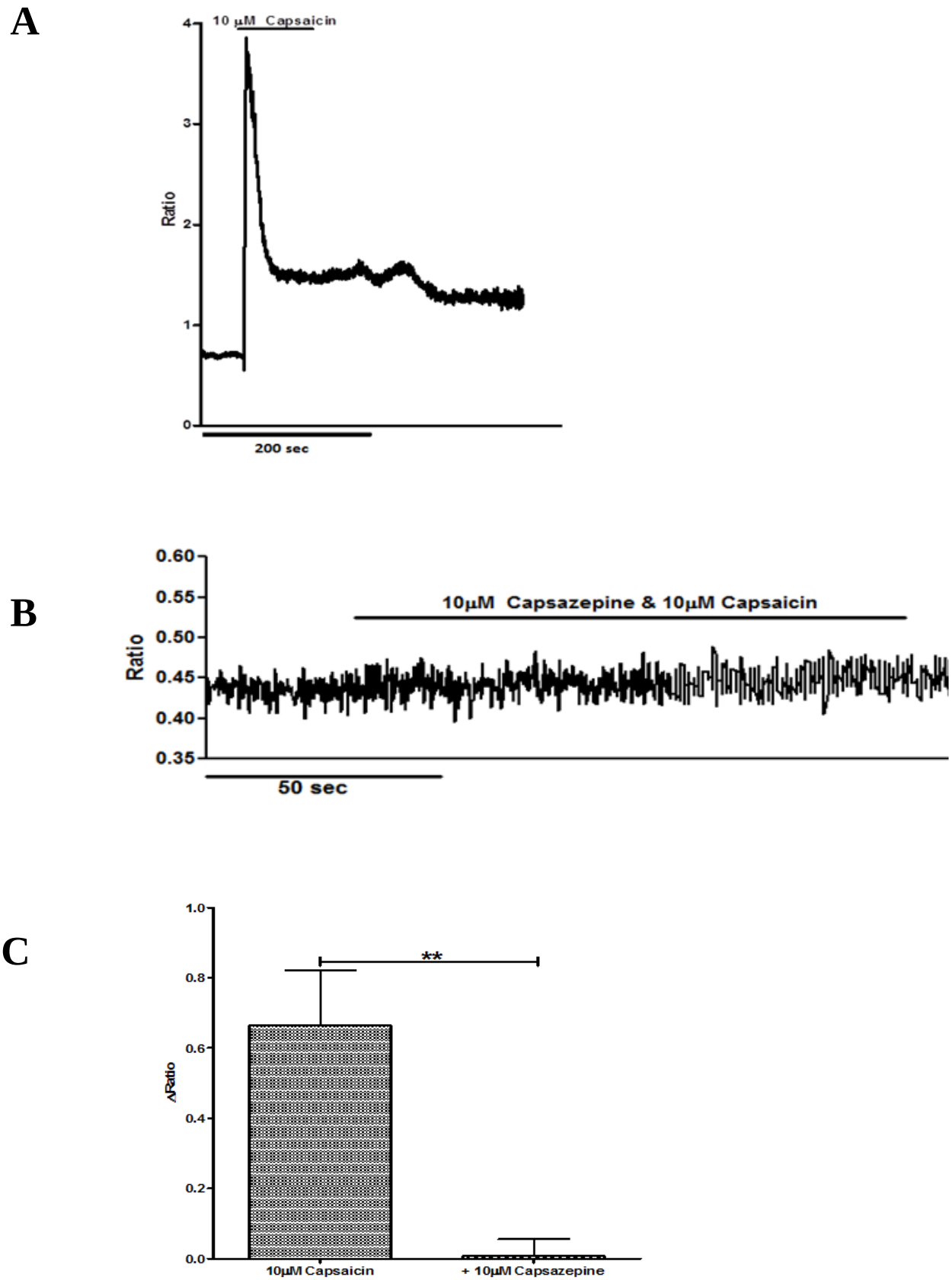

A
10 μM Capsaicin
Ratio
4
3
2
1
0
200 sec
B
10μM Capsazepine & 10μM Capsaicin
Ratio
0.60
0.55
0.50
0.45
0.40
0.35
50 sec
C
**
ΔRatio
1.0
0.8
0.6
0.4
0.2
0.0
10μM Capsaicin
+ 10μM Capsazepine

