The role of neuraminidase 1 and 2 in glycoprotein Ibα-mediated integrin αIIbβ3 activation

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ABSTRACT

Upon vascular injury, platelets adhere to von Willebrand Factor (VWF) *via* glycoprotein Ibα (GPIbα). GPIbα contains many glycans, capped by sialic acid. Sialic acid cleavage (desialylation) triggers clearance of platelets. Neuraminidases (NEU) are responsible for desialylation and so far, NEU1-4 have been identified. However, the role of NEU in healthy platelets is currently unknown. Aim of the study was to study the role of NEU1 and NEU2 in platelet signalling. Membrane association of platelet attached glycans, NEU1 and NEU2 was measured following activation with agonists using flow cytometry. Adhesion on fibrinogen, aggregation and fibrinogen-binding were assessed with/without the NEU-inhibitor, 2-deoxy-2-3-dide-hydro-*N*-acetylneuraminic acid. Cellular localisation of NEU1 and NEU2 was examined by fluorescence microscopy. Desialylation occurred following GPIbα-clustering by VWF. Basal levels of membrane NEU1 were low; glycoprotein Ibα-clustering induced a four-fold increase (n=3, $P<0.05$). Inhibition of $\alpha_{IIb}\beta_3$-integrin prevented the increase in NEU1 membrane-association by ~60%. Membrane associated NEU2 increased two-fold (n=3, $P<0.05$) upon VWF-binding, while inhibition/removal of GPIbα reduced the majority of membrane associated NEU1 and NEU2 (n=3, $P<0.05$). High shear and addition of fibrinogen increased membrane NEU1 and NEU2. NEU-inhibitior prevented VWF-induced αIIbβ3-integrin activation by 50% (n=3, $P<0.05$), however, promoted VWF-mediated agglutination, indicating a negative feedback mechanism for NEU activity. NEU1 or NEU2 were partially co-localised with mitochondria and α-granules respectively. Neither NEU1 nor NEU2 co-localised with lysosomal-associated membrane protein 1. These findings demonstrate a previously unrecognised role for NEU1 and NEU2 in GPIbα–mediated and $\alpha_{IIb}\beta_3$-integrin signalling.

Introduction

Glyocoprotein Ibα (GPIbα), part of the GPIb-V-IX-complex, binds to von Willebrand Factor (VWF), initiating platelet adhesion to the endothelium following vascular injury. GPIbα is heavily glycosyslated,[1] with both O-[2] and *N*-linked glycans.[3] When fully assembled, *N*-linked glycans are complex, branched carbohydrates, capped by sialic acid.[3] The majority of O-linked structures on GPIbα are core 2 and also capped by sialic acid.[4]

Sialic acid can be cleaved from platelet glycoproteins under various conditions, known as desialylation. Desialylation is important for the clearance of senescent platelets.[5] Desialylation also occurs following cold-storage of platelets, which also triggers GPIbα-clustering, resulting in rapid platelet clearance by liver phagocytes.[6]Additionally, desialylation is linked to platelet activation[7] and intrinsic apoptosis.[8,9] In the bleeding disorder immune thrombocytopenia (ITP), platelets are also desialylated and hyper-activated, resulting in clearance by the liver.[10,11]

Desialylation is mediated by neuraminidases (NEU), of which four have been described in mammalian cells,[12] NEU1, NEU2, NEU3 and NEU4. NEU desialylate their substrates at the α2,6 and/or the α2,3 glycan-linkages.[13] NEU differ in their intracellular location as well as their substrate specificity. NEU1 is typically located

within lysosomes and cleaves oligosaccharides and glycopeptides. NEU2 and NEU3 are specific for gangliosides and located in the cytosol and on the plasma membrane respectively,[14,15] whereas NEU4 is located within mitochondria[16] and cleaves all aforementioned substrates. Sialic acid is also present in mitochondria.[17]

Within secretory lysosomes NEU1 is complexed with other degradation enzymes including sulphate 6-sulphatase, β-galactosidase and cathepsin A.[13] NEU are involved in many cell signalling processes: NEU1 binds Toll-like receptors,[18] negatively regulates lysosomal exocytosis[19] and suppresses cell adhesion by interfering with integrin phosphorylation, ERK1/2 and matrix metalloproteinase-7 signalling.[20] NEU3 also interacts with $\alpha_6\beta_4$-integrin, inducing ERK signalling.[21]

Earlier studies have shown upregulation of membrane-associated NEU1 in platelets following cold-storage,[22] and in ITP-patients where anti-GPIbα antibodies were present.[10] An association between sialic acid and platelet activation has been previously observed,[23] whereby surface sialic acid increases following stimulation of platelets by ADP, thrombin and collagen. Additionally, sialic acid is cleaved off the platelet membrane following GPIbα-clustering after binding its ligand VWF.[24] Addition of a NEU inhibitor, 2-deoxy-2-3-didehydro-N-acetylneuraminic acid (DANA), prevents clustering, indicating a potential role for desialylation in GPIbα-mediated platelet activation. While low levels of NEU activity have been demonstrated in platelets,[25] the role of NEU1-4 in the haemostatic function of platelets has not been studied. The aim of this study was therefore to investigate the role of both NEU1 and NEU2 in general platelet function under physiological conditions.

Methods

Ethics

Ethics approval was obtained from Australian Red Cross Blood Service Ethics Committee prior to conducting this study.

Platelet incubations

See the *Online Supplementary Material and Methods* (reagent sources, concentrations and further experimental details). Platelet rich plasma (PRP)/platelets were isolated[10] from whole blood, collected in sodium citrate, diluted ($200x10^9$/L with non-autologous apheresis-derived plasma/HEPES-Tyrode's buffer [Tyrode's] respectively), stimulated with agonists ± inhibitors (Table 1).[7]

Glycan binding lectins

Washed platelets/PRP/apheresis platelets were incubated with fluorescein-conjugated lectins (5 μg/mL), *Ricinus Communis* Agglutinin-1 (RCA-1, 1/500) and Wheat Germ Agglutinin (WGA, 1/1000) to assess galactose/sialic acid exposure respectively by flow cytometry (20 min, 21°C, BD FACSCAnto II, FACS Diva software, San Diego, CA, USA.).

Membrane NEU expression

PRP/washed platelets (± agonists/inhibitors) were diluted 1/2, stained with anti-NEU1, anti-NEU2 or anti-NEU4 (1/60, 30 min at 21°C) followed by anti-goat A488 or anti-rabbit A647 (1/60, 30 min, 21°C) antibodies respectively. Platelets were fixed (1% paraformaldehyde [PFA] prior to flow cytometry. Single platelets were gated; doublets and small aggregates were excluded.

Platelet activation markers

To assess $\alpha_{IIb}\beta_3$-integrin activation, PAC-1-FITC (neat, $2x10^6$ platelets), anti-fibrinogen-FITC was added (1/50) to 50 μL of $2x10^6$/L platelets (15 min, 21°C). Washed platelets were stimulated (VWF+ristocetin; VWF/risto), stained with anti-lysosomal-associated membrane protein 1 (LAMP-1, 1/50, 45 min, 21°C) and anti-mouse A488 or P-selectin-PE (45 min, 21°C).

Aggregation of washed platelets

Aggregation or agglutination (VWF/risto) was performed with indicated agonists using an AggRAM aggregometer (Helena Laboratories, Beaumont, TX, USA), stirring at 600 rpm.

NEU-activity

Activity of NEU in apheresis plasma (1/8 and 1/32 diluted in MQ $H_2$0) was measured using an (adapted) protocol provided by C.A. Foote (Dalton Cardiovascular Research Center and[26], *Online Supplementary Materials and Methods*). Activity of recombinant NEU was measured by Amplex Red Sialidase kit (recNEU, 2.5-40 mU/mL, diluted in reaction buffer) ± fibrinogen, collagen and D-dimer (500 μg/mL). Fluorescence was measured after 30 min, (excitation λ_{ex}=530nm, emission λ_{em}=590nm).

Adhesion of platelets to fibrinogen

Glass coverslips were coated (100 μg/mL fibrinogen 2 hours [h], 21°C) and pre-blocked (1% BSA/PBS, 1 h, 21°C). PRP±DANA (1 mM, 15 min, 37°C), prior to adhesion ($30x10^9$/L; 30 min, 37°C). Platelets were fixed (2% PFA, 20 min, 21°C), permeabilised (0.5% Triton X-100) and blocked (5% BSA) prior to phalloidin-CF594 (1/42; 20 min, 21°C) staining. Fields of view (FOV) were analysed (ImagePro Premier v9.2, Media Cybernetics, Rockville, MD, USA).

