

Supplemental material to:

The Brain-Derived Neurotrophic Factor prompts platelet aggregation and secretion

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Materials and methods

Materials

Anticoagulant Citrate Dextrose Solution Formula A (DIN: 00788139) and eptifibatide (Integrilin, DIN: 02240351) were purchased at the Montreal Heart Institute Pharmacy. Adult human brain cerebral cortex whole tissue lysate (catalog no. NB820-59182) was from Novus Biologicals. U251-MG (glioblastoma cell line) were a gift from Dr Gaëlle Roullin (Université de Montréal) and HEPG2 hepatoma cells were a gift from Dr Gaetan Mayer (Montreal Heart Institute). Prostaglandin E1 (catalog no. 1620), wortmannin (catalog no. 1232), Y-27632 dihydrochloride (catalog no. 1254), AR-C 66096 tetrasodium salt (catalog no. 3321), cyclotraxin B (catalog no. 5062), and GNF5837 (catalog no. 4559) were from Tocris Bioscience. PP2 (catalog no. Ak-60369), PRT318 (catalog no. Ak-198900), and NSC23766 (catalog no. CD0192) were from Cederlane Labs. K252a (catalog no. K-2151) and Dasatinib (catalog no. D-3307) were from LC Laboratories. Sodium arachidonate (catalog no. 10006607) and bisindolylmaleimide I (BIM-I, catalog no. 13298-10) were from Cayman Chemical Co. Aspirin (catalog no. A-

6810) was from Sigma Aldrich and zoledronic acid (catalog no. 6111-10) was from R&D Systems. TRAP-6 amide (catalog no. H-2936.0025BA) was from VWR and collagen type 1 fibrils (catalog no. 385) were purchased from Chrono-Log Corporation. Protease and phosphatase inhibitor mini-tablets (catalog no. A32959) and protein G agarose (catalog no. 16-266) were from Fisher Scientific. Recombinant human neurotrophin-4 (NT-4) protein (catalog no. N-270) and human TrkB-Fc Chimera (catalog no. RPC-001) were from Alomone Labs. Anti-BDNF antibodies (catalog no. 500-P84) were from Peptide. Recombinant human BDNF were from Peptide (catalog no. 450-02-500), Alomone (catalog no. B-250), and Sigma-Aldrich (catalog no. B3795). Anti-BDNF hybridoma (mab #9) was from the Developmental Studies Hybridoma Bank at the University of Iowa. Human TrkB antibodies Clone 7H6E7B3 (catalog no. 10047-MM12) and clone 3D12 (catalog no. M02) were from SinoBiological and Abnova, respectively. β -actin antibody (catalog no. 937215) was from R&D Systems. Alpha-2-Macroglobulin (α 2M) antibody (catalog no. abx132389) was from Abnova Ltd. AffiniPure goat anti-mouse IgG (H+L) (catalog no. 115-005-003) and AffiniPure goat anti-rabbit IgG (H+L) (catalog no. 111-005-003) were from Jackson ImmunoResearch Laboratories. Alexa Fluor 488-conjugated donkey anti-mouse IgG (H+L) (catalog no. A-21202) was from ThermoFisher Scientific. Anti-Akt antibody (catalog no. 9272S), anti-phospho-Akt (Ser-473, catalog no. 4060S), anti-PLC γ 2 (catalog no. 3872T) and anti-phospho-PLC γ 2 (Thr-1217, catalog no. 3871T) were from Cell Signaling Technology. Customized multiplex cytokine detection kits were from AimPlex Bioscience.

Participant selection

This study was approved by the Montreal Heart Institute Scientific and Research

Ethics Committee (REC reference: #2018-2368) and all participants gave written informed consent. Participants were considered healthy if they were aged 18 years or older, did not require long-term medical therapy, had refrained from drugs known to influence platelet function in the previous 2 weeks, had not undergone major surgery in the previous 6 months, did not have a history of bleeding symptoms, and had platelet counts and hemoglobin levels within the normal reference range.

