Proteolytic shedding of the prion protein via activation of metallopeptidase ADAM10 reduces cellular binding and toxicity of amyloid-β oligomers

Heledd H. Jarosz-Griffiths1#, Nicola J. Corbett1, Helen A. Rowland1, Kate Fisher1, Alys C. Jones1, Jennifer Baron2, Gareth J. Howell2, Sally A. Cowley34, Satyan Chintawar56, M. Zameel Cader56, Katherine A. B. Kellett1 and Nigel M. Hooper1*

1Division of Neuroscience and Experimental Psychology, School of Biological Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester Academic Health Science Centre, Oxford Road, Manchester M13 9PT, UK
2Flow Cytometry Facility Laboratory, Faculty of Biology, Medicine and Health, University of Manchester, CTF Building, Oxford Road, Manchester M13 9PT, UK
3Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK
4Oxford Parkinson’s Disease Centre University of Oxford, South Parks Road, Oxford OX1 3QX, UK
5Nuffield Department of Clinical Neurosciences, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, UK
6The Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX1 3QX, UK

Running title: Shedding of PrPc by ADAM10 reduces Aβ binding and toxicity

#Present address: Clinical and Translational Rheumatology, Leeds Institute of Rheumatic & Musculoskeletal Medicine, University of Leeds, Leeds LS9 7TF, UK

*To whom correspondence should be addressed: Nigel Hooper: Division of Neuroscience and Experimental Psychology, School of Biological Sciences, Faculty of Biology, Medicine and Health, AV Hill Building, University of Manchester, Oxford Road, Manchester M13 9PT, UK; nigel.hooper@manchester.ac.uk, Tel: +44 (0)161 306 5765

Key words: metallopeptidase ADAM10, amyloid, induced pluripotent stem cells, prion protein, proteolytic shedding, neurodegenerative disease, Alzheimer’s disease, oxidative stress, cell surface protein.

ABSTRACT

The cellular prion protein (PrPc) is a key neuronal receptor for amyloid-β oligomers (AβO), mediating their neurotoxicity, which contributes to the neurodegeneration in Alzheimer’s disease (AD). Similarly to the amyloid precursor protein (APP), PrPc is proteolytically cleaved from the cell surface by a disintegrin and metalloprotease, ADAM10. We hypothesized that ADAM10-modulated PrPc shedding would alter the cellular binding and cytotoxicity of AβO. Here, we found that in human neuroblastoma cells, activation of ADAM10 with the muscarinic agonist carbachol promotes PrPc shedding and reduces the binding of AβO to the cell surface, which could be blocked with an ADAM10 inhibitor. Conversely, siRNA-mediated ADAM10 knockdown reduced PrPc shedding and increased AβO binding, which was blocked by the PrPc-specific antibody 6D11. The retinoic acid receptor analog acitretin, which up-regulates ADAM10, also promoted PrPc shedding and decreased AβO binding in the neuroblastoma cells and in human induced pluripotent stem cell (iPSC)-derived cortical neurons. Pretreatment with acitretin abolished activation of Fyn kinase and prevented an increase in reactive oxygen species caused by AβO binding to PrPc. Besides blocking AβO binding and toxicity, acitretin also increased the non-amyloidogenic processing of APP. However, in the iPSC-derived neurons, Aβ and other amyloidogenic processing products did not exhibit a reciprocal decrease upon acitretin treatment. These results indicate that by
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Promoting the shedding of PrPC in human neurons, ADAM10 activation prevents the binding and cytotoxicity of AβO, revealing a potential therapeutic benefit of ADAM10 activation in AD.