Intracellular localisation of NEU1 and NEU2

Washed platelets (±VWF/risto, 5 min, 37°C) were fixed (1% PFA, 15 min, 21°C), unreacted aldehyde was neutralised (20 mM NH_4Cl-Tris) and adhered ($200x10^9$/L) to pre-coated microchannel slides (25 μg/mL laminin, 1 h, 37°C). Platelets were permeabilised (0.1% Triton X-100, 10 min, 21°C), blocked (1% BSA, 1 h, 21°C) prior to antibody staining (*Online Supplementary Materials and Methods*). Secondary anti-mouse A488 (1/2000), anti-rabbit A647 (1/800, 1 h, 21°C) and non-fading mounting media were added. Images (FITC and Texas Red filters) were captured with 60X water objective, additional 1.6X optical zoom (10X ocular; 960X total magnification) on Olympus IX71 (Olympus Corporation, Tokyo, Japan) inverted microscope (DP71 CCD camera). All images were taken with the same exposure time across treatments, with exception of NEU2 (1/2 exposure time), due to high fluorescence post-stimulation.

Statistics

Data were analysed using one-way ANOVA or paired *t*-tests using (GraphPad Software, Inc version 7.05.). A *P*-value of <0.05 was considered to be significant.

Results

Desialylation in platelets in presence of plasma

Platelets were stimulated with different agonists to investigate desialylation and cleavage of other glycans, using glycan-binding lectins and flow cytometry. Activation of GPIbα only by VWF (risto), but not ADP, increased desialylation by more than two-fold compared to unstimulated controls, as deduced from RCA-1-bind-

ing (to underlying galactose-residues), (Figure 1A). WGA-binding (to sialic acid and GlcNAc-residues), was decreased by 25% following ristocetin addition, also indicating some desialylation (Figure 1B). Similarly, the proportion of platelets binding RCA-1 increased significantly upon ristocetin stimulation (Figure 1, Table 1).

Additionally, binding to other lectins MAL-1 and ECL (bind to exposed β-galactosidase residues),[27] PNA (binds only to underlying GalNAc-residues on the T-antigen of VWF) and SNA (binds sialic acid) was examined in washed platelets (Figure 1C). MAL-1 and ECL-binding were increased following VWF/risto (Figure 1C), which was consistent with RCA-1 binding, although the

increase was smaller (1.5-fold) when compared to over a two-fold increase on platelets in PRP (Figure 1A).

NEU1 and NEU2 are expressed on the platelet membrane

In order for desialylation to occur, it was hypothesised that the enzymes responsible, NEU1 and/or NEU2 were most likely associated with the plasma membrane. Since clustering of GPIbα specifically induced platelet desialylation, we investigated membrane association of both enzymes following addition of ristocetin. Following ristocetin-stimulation, there was a significant increase in membrane associated NEU1 and NEU2 (Figure 2A-B), demon-

Table 1. Concentrations of platelet agonists and inhibitors.

Platelet rich plasma Compound	Abbreviation	Function	Final concentration
Ristocetin	Risto	facilitates/potentiates vWF binding to GPIbα	3 mg/mL
Adenosine diphosphate	ADP	platelet agonist through binding to P2Y1 and P2Y12	200 μM
Arachidonic acid	AA	platelet agonist through formation of TXA_2	800 μM
O-sialoglycoprotein endopeptidase	OSGE	cleaves all O-linked glycans & GPIbα VWF-binding domain	80 μg/mL
Arg-Gly-Asp-Ser peptide	RGDS	inhibits fibrinogen binding to GPIIb/IIIa integrin	200 μM
N-acetylglucosamine	GlcNAc	inhibits GPIbα clustering	100 mM
Fibrinogen	Fg	binds GPIIb/IIIa integrin	500 μg/mL
2-deoxy-2-3-didehydro-N-acetylneuraminic acid	DANA	neuraminidase inhibitor	1 mM
Calcium chloride	$CaCl_2$	recalcification	1 mM
Collagen	coll	platelet agonist through binding to GPVI	10 μg/mL

Washed platelets Compound	Abbreviation	Function	Final concentration
Ristocetin + von Wilebrand factor	Risto + VWF	Platelet stimulation through activation of GPIbα	1.2 mg/mL + 10 μg/mL
Adenosine diphosphate	ADP	Platelet agonist through binding to P2Y1 and P2Y12	20 μM
Arachidonic acid	AA	Platelet agonist through formation of TXA_2	50 μM
O-sialoglycoprotein endopeptidase	OSGE	Cleaves all O-linked glycans & GPIbα VWF-binding domain	80 μg/mL
Peptide:N-glycosidase F	PNGAse F	Cleaves all N-linked glycans	10,000 U/mL + 1/10 reaction buffer
2-deoxy-2-3-didehydro-N-acetylneuraminic acid	DANA	Neuraminidase-inhibitor	1 mM
Collagen	coll	Platelet agonist through binding to GPVI	10 μg/mL
(Z)-7-[(1S,4R,5R,6S)-5-[(E,3S)-3-hydroxyoct-1-enyl]-3-oxabicyclo[2.2.1]heptan-6-yl]hept-5-enoic acid	U46619	TxA_2 mimetic	1 μM
α-thrombin	thr	Platelet stimulation through activation of GPIbα (low-dose only)	0.1 U/mL
1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester)	BAPTA-AM	Calcium chelator	10 μM
Indomethacin	indo	Blocks TXA_2 formation;	
Cyclo-oxygenase-1 inhibitor	30 μM		
Apyrase		ADP inhibitor: hydrolyses ADP	0.1 U/mL
Calcium chloride	$CaCl_2$	recalcification	1 mM
Fibrinogen	Fg	binds GPIIb/IIIa integrin	500 μg/mL
Monosialodihexosylganglioside	GM3	ganglioside; NEU2 substrate and involved in GPIbα clustering	10 μM
Recombinant neuraminidase (Clostridium)	recNEU	desialylation enzyme	0.2 U/mL

All agonists and metabolic inhibitors used for stimulation or inhibition of platelet rich plasma (PRP) or washed platelets respectively were used at final concentrations as indicated. More details and abbreviations are described in the *Online Supplementary Materials and Methods*.

strating that in platelets from healthy individuals, a proportion of NEU1 and NEU2 is membrane expressed. Secondary antibody-only controls showed little non-specific fluorescence (Figure 2A-B). Blockade of GPIbα-clustering with GlcNAc[28] prevented the increase in NEU1 and NEU2 membrane association.

In addition, removal of the 45 kDa *N*-terminal domain of GPIbα (VWF-binding domain) with OSGE, to mimic GPIbα-deficient platelets,[29,30] prevented the ristocetin-induced increase in NEU1 (Figure 2A-B), suggesting that NEU1 expression is highly dependent on VWF-binding to GPIbα. Furthermore, when fibrinogen binding to α$_{IIb}$β$_3$-integrin was blocked using RGDS peptide, the increase in membrane NEU1 was also significantly reduced by 50% (Figure 2A), and there was also a trend towards decreased NEU2 expression (Figure 2B). Similarly, the proportion of platelets that were positive for NEU1 and NEU2 increased in PRP stimulated with ristocetin, but these changes were not significantly different when compared to unstimulated platelets or those treated with inhibitors (Figure 2C).
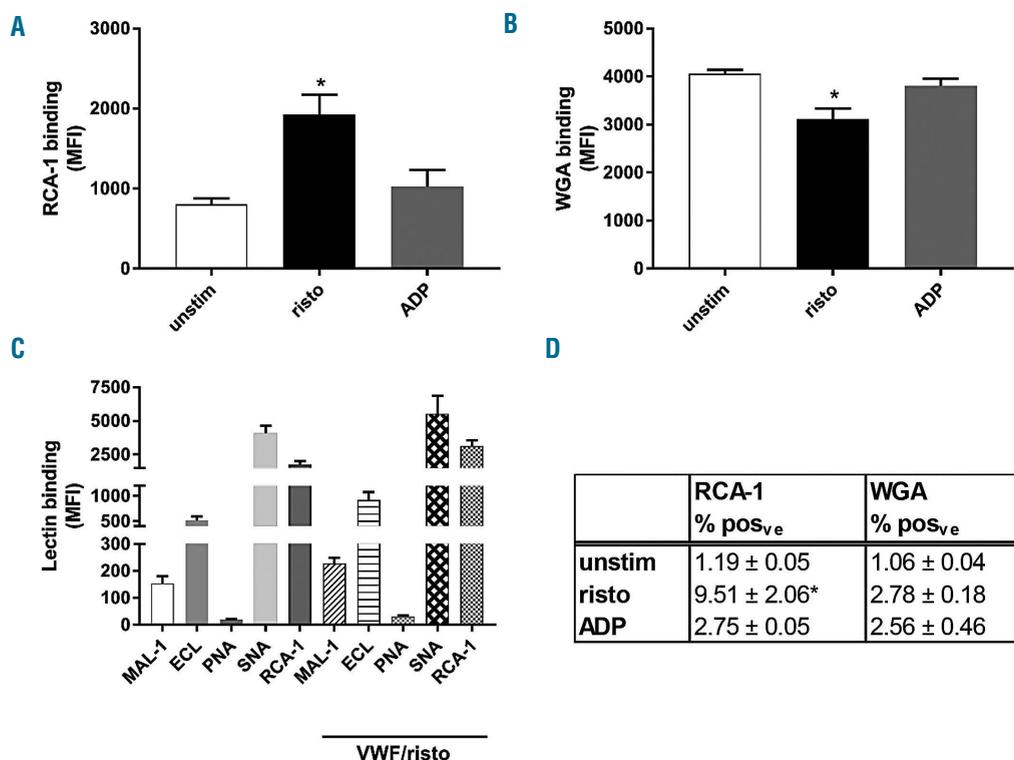
Desialylation in washed platelets

Washed platelets were used to prevent interference by plasma proteins such as fibrinogen. Cleavage of the extracellular domain of GPIbα by OSGE to mimic GPIbα-deficient platelets,[31] reduced binding of all lectins by over 50% and below unstimulated control (*Online Supplementary Figure S1A*), demonstrating the importance of the VWF-binding domain in NEU mediated desialylation. When only *N*-linked glycans were cleaved by PNGase F, binding to most lectins was also reduced, albeit to a lesser degree, indicating desialylation of N-linked glycans (*Online Supplementary Figure S1A*).