Blood collection and platelet isolation

Blood was drawn by venipuncture into syringes containing acid citrate dextrose (ACD-A) in a 1:5 volume ratio (ACD/blood) to prepare washed platelets. Blood was centrifuged at 200 g for 15 min without a brake, and platelet-rich plasma (PRP) was collected. Prostaglandin E1 (1 μ M) was added to PRP prior to centrifugation at 1000 g for 10 min. Plasma was removed and discarded. Pelleted platelets were resuspended in Tyrode's buffer (137 mM NaCl, 11.9 mM NaHCO₃, 0.4 mM NaH₂PO₄, 2.7 mM KCl, 1.1 mM MgCl₂, 5.6 mM glucose, pH 7.4). Platelet counts were determined using a Beckman Coulter hematology analyzer (Ac-T 5diff AL) and adjusted to a final concentration of 2.5×10^8 /mL for platelet aggregation assays or 5×10^8 /mL for protein phosphorylation assays. Platelets were allowed to rest at room temperature for 60 min prior to functional experiments.

Flow cytometry

Washed platelets were fixed with 1% paraformaldehyde for 20 min at room temperature. Platelets were then incubated at room temperature with anti-TrkB primary antibody (R&D Mab397, diluted 1:25) or isotype control (mouse anti-human IgG₁) for 30

min, followed by an Alexa Fluor 488-conjugated donkey anti-mouse secondary antibody (Invitrogen, diluted 1:200) for 30 min, and analyzed using the MACSQuant Analyzer 10 (Miltenyi Biotec, Germany). Peripheral blood mononuclear cells (PBMCs) and U251-MG (glioblastoma cell line) were used as a TrkB-high control and HEPG2 hepatoma cells were used as a TrkB-low control.

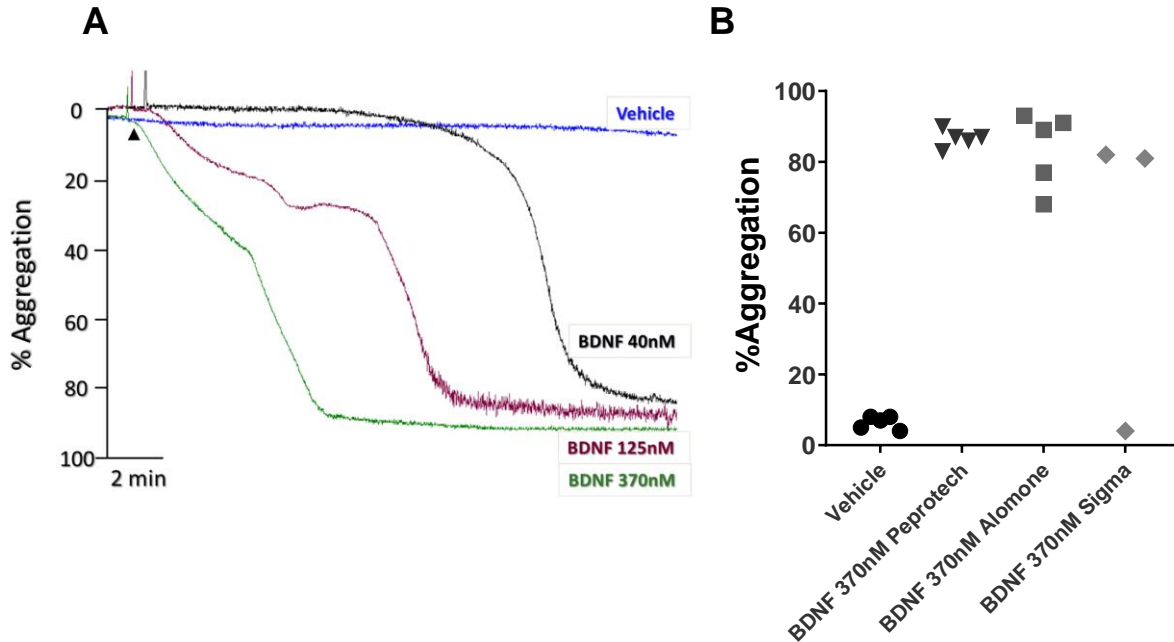
Confocal microscopy

Glass coverslips were pre-coated with 0.1% poly-L-lysine for 15 min. Platelets fixed with 1% paraformaldehyde for 20 min at room temperature and permeabilized with triton 0.1% were transferred onto pre-coated coverslips and allowed to adhere overnight at 4 °C. TrkB labelling was performed using an anti-TrkB primary antibody (SinoBiological MM12, 5 µg) for 90 min at room temperature followed by incubation with an Alexa Fluor 488-conjugated donkey anti-mouse secondary antibody (1:200) for 1 h. Fluorescence was visualized using a Zeiss LSM510 confocal microscope. PBMCs and U251 were used as a TrkB-high control and HEPG2 hepatoma cells were used as a TrkB-low control.