Alzheimer’s disease (AD) is a progressive, age-associated disorder which is characterised by abnormal accumulation of proteinaceous aggregates in the form of amyloid-β (Aβ) containing plaques and neurofibrillary tangles composed of hyperphosphorylated tau (1,2). Oligomers of Aβ (AβO) appear to be the most neurotoxic species, binding to receptors on the surface of neurons and triggering a variety of downstream signalling pathways that negatively impact on neuronal function and survival (reviewed in (3,4)). A substantial portion of AβO toxicity in AD is mediated following the initial interaction with the cellular prion protein (PrPC) which resides in cholesterol-rich lipid rafts at the neuronal surface (4,5). AβO binding to PrPC mediates inhibition of long-term potentiation (LTP) in hippocampal slices (6) and memory and behavioural impairments in multiple AD mouse models (7,8). The binding of AβO to PrPC leads to activation of Fyn kinase, a loss of surface N-methyl-D-aspartate (NMDA) receptors and subsequent phosphorylation of tau (9-11). AβO also cause increases in reactive oxygen species (ROS) which contribute to the neurodegeneration (reviewed in (12)).

Given the central role of PrPC in mediating the toxicity of AβO, targeting PrPC has potential for AD therapy (reviewed in (4)). Immunotoargeting, for example using the anti-PrPC monoclonal antibody 6D11 to block the AβO binding site on PrPC, prevented the impairment in LTP caused by AβO derived from AD brain extracts (13,14) and blocked Aβ synaptotoxicity following peripheral administration (15). Altering the conformation of AβO, disrupting AβO binding to PrPC or displacing PrPC from lipid rafts blocked downstream cellular toxicity (11,16). Several of the actions of AβO, including activation of Fyn, dendritic spine loss and tau phosphorylation, are mediated by PrPC coupling to mGluR5 (17-19) and pharmacological inhibition or allostERIC modulation of mGluR5 reduced pathogenesis in AD mouse models (20,21). Another approach has been to target Fyn directly with a specific inhibitor to rescue the memory deficits in an AD mouse model (22). These approaches highlight that targeting PrPC or other components of the AβO-PrPC signalling complex may have therapeutic potential in AD.

Aβ peptides are generated when the amyloid precursor protein (APP) is cleaved by the sequential action of the β-secretase (β-site APP cleaving enzyme 1; BACE1) and the multi-subunit γ-secretase complex in the amyloidogenic pathway (23). β-secretase cleavage of APP also releases the large soluble ectodomain fragment sAPPβ. Alternatively, APP can be cleaved via the non-amyloidogenic pathway through the action of the α-secretase, a disintegrin and metalloprotease ADAM10, precluding the formation of the Aβ peptide and generating an alternative soluble fragment sAPPα that has neuroprotective and neurotrophic properties (23). It is generally assumed that there is competition between the α- and β-secretases for their substrate APP, resulting in a reciprocal relationship between the amyloidogenic and non-amyloidogenic APP processing pathways. In support of this reciprocal relationship, neuronal overexpression of ADAM10 in APPV717I transgenic mice increased the secretion of sAPPα and reduced the formation of Aβ peptides (24), while in human induced-pluripotent stem cell (iPSC)-derived neurons, inhibition of BACE1 reduced sAPPβ and Aβ and increased sAPPα (25).

The ectodomain shedding of multiple cell surface proteins can be promoted by a variety of compounds. For example, activators of protein kinase C and the muscarinic agonist carbachol promote the shedding of APP (26-29). The vitamin A analog, acitretin, promoted the α-secretase cleavage of APP by stimulating the transcription of ADAM10 via interaction with retinoic acid responsive elements within the ADAM10 promoter (30). As ADAM10 also cleaves and sheds the ectodomain of PrPC from the cell surface (31-33), we hypothesised that modulating ADAM10 activity, thereby altering the shedding and thus the amount of PrPC at the
cell surface, would modulate the binding and toxicity of AβO.

Here, we have used human neuroblastoma cells and iPSC-derived cortical neurons to show that carbachol and acitretin promote the shedding of cell surface PrP^C through activation of ADAM10. The resulting reduction of cell surface PrP^C leads to a concomitant reduction in the binding of AβO. Conversely, siRNA knockdown of ADAM10 resulted in increased cell surface PrP^C and a corresponding increase in AβO binding which could be blocked with the PrP^C antibody, 6D11. AβO binding to PrP^C activated Fyn kinase and caused an increase in ROS which could be blocked by promoting the shedding of PrP^C with acitretin. We also report that although acitretin reciprocally modulated the amyloidogenic and non-amyloidogenic processing of APP in neuroblastoma cells and rat hippocampal neurons, no such reciprocal relationship was observed in the human iPSC-derived neurons.