Recombinant NEU (recNEU), which desialylates sialic acid at α2,3, α2,6, or α2,8-linkages was used as a positive control to achieve complete desialylation. RecNeu alone significantly increased binding of MAL-1, ECL and PNA (not shown). RecNEU treatment further increased RCA-1 binding (*Online Supplementary Figure S1B*), when compared to VWF/risto alone (Figure 4D), albeit to a lesser extent than ECL-binding. PNA-binding was significantly increased (*Online Supplementary Figure S1B*), demonstrating potential desialylation of VWF itself. SNA-binding however was insensitive to VWF/risto+recNEU, indicating different and more extensive desialylation by recNEU (*Online Supplementary Figure S1A*). As a negative control, washed platelets incubated with ristocetin only, which showed no increase in membrane NEU1 and NEU2 (*Online Supplementary Figure S1C*).

To further confirm the role of GPIbα-clustering in translocation of NEU to the membrane, washed platelets were activated with various agonists (VWF/risto, collagen, thrombin, AA, ADP and U46619). Only clustering of GPIbα by VWF/risto, and to a lesser extent, arachidonic

	RCA-1 % pos$_{ve}$	WGA % pos$_{ve}$
unstim	1.19 ± 0.05	1.06 ± 0.04
risto	9.51 ± 2.06*	2.78 ± 0.18
ADP	2.75 ± 0.05	2.56 ± 0.46

Figure 1. Platelet desialylation is induced by von Willebrand factor-mediated glycoprotein Ibα clustering. Platelets in platelet rich plasma (PRP) (200 x 10^6/mL) were stimulated with ristocetin (3 mg/mL) and ADP (200 μM), then stained with fluorescein-conjugated lectins (A) RCA-1 or (B) WGA; 10,000 single platelets (doublets and small aggregates were excluded from analysis) were measured by flow cytometry (n=3). Washed platelets were stimulated (VWF/risto: 10 μg/mL VWF/ 1.2 mg/mL ristocetin), and (C) binding to fluorescein-conjugated MAL-1, ECL, PNA, SNA and RCA-1 lectins was measured by flow cytometry (n=3). Mean fluorescent intensities (MFI) ± standard error of mean (SEM). *$P<0.05$ significant when compared to unstimulated controls (unstim) using a one-way ANOVA. (D) Lectin binding in unstimulated platelets was set at ~1% positive and samples were analysed following risto or ADP-addition. Data ± SEM. *$P<0.05$ significant when compared to unstimulated controls (t-test). VWF: von Willebrand factor; GPIbα: glycoprotein Ibα.
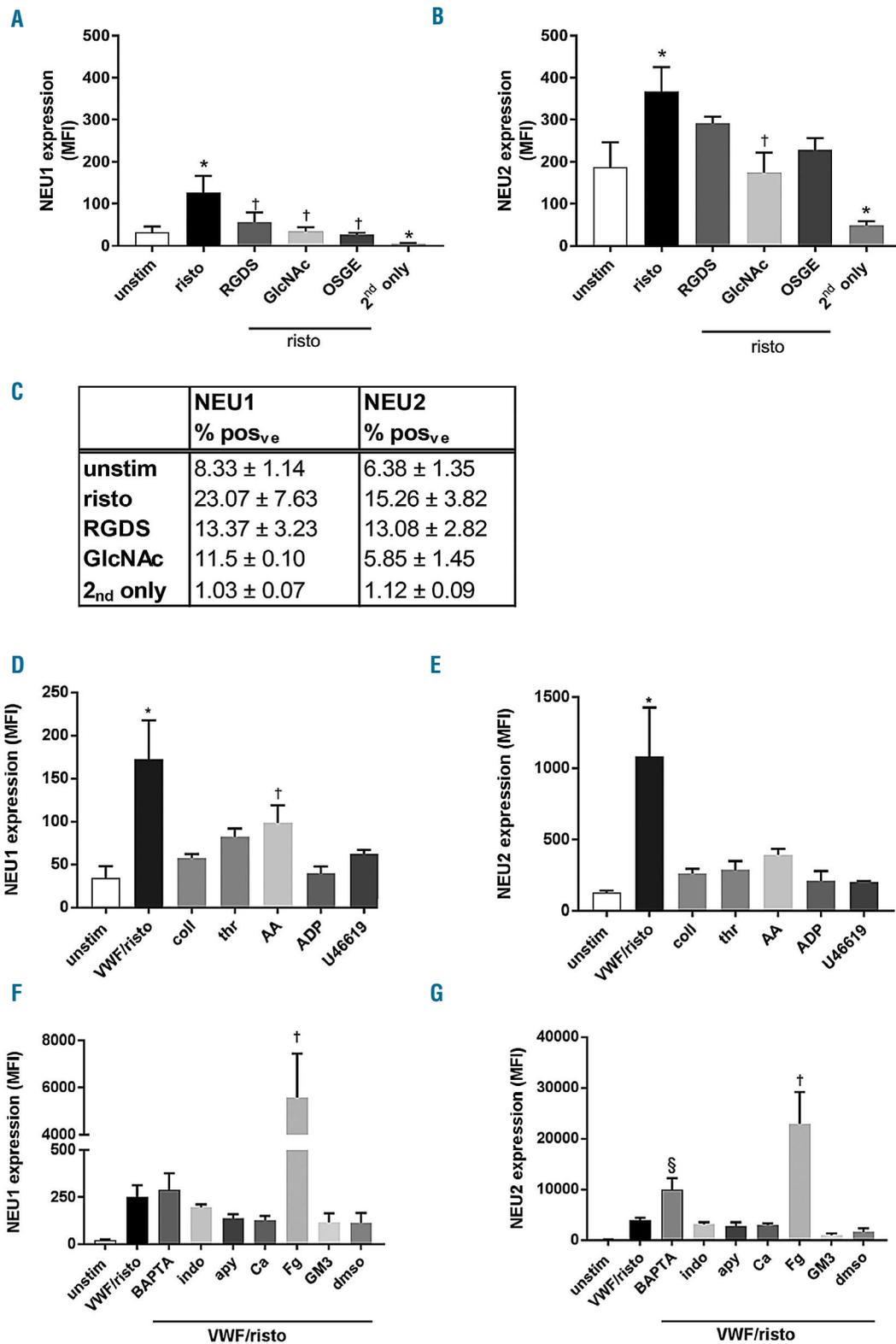
Figure 2. NEU1 and NEU2 membrane expression is mediated by von Willebrand factor-binding to glycoprotein Ibα. Platelets in platelet rich plasma (PRP) (200 x 10^6/mL) were pre-incubated with RGDS peptide (Arg-Gly-Asp-Ser, 200 μM), *N*-acetyl-*D*-glucosamine (GlcNAc, 100 mM) and *O*-sialo-glycoprotein endopeptidase (OSGE, 80 μg/mL) *prior* to ristocetin stimulation (3 mg/mL) and membrane association of (A) NEU1 and (B) NEU2 were measured by flow cytometry using NEU1 or NEU2 antibodies followed by fluorescently conjugated secondary antibodies. *P<0.05 was considered significant when compared to unstimulated (unstim)*, †: significant against risto (one-way ANOVA, n=3). (C) Secondary antibody-only controls (unstim platelets) were used to set a gate (~1%) and everything above this was considered positive (% pos_ve). Washed platelets were stimulated with VWF/risto (10 μg/mL VWF+ 1.2 mg/mL risto), collagen (10 μg/mL), thrombin (0.1 U/mL), AA (50 μM), ADP (20 μM), and U46619 (1 μM) *prior* to measurement of (D) NEU1 (n=4) or (E) NEU2 (n=4) membrane association by flow cytometry. *Prior* to VWF/risto stimulation, platelets were treated with the indicated inhibitors (n=4) for calcium (BAPTA-AM, 10 μM), TXA₂ (indomethacin, indo, 30 μM) and ADP (apyrase, 0.1 U/mL). As indicated calcium (1 mM), fibrinogen (500 μg/mL), GM3 (10 μM) were also used. (F) NEU1 or (G) NEU2 membrane association was measured. Results are shown as mean fluorescent intensities (MFI) ± standard error of mean (SEM). *P<0.05 significant against unstimulated (unstim); †: significant against VWF/risto, §: significant against vehicle (dmso, one-way ANOVA); VWF: von Willebrand factor; GPIbα: glycoprotein Ibα.