Statistical analyses

All data sets passed the Shapiro-Wilk test for normality. Repeated-measures analysis of variance (ANOVA) with Geisser-Greenhouse correction for sphericity and Dunnett's correction for multiple comparisons was performed using GraphPad Prism Software version 8 for Windows (GraphPad Software, San Diego, CA, USA). A multiplicity-adjusted p-value < 0.05 was considered significant. Continuous variables are presented as median ± interquartile range (IQR). N refers to the number of independent experiments with each experiment representing a different biological sample.

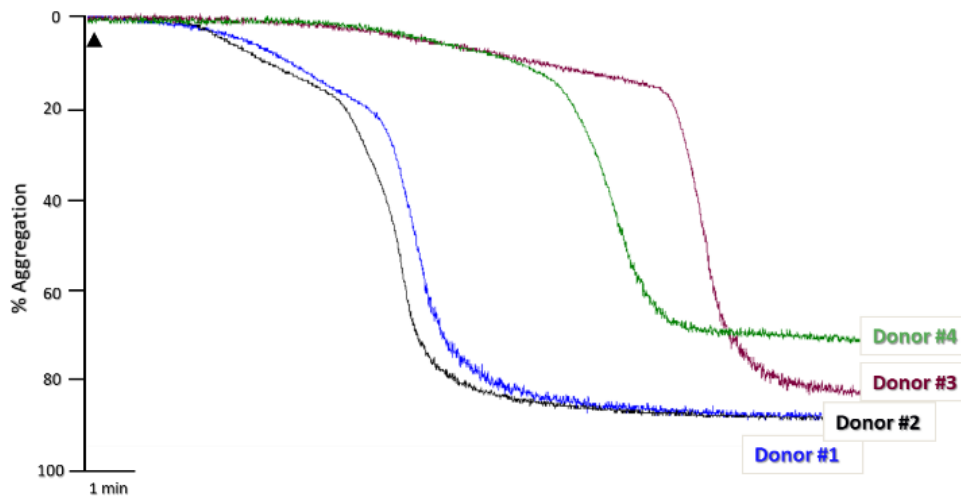
Supplemental figures:



Supplemental figure 1: Comparison of platelet aggregation in response to BDNF from different sources.

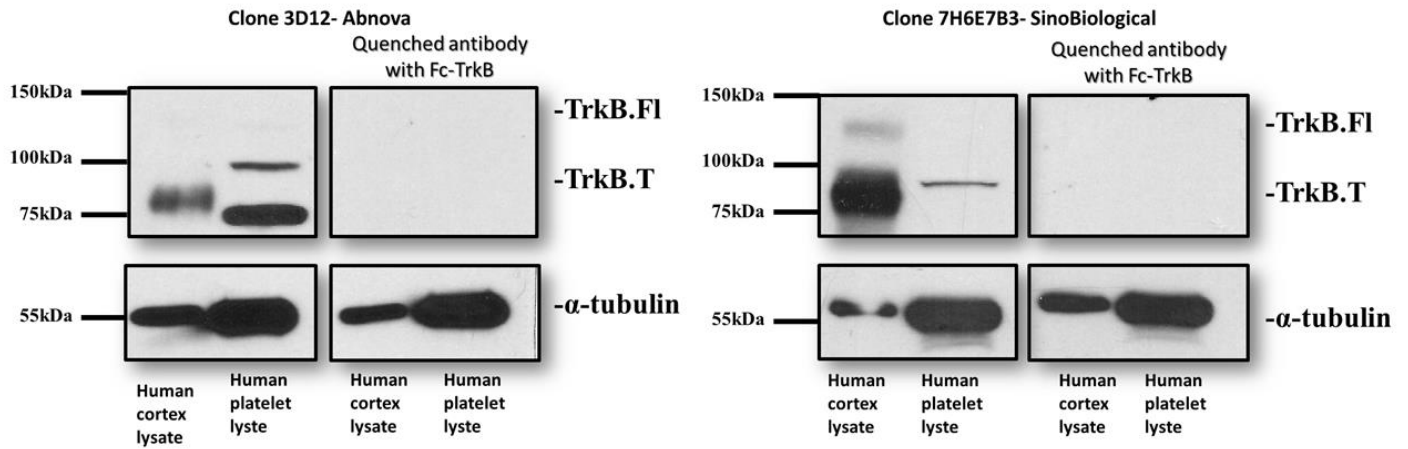
(A) Representative traces of platelet aggregation in response to 40 nM, 125 nM and 370 nM BDNF from Alomone. Arrowheads indicate the time point at which the agonist was added

(B) Quantification histogram of platelet aggregation in response to 370 nM BDNF from Peprotech, 370 nM BDNF from Alomone and 370 nM BDNF from Sigma compared to vehicle. Results are representative of at least 5 independent experiments except for BDNF from Sigma (n=3).