Results

Promoting the shedding of PrP^C decreases the cell surface binding of AβO

As ADAM10 mediates the shedding of PrP^C from the cell surface (31,32), we hypothesised that activation of ADAM10 would reduce AβO binding to cells due to shedding of its cell surface receptor PrP^C. Initially the muscarinic agonist carbachol, which increases the shedding of multiple cell surface proteins, including APP, was employed (28). The effect of carbachol on PrP^C and APP shedding was monitored by detection of the soluble fragments, sPrP^C and sAPPα, respectively, in the media fraction. Treatment of human SH-SY5Y cells expressing PrP^C with carbachol promoted the shedding of full length glycosylated PrP^C by 1.4-fold and the α-secretase cleavage of APP by 1.5-fold (Fig. 1A-C). To establish that carbachol was acting via activation of ADAM10, the cells were preincubated with the ADAM10 selective inhibitor, GI254023X (34). On its own the ADAM10 inhibitor significantly reduced the amount of sPrP^C in the media by 83% (Fig. 1A and B), indicating that carbachol was promoting the shedding of PrP^C via activation of ADAM10. Similar to its effect on the shedding of PrP^C, the ADAM10 inhibitor significantly reduced sAPPα in the media by 85% and blocked the increase in sAPPα caused by carbachol (Fig. 1A and C). Treatment with carbachol and the GI inhibitor did not significantly alter ADAM10 expression (Fig. 1D). Consistent with increasing the shedding of PrP^C, carbachol treatment resulted in a decrease in cell surface expression of PrP^C evaluated by ImageStream imaging cytometry (Fig. 1E). This analysis demonstrated a decrease in high-expressing PrP^C cells in a population of cells treated with carbachol when compared to the control population. The decrease in cell surface PrP^C was confirmed using immunofluorescence microscopy. Treatment of the cells with carbachol resulted in a significant decrease (40%) in the amount of PrP^C localised to the cell surface (Fig. 1F and G). AβO were prepared and characterised as previously described (11) and their binding to the cells was monitored by immunofluorescence microscopy. Treatment of the cells with carbachol resulted in a significant decrease (62%) in the amount of AβO bound to the cell surface (Fig. 1F and G).

In addition to promoting the shedding of the entire ectodomain of PrP^C through cleavage near to the site of glycosyl-phosphatidylinositol (GPI) anchor attachment (31), ADAM10 has also been reported to cleave PrP^C within the middle of the ectodomain (sometimes referred to as α-cleavage) (35). The α-cleavage of PrP^C releases an N-terminal fragment, termed N1, and leaves a C-terminal fragment, C1, tethered to the membrane via the GPI anchor. To establish whether carbachol also promoted the cleavage of PrP^C within its ectodomain, samples were deglycosylated so that the C1 fragment could be detected and distinguished from unglycosylated full length PrP^C. There was no change in the ratio of full-length PrP^C to C1 fragment when cells were incubated with carbachol (Fig. 1H and I), indicating that carbachol reduces AβO binding by promoting the shedding of full length PrP^C and not by promoting cleavage within its ectodomain.
Knockdown of ADAM10 increases Aβ oligomer binding in a PrP-dependent manner