acid, increased membrane association of NEU1 (Figure 2D) and was even more pronounced for NEU2 (Figure 2E). Although collagen binds to VWF upon vascular injury, it did not induce NEU membrane translocation. AA also significantly increased NEU1, which is in line with earlier findings that AA induced clustering of GPIbα, which may lead to subsequent desialylation (Figure 2D).[32] The VWF-induced increase in membrane NEU was less pronounced in PRP (Figure 2A-B) when compared to washed platelets (Figure 2D-E), suggesting a potential inhibitory effect of plasma proteins on NEU activation. Risto-only controls (without VWF-addition) did not induce NEU membrane expression (*Online Supplementary Figure S1C*). GPIbα is also clustered by low concentrations of thrombin,[33] however thrombin-stimulated washed platelets were not desialylated (*Online Supplementary Figure S1D*). Binding of all lectins was similar to unstimulated controls; demonstrating desialylation is specific for the VWF-GPIbα interaction. This data demonstrate that desialylation occurs upon specific clustering of GPIbα through binding to its ligand VWF, which in turn triggers membrane association of NEU1 and NEU2.

Signalling pathways involved in NEU expression

To further investigate the signalling pathways involved in NEU membrane association, platelets were incubated with metabolic inhibitors prior to VWF/risto-stimulation (Figure 2F-G). A negative feedback role of calcium was demonstrated as calcium-chelation by BAPTA-AM significantly increased NEU2 membrane-association (Figure 2G), while addition of calcium slightly decreased NEU2 (Figure 2G). GPIbα-clustering is known to trigger AA-release, leading to thromboxane (TX) A_2-formation.[34] Inhibition of TXA_2-formation with indomethacin reduced NEU2 membrane association slightly, as did apyrase, which hydrolyses ADP (Figure 2G). In contrast, addition of fibrinogen significantly increased membrane association of both NEU1 and NEU2 (Figure 2F-G) following VWF/risto-stimulation, demonstrating an important role for fibrinogen. Gangliosides (GM) (GM3: sialic acid-α2,3Galβ1,4Glcβ1,1Cer, a known substrate for NEU2) are able to inhibit platelet adhesion and aggregation[35] and bind to GPIbα following its clustering. Incubation of platelets with GM3 reduced membrane association of NEU2, and to a lesser extent, NEU1 (Figure 2F-G).

As the GPIbα-VWF interaction is highly dependent on shear stress, apheresis platelets were stimulated with ristocetin in combination with shear. NEU1 and NEU2 membrane association were both significantly higher when risto-stimulated platelets were subjected to high shear of $10,000s^{-1}$ (Figure 3A-B), confirming the link between GPIbα-signalling and NEU-translocation. At this stage, we started to look into NEU4, as this might also have a role in platelets as it cleaves gangliosides.[16] NEU4 was also membrane-associated following VWF/risto and increased further by shear, but not significantly (Figure 3C). Shear alone did increase NEU1 slightly, but did not affect NEU2 or NEU4 membrane-association (Figure 3A-C).

The data so far demonstrates that NEU1, NEU2 and even NEU4, may be released from their intracellular stores upon GPIbα-clustering, as granule/lysosome content may also be released.[36] In general, VWF/risto-stimulation without shear does not induce secretion in washed platelets.[27] However, since the indirect NEU staining protocol used for flow cytometry involved an incubation time of 90 min,

which might potentiate α-/δ-granule/lysosome-release, these were examined following VWF/risto-stimulation. Both P-selectin and LAMP-1 surface expression following VWF/risto-stimulation were increased as a consequence of the longer incubation times (*Online Supplementary Figure*
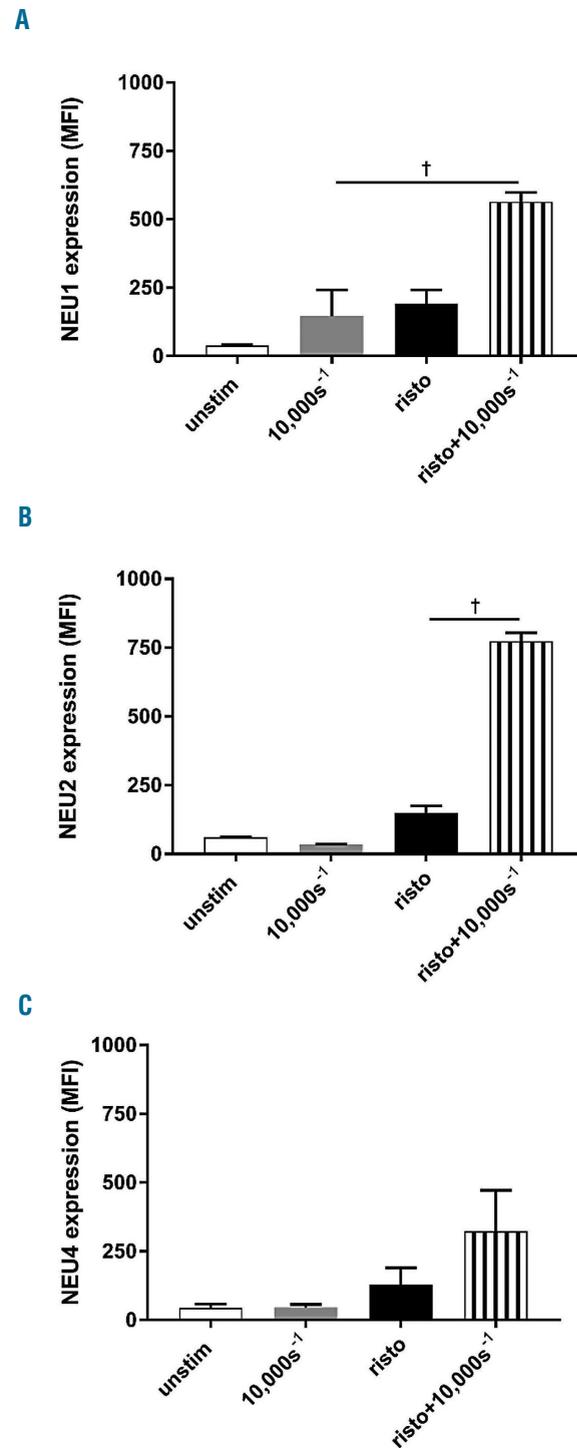
Figure 3. NEU1, NEU2 and NEU4 are membrane expressed following high shear. Apheresis platelets were treated with risto, high shear ($10,000s^{-1}$) or both and membrane expression of (A) NEU1 and (B) NEU2 were measured by flow cytometry. †Significant against shear or risto-only. *$P<0.05$ significant against unstimulated (unstim), one-way ANOVA. (C) NEU4 was measured by flow cytometry. Results are shown as mean fluorescent intensities (MFI) ± standard error of mean (SEM).

S2A-B). RecNEU significantly enhanced LAMP-1 surface expression, P-selectin and PAC-1 binding relative to unstimulated platelets (*Online Supplementary Figure S2A-C*). Fibrinogen also potentiated LAMP-1 (*Online Supplementary Figure S2A*) when compared to VWF/risto-stimulation alone, while PAC-1 binding (*Online Supplementary Figure S2C*) was slightly decreased, as expected. As anticipated, addition of calcium increased PAC-1 and P-selectin (*Online Supplementary Figure S2B-C*), whereas OSGE abolished the VWF-induced increase in LAMP-1 and PAC-1-binding (*Online Supplementary Figure S2A, C*) indicating that dense granule/lysosome secretion and fibrinogen-binding do not occur when GPIbα is removed.

In summary, these findings demonstrate that NEU translocate to the plasma membrane following clustering of GPIbα by VWF, and NEU2 membrane expression is negatively controlled by high calcium concentrations. More importantly, fibrinogen-binding following platelet activation by VWF potentiated the NEU-translocation.

The role of NEU-activity in αIIbβ3-integrin activation

The data presented thus far demonstrate that NEU1 and NEU2 are specifically translocated to the membrane following VWF-mediated GPIbα-clustering, and is downstream of secondary signalling, leading to desialylation. To further investigate a potential role for NEU activity in platelet activation, a NEU-inhibitor DANA[10,22] was used. DANA inhibited desialylation, as RCA-1-binding was significantly decreased (approximately two-fold decrease), but not completely inhibited, following recNEU-treatment (*Online Supplementary Figure S3*).