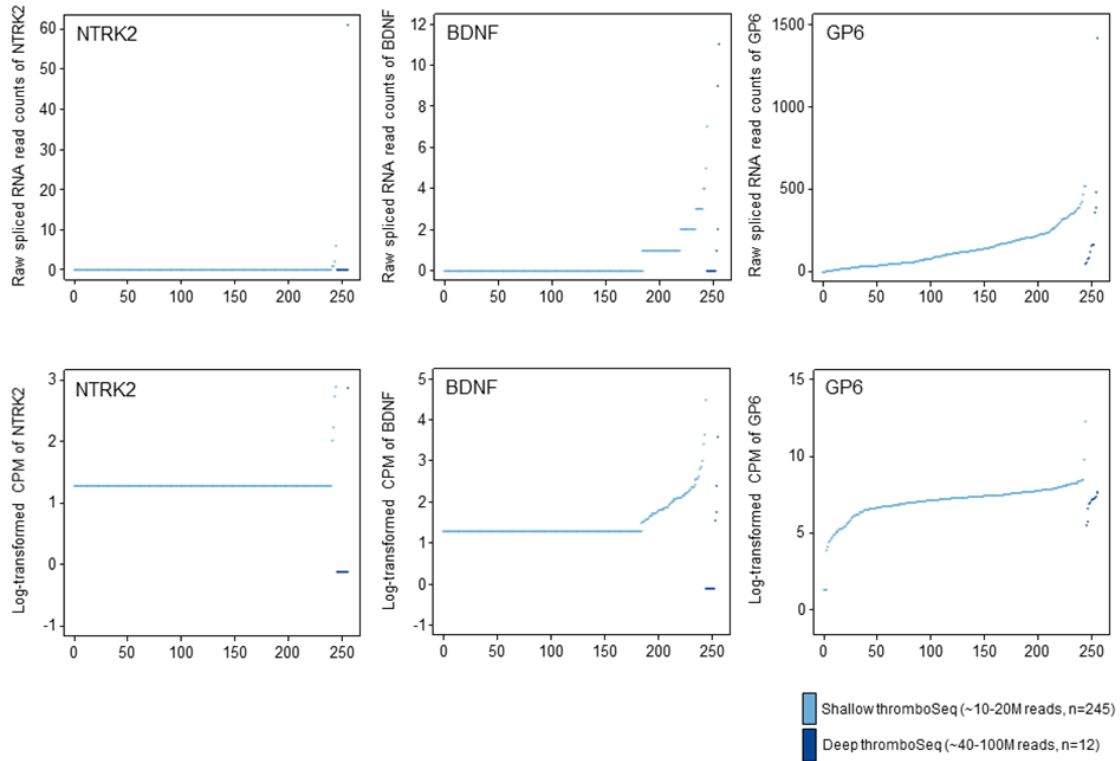
Supplemental figure 2: Individual traces of BDNF-induced aggregation showing varying lag times between BDNF addition and start of aggregation.

Example traces of BDNF (370 nM)-induced aggregation in independent healthy volunteers. Whereas full biphasic and irreversible aggregation was seen in all experiments, the time from BDNF addition to primary wave aggregation was variable between individuals.