To confirm that ADAM10 was responsible for altering AβO binding through modulating the cell surface level of PrPC, ADAM10 was knocked down using siRNA in the SH-SY5Y cells expressing PrPC. For these experiments we used both a SmartPool siRNA containing 4 target siRNAs (Dharmacon) shown in Figure 2 and a single, independent siRNA (Ambion) shown in Figure 3. In the ADAM10 siRNA treated cells there was a significant reduction in the pro and mature forms of ADAM10 of 69% (Fig. 2A and B) and 68% (Fig. 3A and B) in Dharmacon treated and Ambion siRNA treated cells, respectively. Following ADAM10 siRNA knockdown, the amount of sPrPC was significantly reduced by 48% (Fig. 2A and C) and by 30% (Fig. 3A and C) and the amount of sAPPα was also significantly reduced, by 67% (Fig. 2A and D) and 85% (Fig. 3A and D). ADAM10 siRNA caused a 2.2-fold (Fig. 2E and F) and a 1.3-fold (Fig. 3E and F) increase in cell surface PrPC relative to non-targeting siRNA as assessed by immunofluorescence microscopy and the binding of AβO to the cells was increased by 1.7-fold (Fig. 2E and G) and 1.6-fold (Fig. 3E and G) in the presence of ADAM10 siRNA relative to non-targeting siRNA. To establish whether this increased binding of AβO was due to the increased cell surface level of PrPC, cells were pre-incubated with the PrPC antibody, 6D11, which blocks the AβO binding site on PrPC (6). Incubation of the cells with the 6D11 antibody had no significant effect on the amount of PrPC at the cell surface (Fig. 2E and F, Fig. 3E and F). However, the 6D11 antibody significantly reduced AβO binding to the cells by 77% (Fig. 2E and G) and by 60% (Fig. 3E and G). In the presence of the 6D11 antibody, siRNA knockdown of ADAM10 failed to increase AβO binding (Fig. 2E and G, Fig. 3E and G), indicating that the increased binding of AβO upon ADAM10 knockdown was due to increased cell surface PrPC.

To confirm that ADAM10 modulated AβO binding in a PrPC-dependent manner, SH-SY5Y cells lacking PrPC (36) were treated with ADAM10 siRNA and AβO binding assessed. In these cells, ADAM10 siRNA treatment significantly reduced the pro and mature forms of ADAM10 and the amount of sAPPα in the media (Fig. 4A and B). In the absence of PrPC, AβO binding was <15% (Fig. 4C) of that observed in cells expressing PrPC (Fig. 2E and 3E) and this residual binding was unchanged following knockdown of ADAM10 (Fig. 4C and D). Together these data indicate that ADAM10 modulates AβO cell surface binding in a PrPC-dependent manner.

Activation of ADAM10 with acitretin increases the shedding of PrPc and decreases AβO binding

Acitretin, a synthetic retinoid, increases the expression of ADAM10 in cell culture and animals, leading to an increase in sAPPα (30). As acitretin may have potential as a novel therapeutic drug for AD due to its ability to increase sAPPα in the CSF of AD patients (37), we investigated whether acitretin, via activation of ADAM10, would increase the shedding of PrPC and decrease AβO binding. Acitretin treatment of the SH-SY5Y cells expressing PrPC increased the expression of mature ADAM10, and the shedding of PrPC and sAPPα by 1.2-fold (Fig. 5A-C). In the presence of the ADAM10 inhibitor GI254023X, acitretin failed to induce a significant increase in the amount of sPrPC (Fig. 5A and B) or of sAPPα in the media (Fig. 5A and C), indicating that it was acting via ADAM10. Treatment with acitretin caused an increase in ADAM10 protein (Fig. 5A and D) and mRNA (Fig. 5E) expression. Treatment with acitretin resulted in a decrease in cell surface PrPC as evaluated by ImageStream imaging cytometry (Fig. 5F). This analysis demonstrated a decrease in high-expressing PrPC cells in a population of cells treated with acitretin when compared to the control population. The decrease in cell surface PrPC was confirmed using immunofluorescence microscopy, where acitretin treatment of the cells caused a significant decrease (81%) in the amount of PrPC localised to the cell surface (Fig. 5G and H). This decrease in cell surface PrPC with acitretin resulted in a reduction of the amount of AβO bound to the surface of the cells by 86% (Fig. 5G and H). These data indicate that activation of ADAM10 with the retinoic acid analog acitretin increases the shedding of...
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PrPC and decreases the binding of AβO to the cells.