Since our findings indicated that fibrinogen binding potentiated the membrane association of NEU1 in particular, the role of NEU activity in αIIbβ3-integrin activation was further studied using PAC-1 and fibrinogen antibodies. Incubation of PRP with DANA prior to addition of ristocetin significantly reduced fibrinogen binding (Figure 4A), suggesting NEU activity plays a role in fibrinogen binding. Furthermore, the partial inhibition of fibrinogen binding by RGDS was not observed in the presence of
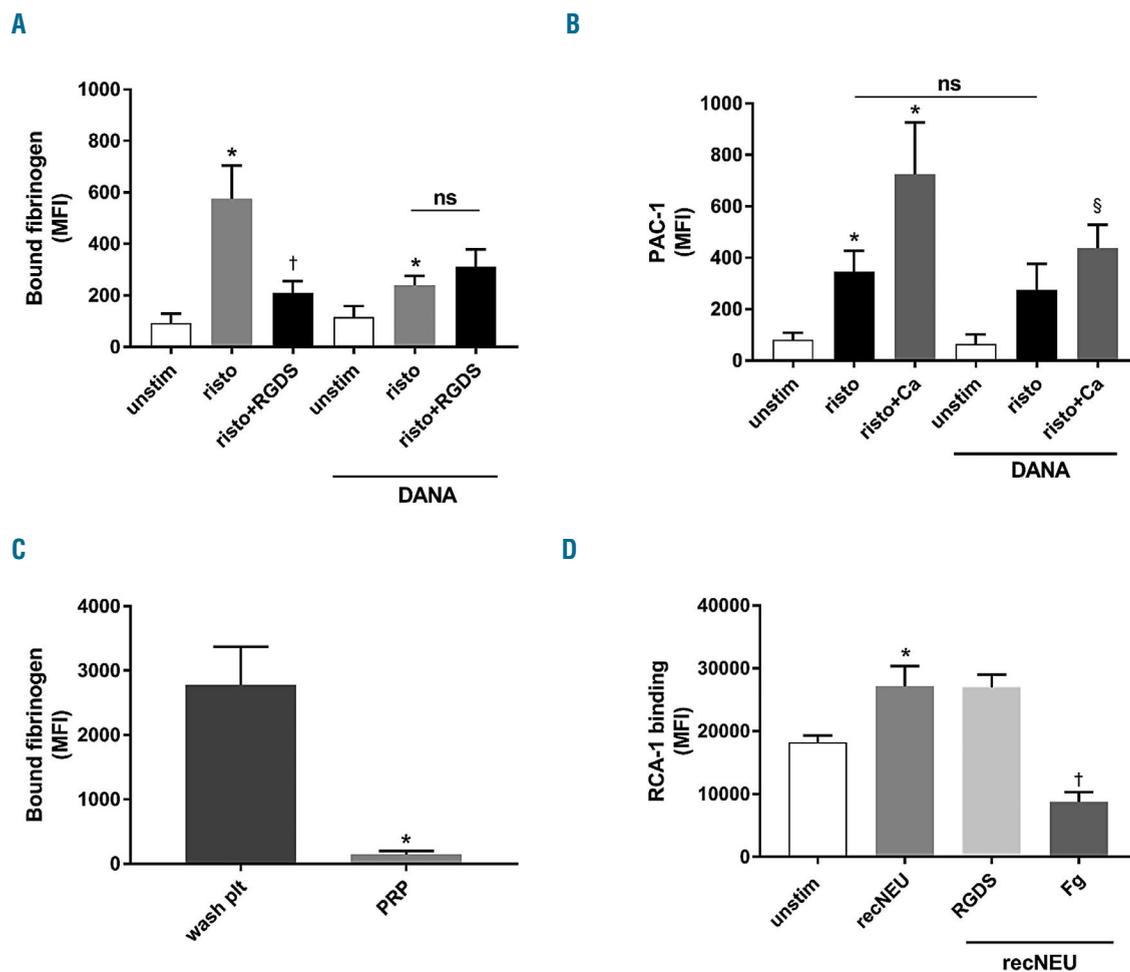
Figure 4. Neuramidase-inhibition reduces fibrinogen binding. Platelets in platelet rich plasma (PRP) were pre-incubated with neuramidase (NEU) inhibitor 2-deoxy-2-3-didehydro-N-acetylneuraminic acid (DANA) and/or +/- αIIbβ3 integrin inhibitor RGDS prior to stimulation with ristocetin (3 mg/mL) and then stained with (A) fibrinogen-FITC or (B) FITC-conjugated PAC-1 antibodies respectively. 10,000 single platelets were measured by flow cytometry. Data represents the mean fluorescent intensities (MFI) ± standard error of mean (SEM), n=3. *P<0.05, *: significant against unstimulated (unstim), §: significant against risto + calcium (Ca) (one-way ANOVA). (C) Unstimulated washed platelets (wash plt) or PRP were stained with fibrinogen-FITC antibody. (D) PRP was treated with RGDS, or fibrinogen, prior to recNEU-treatment (Table 1), then stained with fluorescein-conjugated RCA-1 (n=5). Data represents mean ± standard error of mean (SEM). *P<0.05 significant against unstimulated (unstim), †: significant against risto using a one-way ANOVA.

DANA, suggesting potential competition for the same binding site by NEU and RGDS, further linking $\alpha_{IIb}\beta_3$ activation and NEU activity. Cations are essential for complete fibrinogen-binding to $\alpha_{IIb}\beta_3$.[37] Ristocetin induced PAC-1 binding and was only sensitive to DANA-inhibition upon addition of calcium (Figure 4B). Similarly, PAC-1 binding was also further increased by calcium addition, confirming the importance of calcium in NEU activity (Figure 4B). In contrast, ADP-induced PAC-1 binding was unaffected by DANA treatment, demonstrating again that NEU activity is GPIbα-VWF specific (data not shown). Basal levels of fibrinogen binding in washed platelets were much greater than in PRP (Figure 4C), indicating some activation had occurred that may have led to higher basal NEU1 and NEU2 membrane association. Remarkably, the recNEU induced RCA-1 binding was significantly reduced by additional fibrinogen, due to saturation of $\alpha_{IIb}\beta_3$-integrin's fibrinogen binding site (Figure 4D); however fibrinogen-binding kinetics will be different during aggregation/adhesion.

These findings emphasise the importance of calcium in modulating surface bound NEU expression following GPIbα-clustering. This facilitates $\alpha_{IIb}\beta_3$ activation, which in turn potentiates NEU activity and is downregulated again by fibrinogen-binding.

NEU-activity and platelet aggregation

To further examine GPIbα-mediated signalling without plasma in a buffered system, washed platelets were stimulated by VWF/risto and agglutination was measured. DANA treatment increased agglutination, which was further increased by addition of fibrinogen (Figure 5A). When binding of fibrinogen to platelets was blocked with RGDS, agglutination was only slightly reduced. These data indicate an inhibitory role of NEU activity in VWF-mediated agglutination. Additionally, DANA treatment increased fibrinogen-binding to $\alpha_{IIb}\beta_3$ (Figure 5B). To investigate the role of NEU in other activation pathways, washed platelets were pre-incubated with DANA prior to addition of collagen and AA. DANA had no effect on platelet aggregation in response to these agonists (Figure 5C). Static adhesion and spreading of platelets to a fibrinogen coated surface was also unaffected by DANA (Figure 5D). It is important to note that additional platelet adhesion receptors and mechanisms are involved in platelet adhesion when compared to platelets in suspension. As fibrinogen binding appeared to be linked with NEU-activity, recNEU was incubated with fibrinogen and in line with the previous results; fibrinogen enhanced the activity of recNEU (Figure 5E). In contrast, when using control proteins of similar molecular weights, NEU-activity was completely abolished by collagen, while D-dimer showed inhibition by ~50% (Figure 5E). NEU activity in plasma (n=4) was 187.47±22.81 mU/mL (1/32 dilution), while only 84.28±11.26 mU/mL was found when a dilution of 1/8 was used, indicating an inhibitory effect by plasma factors. The maximum platelet activity of 80 mU/mL was reached following platelet permeabilisation by Triton X-100: using 400×10^6/mL platelets. When NEU activity was measured without Triton X-100 (Amplex Red assay), only 35.45±3.51 mU/mL (1/8 dilution), which was ~40%.

Intracellular NEU localisation

It was hypothesised that in order for NEU to cleave their substrates, the enzymes would need to be localised on or within the platelet membrane. Previous findings in cold-stored[22] or platelets from ITP-patients[10] showed that intracellular stores of NEU1 appear to be localised in 'granule'-like organelles; however the actual location was not shown. Therefore, the intracellular origin of NEU was investigated. In permeabilised unstimulated platelets, NEU1 stained in a punctate pattern within the cytoplasm and on the cellular periphery, while NEU2 staining was mostly cytoplasmic and punctate (Figure 6A-B). NEU1 did not co-localise with the lysosomal/δ-granule markers LAMP-1 and β-galactosidase as initially anticipated, nor with an α-granule markers coagulation factor V (FV) (Figure 6A) and P-selectin (Figure 6C). Upon further investigation, NEU1 did however appeared to co-localise with mitochondria to a limited extent (Figure 6A) in approximately 20% of permeabilised platelets. Within these, 10-100% of NEU1 co-localised with mitochondria, whereas the remaining NEU1 was sequestered in other locations. Mitochondria did not stain in unstimulated and stimulated non-permeabilised platelets (*Online Supplementary Figure S4*), while granule and lysosome contents were released following VWF/risto incubation (*Online Supplementary Figure S2A*), in line with the flow cytometry data. Although mitochondria are potentially releasing NEU1, the mitochondrial protein was not retained on the platelet membrane (Figure 6A). NEU2 staining was mostly cytoplasmic and punctate (Figure 6B). Following GPIbα activation (stimulated), NEU2 surface localisation was significantly enhanced. Difficulties were encountered visualising this localisation and due to the large increase in fluorescence (Fig. 6B), the exposure time had to be halved. As with NEU1, NEU2 failed to co-stain with LAMP-1 (data not shown). In contrast to NEU1, NEU2 co-localised with P-selectin (Figure 6C), which is in line with results using DANA, whereby DANA partially reduced expression of P-selectin following ristocetin-stimulation (Figure 6D).