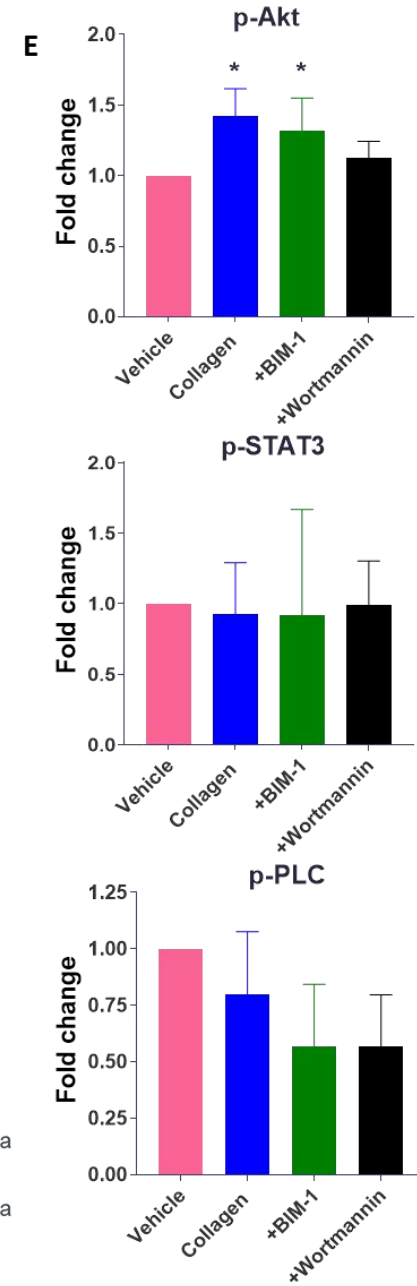
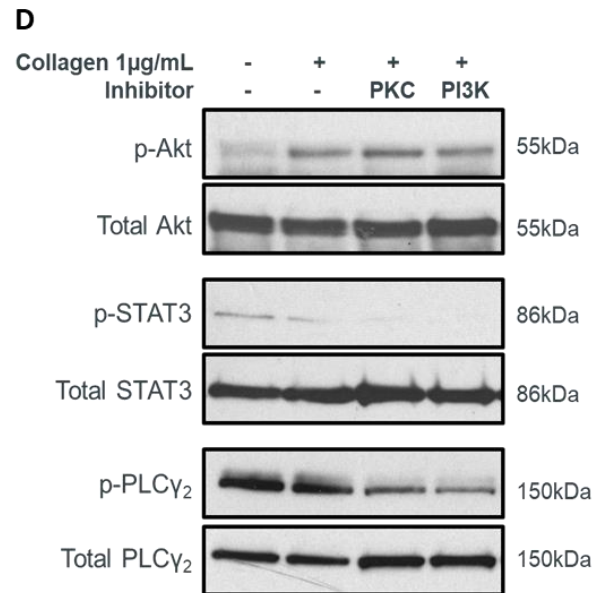
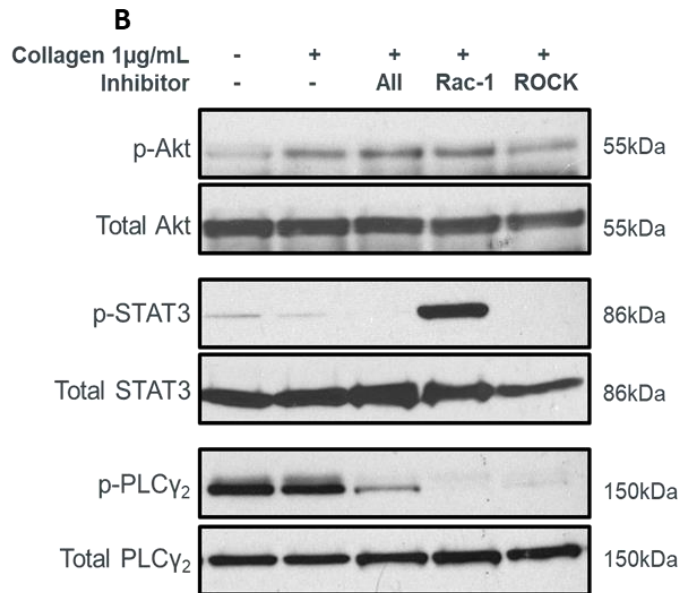
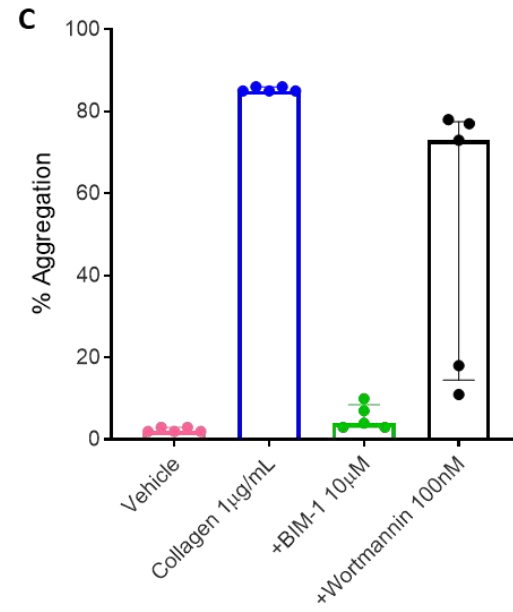
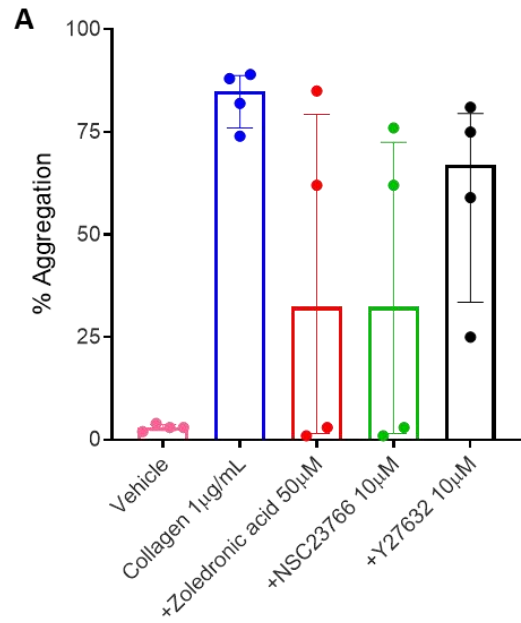
Supplemental figure 3: Competition assays for the TrkB antibodies used