Figure S5

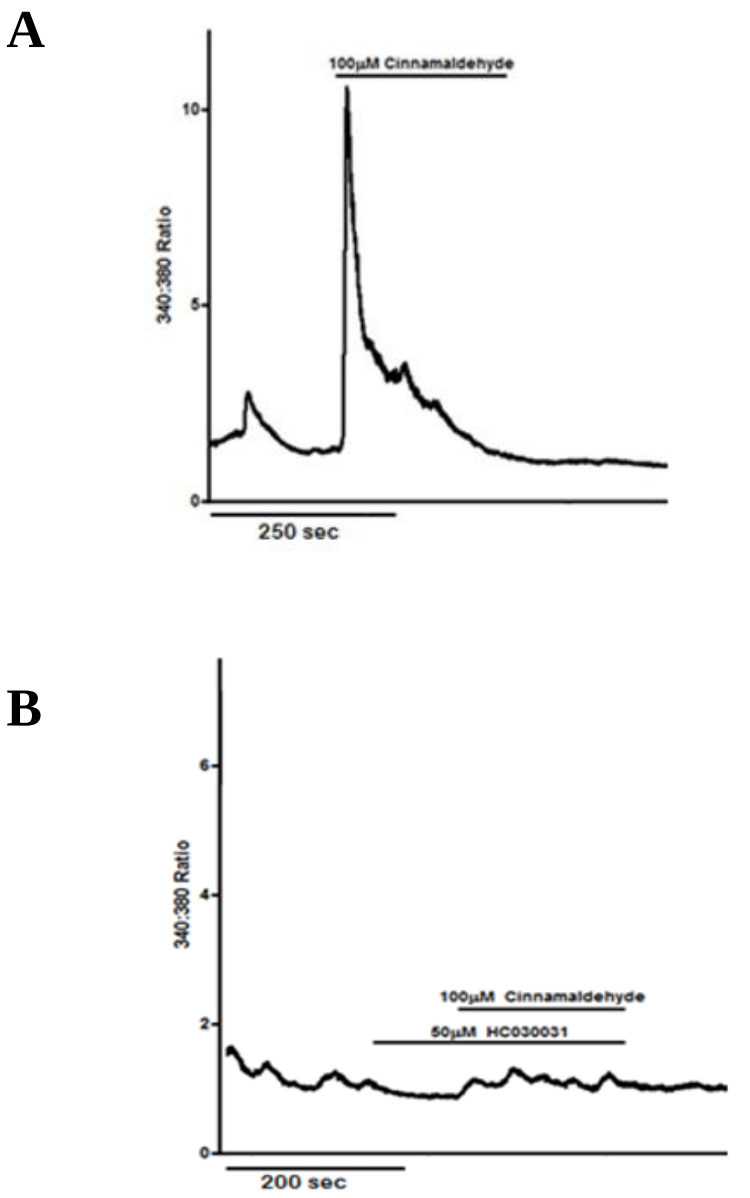

A
100μM Cinnamaldehyde
340:380 Ratio
10
5
0
250 sec
B
340:380 Ratio
6
4
2
0
100μM Cinnamaldehyde
50μM HC030031
200 sec
C
**
ΔRatio
10
8
6
4
2
0
100μM Cinnamaldehyde
+ 50μM HC030031

Figure S6

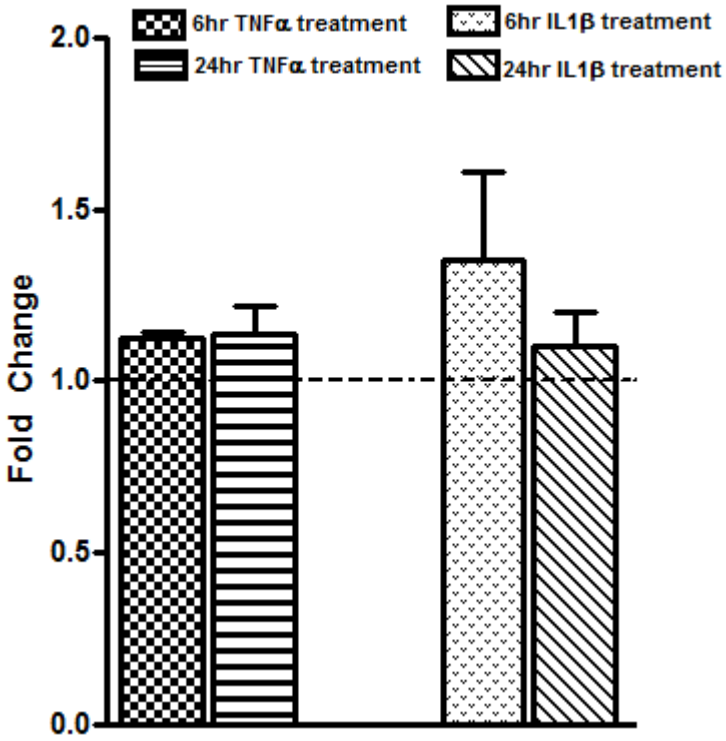
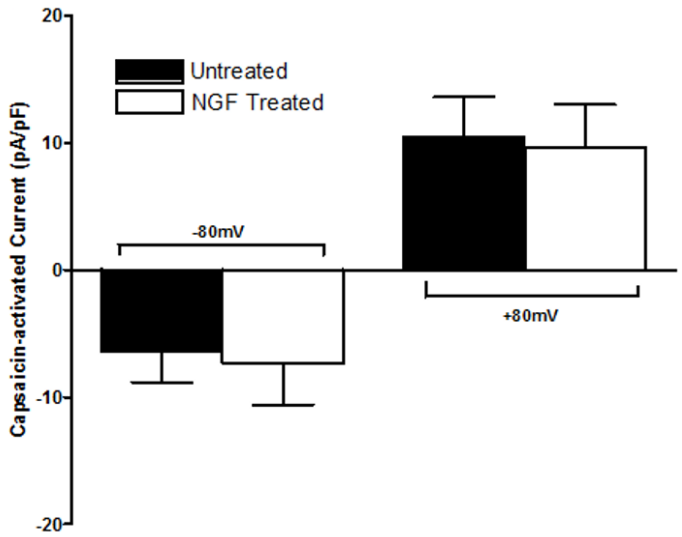