When using a general membrane dye (Figure 6E), some co-localisation was observed, although not 100%. As a control for non-specific staining, platelets were incubated with a secondary antibody only, and no fluorescence was observed (*not shown*). The overall findings from this study and a proposed model of NEU activity are presented in Figure 7.

Discussion

In this study, we have demonstrated a novel role for NEU1 and NEU2 in platelets, which is highly dependent on VWF-GPIbα and consequent $\alpha_{IIb}\beta_3$-integrin activation. Specifically, we have demonstrated that NEU1, NEU2 and NEU4 are present on the plasma membrane of unstimulated platelets. Specific clustering of GPIbα by VWF triggers increased membrane association of NEU1 and NEU2, partially from their respective intracellular stores mitochondria and α-granules, which is even more pronounced under high shear conditions. Membrane association of NEU is highly regulated by mechanisms different for NEU1 and NEU2.

GPIbα is heavily glycosylated, containing *N-* and *O-*linked glycans, capped by sialic acid. Desialylation has been studied before in cold-stored platelets and ITP.[6,10,38] The glycan changes in platelets under these conditions are similar to those observed following activation by VWF,[8,9,27,32,39] as clustered GPIbα leads to various degrees of glycan cleavage (*e.g.* sialic acid and/or galactose). To date,

the enzymes responsible for these changes, mammalian NEU (NEU1-4), have not been investigated in healthy platelets.
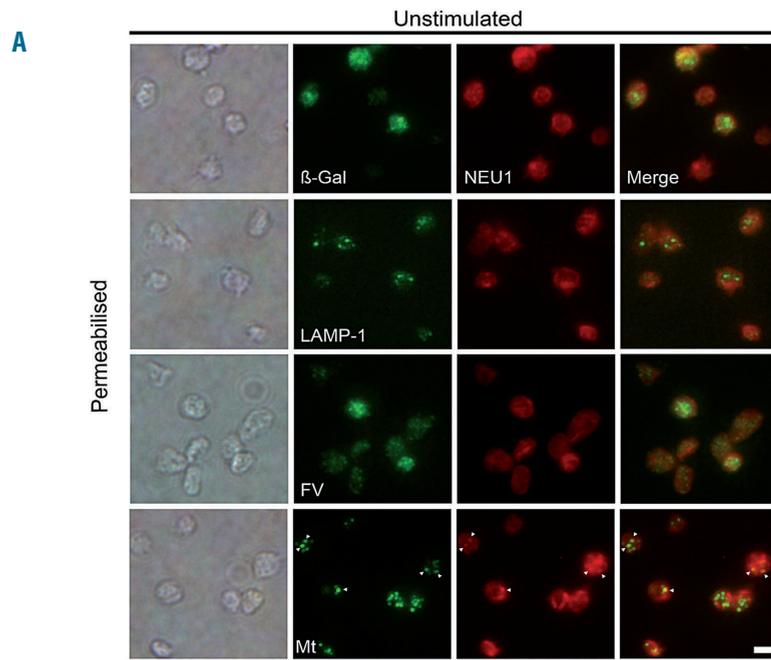
In this study, we did not discriminate between *N*- and *O*-linked glycans. Recent work has shown the importance of *N*-linked glycans in VWF-binding and its clearance.[40,41]

Additionally, *O*-linked glycans have been implicated in both VWF-clearance[42] and binding.[43]

On platelets, *N*- and *O*-linked glycans are covalently bound *via* asparagine residues and capped by sialic acid.[2-4] The T-antigen (*O*-linked (sialic acid(α2-3)Gal-(β1-3)-[sialic acid(α2-6)]GalNAc) is present on VWF.[44] *O*-linked glycans
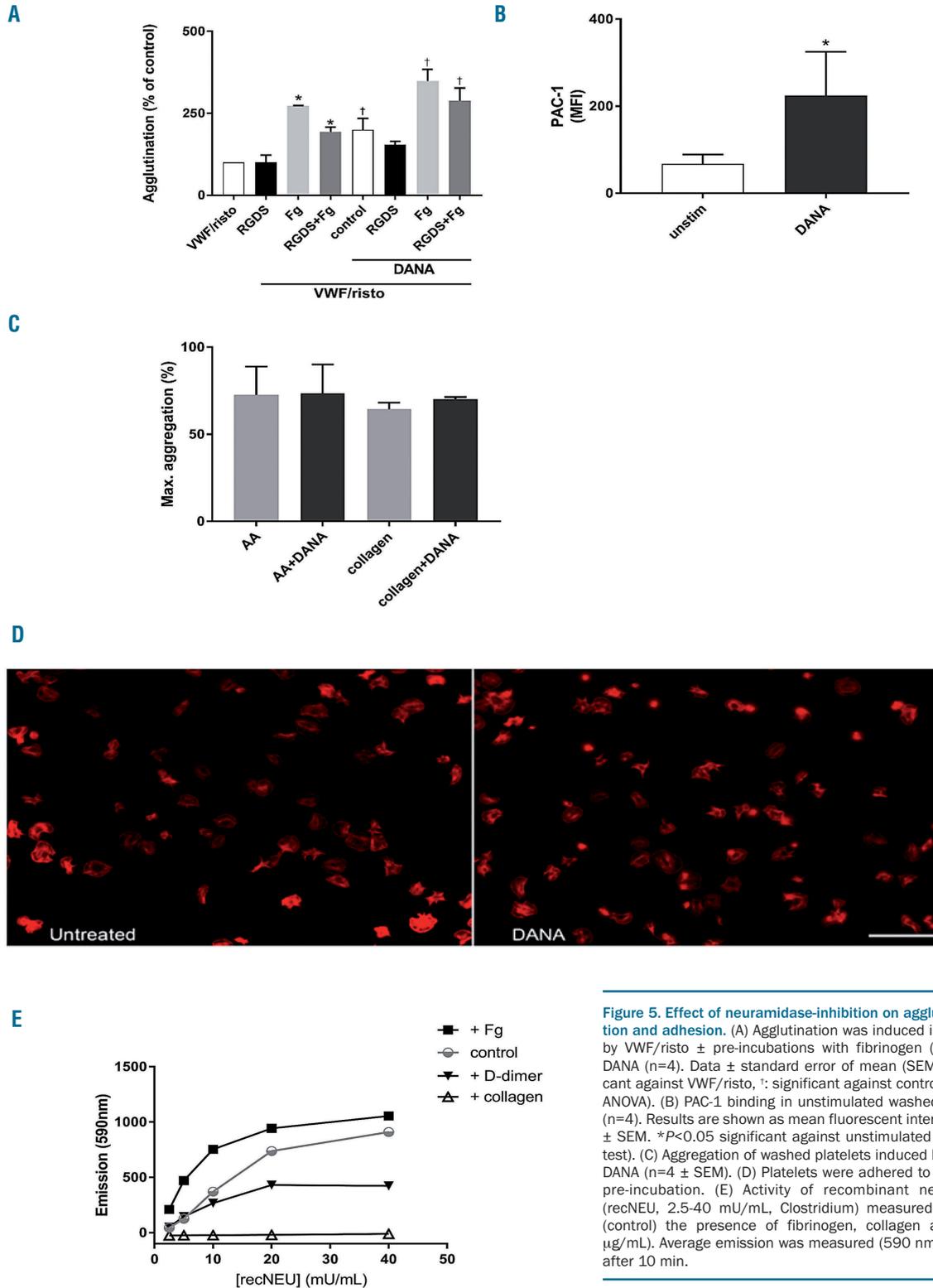
Figure 5. Effect of neuramidase-inhibition on agglutination, aggregation and adhesion. (A) Agglutination was induced in washed platelets by VWF/risto ± pre-incubations with fibrinogen (Fg), RGDS and/or DANA (n=4). Data ± standard error of mean (SEM). *P<0.05 significant against VWF/risto, †: significant against control + DANA (one-way ANOVA). (B) PAC-1 binding in unstimulated washed platelets ± DANA (n=4). Results are shown as mean fluorescent intensities (MFI) values ± SEM. *P<0.05 significant against unstimulated (unstim) (paired t-test). (C) Aggregation of washed platelets induced by AA or collagen ± DANA (n=4 ± SEM). (D) Platelets were adhered to fibrinogen ± DANA pre-incubation. (E) Activity of recombinant neuramidase (NEU) (recNEU, 2.5-40 mU/mL, Clostridium) measured with and without (control) the presence of fibrinogen, collagen and D-dimer (500 µg/mL). Average emission was measured (590 nm) at each [recNEU] after 10 min.