Immunoblotting of TrkB from human platelet lysates (60 μ g for SinoBiological Clone 7H6E7B3 and 30 μ g for Abnova Clone 3D12) along with human cortex whole cell lysate as a positive control (3 μ g, expected molecular weight of truncated TrkB and the full-length TrkB receptor are 95 and 140 kDa, respectively). α -tubulin was used a loading control. Two different antibodies (clone 7H6E7B3 and clone 3D12) targeting the extracellular domain of TrkB were used. Left: blots blocked with 1% BSA for 1h followed by primary antibody. Right: blots blocked in 1% BSA and 30 μ g/ml of TrkB blocking peptide followed by the indicated primary antibody.



Supplemental figure 4: Very low counts of NTRK2 mRNA can be detected in human platelets

Dot plot of spliced RNA reads (upper row) and corresponding log-transformed counts-per-million (lower row) mapping to the gene NTRK2, BDNF, and GPVI. 245 healthy individuals were sequenced according to the shallow thromboSeq protocol (~10-20 million total RNA-seq reads per sample), and 12 healthy individuals were subjected to deep thromboSeq (~40-100 million total RNA-seq reads per sample). Samples were ranked according to shallow (light blue dots) and deep (dark blue dots) thromboSeq mode, and the number of spliced RNA reads respectively log-transformed CPM-values mapping to both genes, as indicated on the x-axis.



Supplemental figure 5: Collagen-induced platelet activation with Rho GTPase inhibitors and PKC/PI3K inhibitors

(A) Collagen-induced aggregation in the presence of rho GTPase inhibitors (zoledronic acid 50 μ M, NSC23766 10 μ M and Y27632 10 μ M; n=5). Repeated-measures ANOVA, $p>0.05$; inhibition of collagen with zoledronic acid, NSC23766 $p=0.005$ and Y27632 were not significant $p>0.05$.