6hr TNFα treatment
24hr TNFα treatment
6hr IL1β treatment
24hr IL1β treatment
Fold Change
2.0
1.5
1.0
0.5
0.0

Figure S7

A


Capsaicin-activated Current (pA/pF)
20
10
0
-10
-20
Untreated
NGF Treated
-80mV
+80mV

B

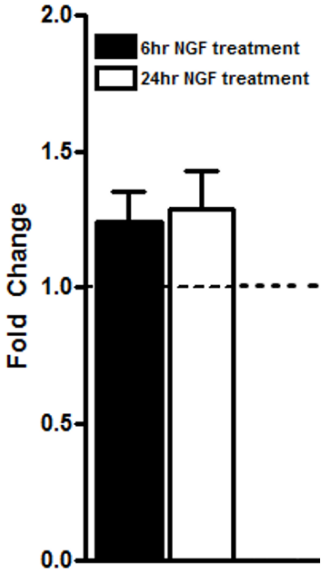

Fold Change
2.0
1.5
1.0
0.5
0.0
6hr NGF treatment
24hr NGF treatment

Figure S8

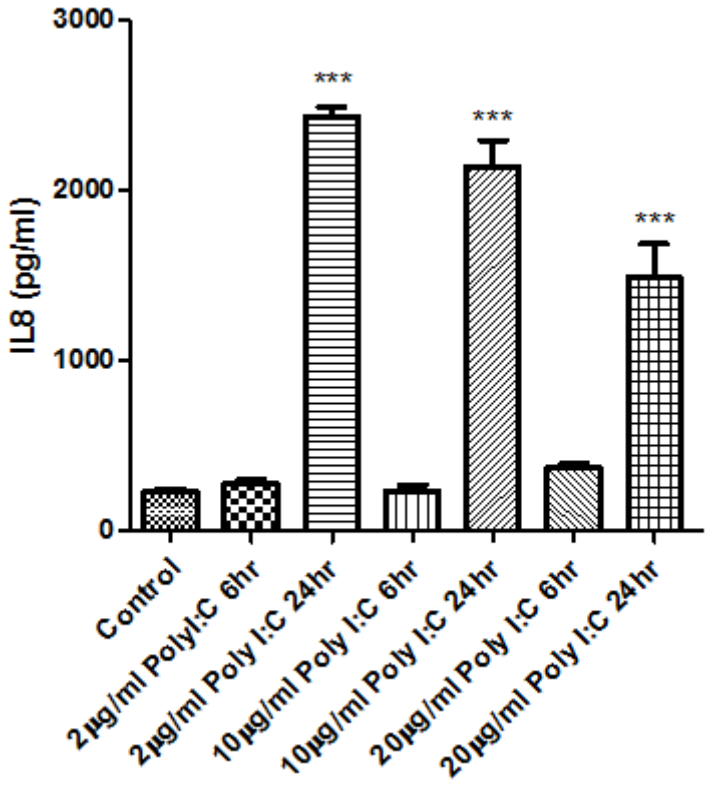

3000
2000
1000
0
IL8 (pg/ml)
***
***
***
Control
2µg/ml PolyI:C 6hr
2µg/ml Poly I:C 24hr
10µg/ml Poly I:C 6hr
10µg/ml Poly I:C 24hr
20µg/ml Poly I:C 6hr
20µg/ml Poly I:C 24hr

Figure S9