A Unstimulated

Permeabilised

ß-Gal | NEU1 | Merge

LAMP-1

FV

Mt

B Unstimulated Stimulated

Perm

Non-perm

NEU2

C

P-Sel | NEU1 | Merge

NEU2 | P-Sel | Merge

D

E Unstimulated Stimulated

Perm

Non-perm

NEU2 | CellBrite 640 | Merge

Figure 6. Membrane association and intracellular location of NEU1 and NEU2. NEU1 (red) and intracellular protein staining (green) for β-Galactosidase (β-Gal), LAMP-1, factor V (FV), mitochondria (Mt) were visualised by fluorescence microscopy and merged in (A) unstimulated + permeabilised washed platelets. (B) Unstimulated and stimulated (VWF + risto) washed platelets ± permeabilisation, were stained with NEU2 (green). Due to high fluorescence in stimulated platelets, ½ exposure time was used. (C) Unstimulated (non-) permeabilised washed platelets were stained for NEU1 (red), NEU2 (green) and P-selectin (P-Sel). (D) P-selectin expression was measured in unstimulated PRP *versus* risto ± DANA (n=4). Results are shown as mean fluorescent intensities (MFI) values ± standard error of mean (SEM). (E) unstimulated platelets and following VWF/risto (stimulated) were stained with NEU2 (green) and membrane dye CellBrite 640 (red). Exposure time 1/3.0 sec for unstimulated samples, and 1/6.0 sec exposure for stimulated samples due to high fluorescence. A total 960X magnification was used. Scale bar is 10 μm. VWF: von Willebrand factor.
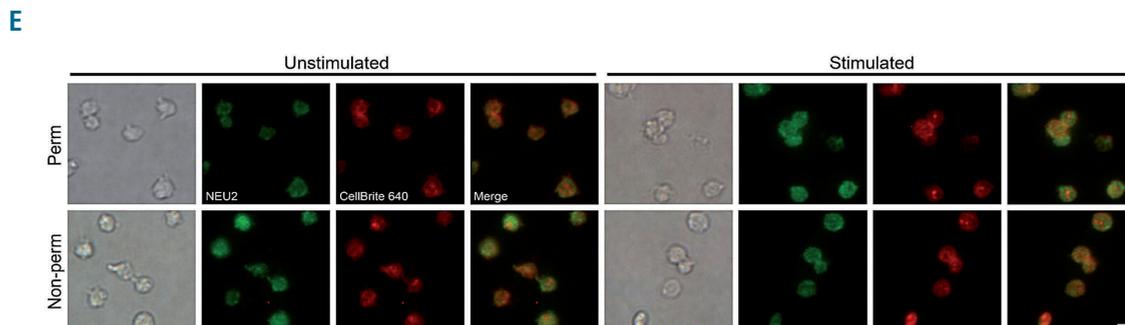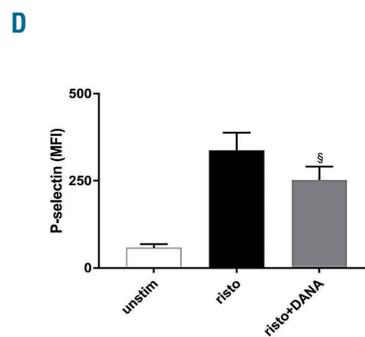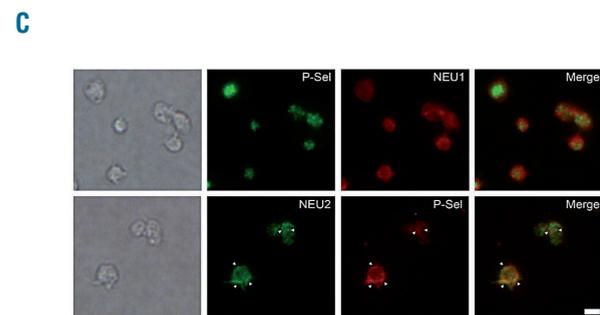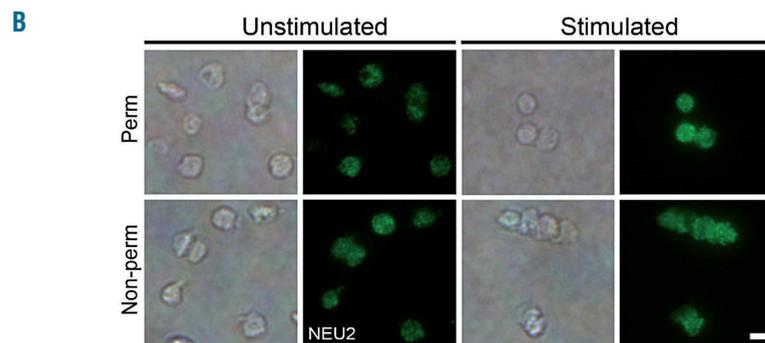
in the A1 domain of VWF are critical for binding to GPIbα.[43] When sialic acid is cleaved from *O*-linked glycan structures, galactose-residues originally bound to GalNAc and GlcNac-residues become exposed, in contrast to N-linked glycans, where sialic acid is attached only to galactose residues. Additionally, the β_3-domain of $\alpha_{IIb}\beta_3$-integrin

also contains *N*-linked glycans[45] and the majority of these structures are rich in mannose. It is currently unclear whether other platelet glycoproteins or plasma proteins (*e.g.* alpha2 macroglobulin) are affected by NEU. However platelet stimulation with other agonists did not lead to an increase in membrane-associated NEU.
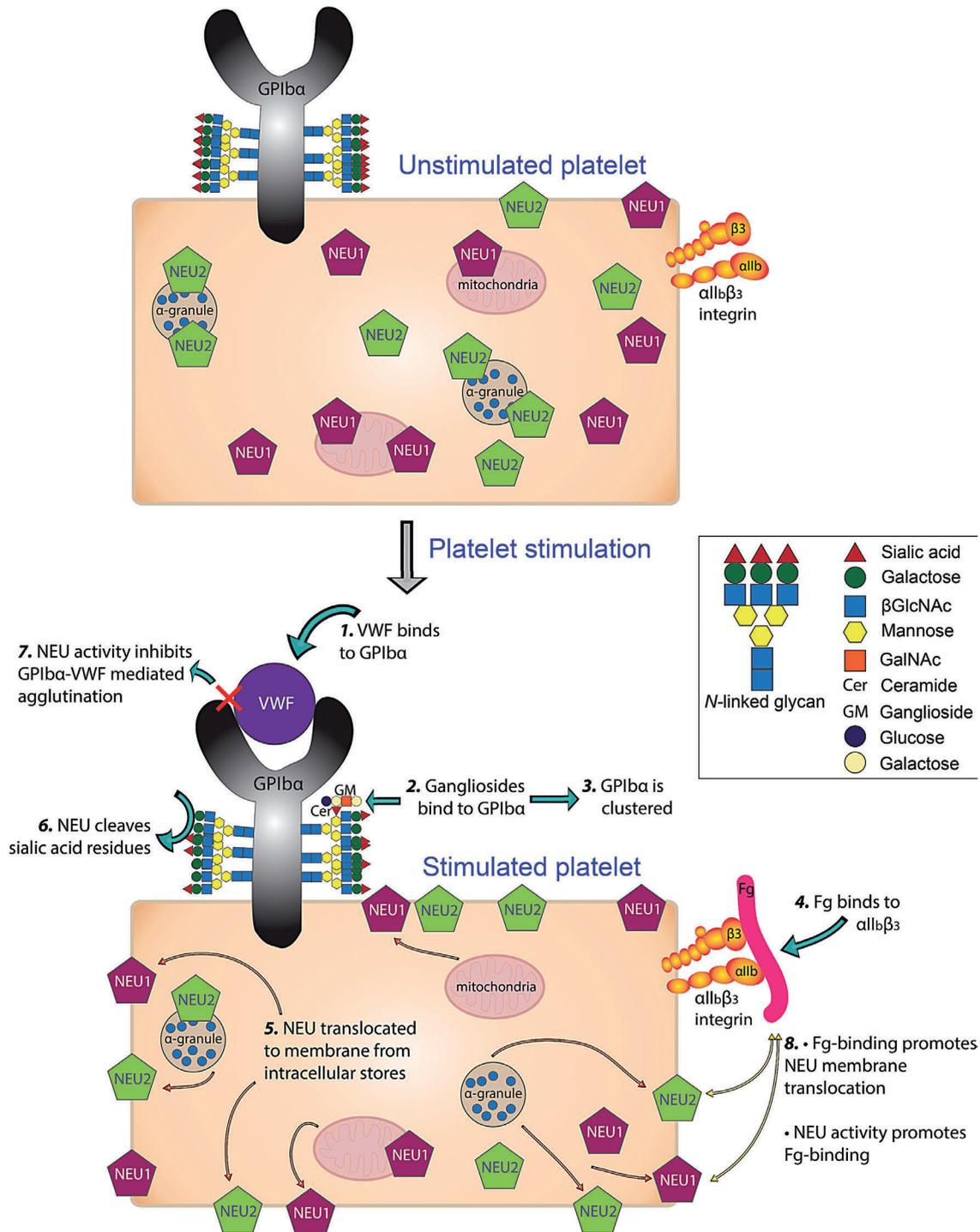
Figure 7: Model of neuramidase membrane-association, activity and feedback to fibrinogen binding. Based on the findings of this study, the following model is proposed: 1) Binding of von Willebrand factor (VWF) to glycoprotein Ibα (GPIbα) potentially leads to 2) gangliosides (GM) binding, 3) GPIbα clusters, leading to 4) fibrinogen binding to $\alpha_{IIb}\beta_3$-integrin, 5) NEU1 and NEU2 translocated to the plasma membrane from mitochondria or α-granules respectively, 6) neuramidase (NEU) cleaves sialic acid, 7) NEU activity inhibits VWF-mediated agglutination and 8) fibrinogen promotes further NEU membrane association in a feedback loop.