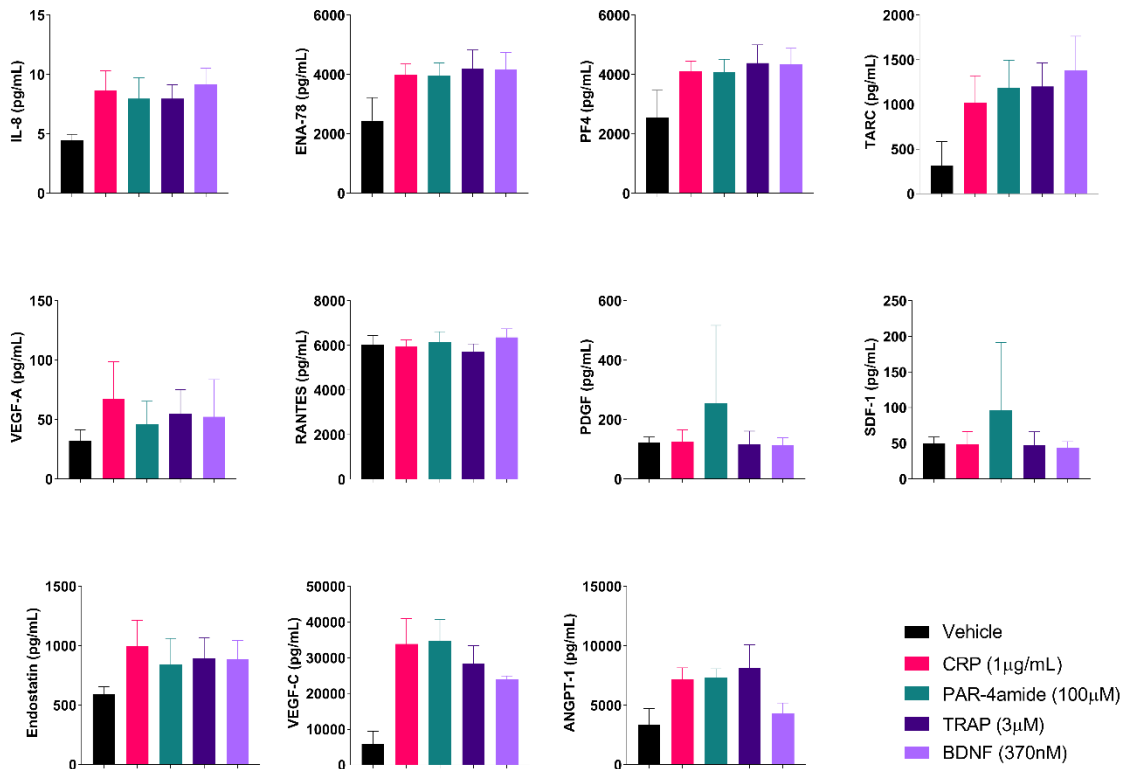
(B) Collagen-induced phosphorylation of Akt, STAT3 and PLC- γ_2 in the presence of Rho GTPase inhibitors (zoledronic acid 50 μ M, NSC23766 10 μ M and Y27632 10 μ M).

(C) Collagen-induced aggregation in the presence of PI3K and PKC inhibitors (wortmannin 100 nM, BIM-1 10 μ M, n=5). Repeated-measures ANOVA, $p<0.0109$; inhibition of collagen with BIM-1 and wortmannin were not significant $p>0.05$

(D) Collagen-induced phosphorylation of Akt, STAT3 and PLC- γ_2 in the presence of PI3K and PKC inhibitors (wortmannin 100 nM and BIM-1 10 μ M).

(E) Quantification of collagen-induced phosphorylation of Akt, STAT3 and PLC- γ_2 in the presence of PI3K and PKC inhibitors (wortmannin 100 nM and BIM-1 10 μ M). One-way ANOVA; compared to vehicle: phosphorylation of Akt by collagen is increased $p<0.05$, with collagen in presence of BIM-1 $p<0.05$ and no significant difference with wortmannin. Phosphorylation of STAT3 by collagen alone or in presence of with BIM-1 and wortmannin was not significant $p>0.05$. Phosphorylation of PLC- γ_2 by collagen alone or in presence of BIM-1 and wortmannin was not significant $p>0.05$. Density was measured with ImageJ and is expressed as a ratio of density of phosphorylated protein/density of total protein and standardized to the vehicle control.

Functional data and phosphoblots are representative of 3 or more independent experiments.



Supplemental figure 6: Platelets release inflammatory and angiogenic cytokines in response to BDNF.

Profiles of cytokine release by platelets activated with CRP (1 μg/ml), PAR-4 amide (100 μM), TRAP (3 μM), or BDNF (370 nM). Vehicle was used as a shear stress control. IL-8, interleukin 8; ENA-78, epithelial neutrophil-activating protein 78; PF4, platelet factor 4; TARC, thymus-and activation-regulation chemokine; VEGF-A, vascular endothelial growth factor A; VEGF-C, vascular endothelial growth factor C; RANTES, regulated on activation, normal T cells expressed and secreted; PDGF, platelet-derived growth factor; SDF-1, stromal cell-derived factor 1; ANGPT-1, angiopoietin 1. Shown are mean ± SD of data from platelets isolated from 4 healthy volunteers.