PNGase digestion did not affect SNA-binding, demonstrating that some sialic acid was still present on remaining *O*-linked glycans, potentially those on VWF, as shown by the small increase in PNA-binding to the VWF T-antigen. However, as SNA-binding was unchanged following VWF/risto-stimulation, these α2,3-linked glycans are not NEU1 and NEU2 substrates. In this study, we did not investigate whether VWF or GPIbα originated from a formerly internalised pool and was re-expressed on the membrane.

NEU membrane association is highly dependent on VWF-binding to GPIbα, as GPIbα removal by OSGE or inhibition by GlcNAc prevented membrane association. α2,3-linked sialic acid has been earlier described to be insensitive to OSGE-cleavage, indicating these structures might be attached to VWF or other platelet glycoproteins.[42] Control experiments with recNEU, which cleaves α2,3, α2,6 and α2,8-linked sialic acid, showed more binding to MAL-1, ECL and RCA-1 when compared to VWF/risto alone, demonstrating that more pronounced desialylation had occurred.

In general, platelet granule contents are not released following stimulation of GPIbα by VWF/risto without shear. However, this study showed that VWF-stimulation triggers P-selectin release as well as increased LAMP-1 membrane association, indicating release of α, δ-granule and lysosome content. This is consistent with the co-localisation of NEU2 with P-selectin. However, this can also be partly attributed to some degree of pre-activation due to the long incubation times required for NEU staining, as LAMP-1 membrane-association is dependent on platelet activation.[36]

This study demonstrates that a negative feedback loop exists between the activity of NEU and platelet agglutination, as inhibition of NEU activity by DANA potentiates platelet VWF-mediated agglutination. Following desialylation, the underlying glycans on GPIbα, including βGlcNAc-residues, are more prone to further cleavage, which was previously found to reduce VWF-binding to platelets.[3] NEU inhibition is described to block both desialylation and consequent degalactosylation by β-galactosidase, which was described as the first step in GPIbα-clustering.[32] Interestingly, NEU1 activity was inversely correlated with integrin-mediated adhesion to laminin.[20] Recent findings showed that pneumococcal NEUA induced platelet hyperactivity through desialylation, which was dependent on ADP-secretion.[46]

GM are glycosphingolipid-containing glycans involved in cell-cell recognition, adhesion and signal transduction, and may be another substrate for NEU1. GM3 blocks GPIbα-clustering by preventing linking with lipid rafts[24] and GM blocks the second wave of aggregation by ADP.[35] Our results demonstrate that GM might be important for NEU2 membrane association, and are potentially involved in the negative feedback loop between NEU activity and VWF-induced agglutination.

The results presented here also indicate that calcium inhibits NEU2 membrane association but promotes its activity, as treatment with DANA prevented the calcium-potentiated increase in PAC-1-binding. NEU activity is also important for integrin activation, as there was no further inhibition of fibrinogen binding by DANA in the presence of RGDS. Previous studies have shown that chelation of cations by EDTA generally inhibited the enzyme important for sialic acid metabolism, sialyl-transferase, as does ADP, and similar mechanisms might be important for NEU activity.[25] Also, full activity of NEU (*Vibrio cholera*) was induced by calcium (1 mM[47]), which is in line with our findings, whereby DANA only inhibited PAC-1 in the presence of calcium. DANA was able to inhibit fibrinogen-binding and consequent activation of $\alpha_{IIb}\beta_3$, although this inhibition was only partial. This could be due to DANA being more specific for NEU1 and less effective in NEU2 inhibition, as another NEU inhibitor, Zanamivir, is more specific for NEU2.[48] However, it is not known which NEU-inhibitor is most effective in blocking NEU activity in platelets. Also, NEU3 and NEU4 might also play a role in healthy platelets, further contributing to desialylation.

Following GPIbα-clustering, fibrinogen binds to $\alpha_{IIb}\beta_3$-integrin, a crucial step for platelet-platelet interactions and aggregation. Interestingly, in the presence of plasma, at least 50% of membrane expressed NEU1 was dependent on fibrinogen binding, as demonstrated by the RGDS blockade, while NEU2 was unaffected. Both NEU become highly membrane-bound when high concentrations of fibrinogen are present. Of interest, the amino acid sequence of NEU2 contains a RGD-motif, which could potentially interfere with fibrinogen-binding. Additionally, calcium signalling plays an important role as its chelation by BAPTA-AM enhanced membrane association of NEU2. The need for a fibrinogen binding conformation of $\alpha_{IIb}\beta_3$ has also been demonstrated, as PAC-1-binding following VWF/risto-stimulation is low in the absence of calcium.[27] Following VWF/risto-stimulation in the presence of saturating levels of fibrinogen, NEU1 and NEU2 became highly associated with the plasma membrane as shown by flow cytometry, potentially through their trans-membrane domain(s).[49] Similar results were found by microscopy for NEU2, which was more highly expressed on the platelet surface following VWF/risto-stimulation, even without additional fibrinogen.

Notably, washed platelets had a significantly higher MFI for RCA-1 binding in comparison to platelets from PRP, both pre- and post-stimulation with VWF/risto, correlating with higher fibrinogen binding due to washing and longer incubation times. Fibrinogen also increased recNEU activity. Earlier findings showed that two-thirds of asialo-VWF binds to GPIbα, while the remainder binds to $\alpha_{IIb}\beta_3$ in the presence of fibrinogen. Without fibrinogen, asialo-VWF binds exclusively to $\alpha_{IIb}\beta_3$.[50] When NEU becomes membrane-bound, it could potentially cleave platelet-bound VWF, thus enhancing its binding to GPIbα. In addition to cleavage of GPIbα itself, desialylation of glycosylated VWF and/or fibrinogen cannot be excluded, as desialylation also affects their platelet binding properties. Further to this, desialylated fibrinogen has a higher affinity for $\alpha_{IIb}\beta_3$[51,52] and platelet aggregation in response to asialo-VWF is approximately 60% lower than native VWF.[53] However, other studies have demonstrated spontaneous binding of asialo-VWF to GPIbα, in which was able to potentiate aggregation in the presence of fibrinogen.[54] These studies have established that the presence of plasma proteins including fibrinogen affect platelet desialylation and thereby also the VWF-binding potential. Whether NEU membrane expression is important for VWF clearance is currently unknown, however a recent study demonstrated a link between VWF-desialylation (terminal α(2-6)-linked sialic acid) and its clearance in low-VWF patients,[55] however no significant changes in SNA were observed.

This study shows for the first time that NEU1 co-localises with some but not all mitochondria within platelets, while it does not co-localise with LAMP as previously demonstrated.[56] In line with our NEU1 findings, NEU4 is located within mitochondria in other nucleated cells[16] and NEU4 also translocates to the platelet membrane following VWF addition under shear. Moreover, in our hands, NEU1 did not co-localise with β-galactosidase in unstimulated healthy or stimulated platelets, despite previous findings indicating these are both present in lysosomes, and not in platelet α and δ-granules.[36] This is in contrast with stored platelets, wherein some diffuse co-localisation of NEU1, with β-galactosidase was observed,[22] although this localisation was partial and mostly cytoplasmic. NEU2 however, was co-localised with the α-granule protein P-selectin, which is not surprising as P-selectin is sialylated[57] and DANA showed a trend towards inhibition of P-selectin expression post-ristocetin treatment.

The findings presented here demonstrate novel roles for NEU1 and NEU2 in healthy platelets, which are well regulated down-stream of GPIbα following VWF-binding, negatively by calcium (NEU2 only) and increased in presence of fibrinogen. Fibrinogen-binding is required for NEU1 and NEU2 membrane association, enhancing their activity. However when platelet $\alpha_{IIb}\beta_3$ is fully occupied with fibrinogen, NEU activity is inhibited.

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