**Activation of ADAM10 prevents AβO mediated activation of Fyn kinase and rescues AβO mediated increase in ROS**

To establish whether promoting the shedding of PrPC through activation of ADAM10 could decrease AβO cytotoxicity, we monitored downstream activation of Fyn kinase and ROS production in cells. Fyn kinase is a member of the Src family kinases (SFK) and AβOs activate Fyn kinase by phosphorylation at Tyr416 (10,11). Treatment of cells with AβO increased pSFK416 levels 1.3-fold (Fig. 5I and J). Pre-treatment with actretin abolished the AβO-mediated increase in pSFK (Fig. 5I and J). To monitor the effect of AβO on ROS, cells were primed with menadione to reduce mitochondrial production of NAD(P)H and increase ROS (38). Addition of AβO increased ROS by 1.2-fold in cells expressing PrPC (Fig. 5K) but pre-treatment with actretin abolished the increase in ROS mediated by AβO (Fig. 5K). Together, these data indicate that promoting the ADAM10-mediated shedding of PrPC blocks both the cellular binding and downstream cytotoxicity of AβO.

**Activation of ADAM10 increases the shedding of PrPC and rescues AβO mediated increase in ROS in human iPSC-derived cortical neurons**

To determine whether activation of ADAM10 would lead to enhanced shedding of PrPC and reduce AβO toxicity in human neurons, we used iPSC-derived cortical neurons. The iPSC line OX1-clone 19 (OX1-19) was verified for pluripotency by the presence of Oct4, SSEA-4 and nanog and the absence of Pax6 using immunofluorescence microscopy (Fig 6A). The iPSCs were differentiated into cortical neurons using the method of Shi et al. (39). The efficiency of cortical induction was calculated using Pax6 expression (39). This demonstrated that the differentiation efficiency of the OX1-19 and SBAD iPSCs was 92.3±7.6% and 85.9±1.9%, respectively (data described as mean±SEM for 4 cortical inductions of the OX1-19 cell line and for 2 cortical inductions of the SBAD cell line). The neuronal marker MAP2 was used along with the marker Tbr1 to confirm the presence of secondary progenitor cells and Satb2 to confirm the presence of upper layer neurons (Fig. 6B). Immunoblotting of the cortical neurons revealed the presence of APP, ADAM10 and PrPC (Fig. 6C).

Acitretin treatment of the iPSC-derived cortical neurons at day 65 increased sAPPα in the media by 1.2-fold (Fig. 6D). In the presence of the ADAM10 inhibitor, acitretin failed to induce an increase in the amount of sAPPα in the media (Fig. 6D). Treatment with acitretin decreased cell surface PrPC by 87% (Fig. 6E and F). AβO treatment increased ROS in the iPSC-derived cortical neurons (Fig. 6G) and this increase was blocked by pretreatment of the neurons with actretin (Fig. 6G). These data indicate that actretin, through increasing ADAM10 activity and the shedding of PrPC, blocks the toxicity of AβO in human neurons.

**Activation of ADAM10 increases the neuroprotective sAPPα but does not decrease Aβ production in human neurons**

Activation of ADAM10 has been reported to increase the production of the neuroprotective sAPPα and, reciprocally, decrease Aβ production (30). However, whether this reciprocal relationship occurs in human neurons upon activation of ADAM10 has not been reported. To determine this, we assessed the effect of acitretin on the relative amounts of sAPPα, sAPPβ and Aβ peptides in both the SH-SY5Y cells and the iPSC-derived neurons. In the SH-SY5Y cells, acitretin caused a significant increase in sAPPα (Fig. 7A) and a reciprocal significant decrease in sAPPβ (Fig. 7A). The decrease in sAPPβ was paralleled by a decrease in both Aβ40 and Aβ42 following acitretin treatment (Fig. 7B and C). However, although acitretin increased sAPPα in the iPSC-derived neurons (Fig. 6C), there was no reciprocal decrease in sAPPβ or Aβ (Fig. 7D-F). To ascertain whether this lack of reciprocal effect of acitretin on the non-amyloidogenic and amyloidogenic pathways was a feature of neurons, the effect of acitretin on rat primary hippocampal neurons was investigated. Acitretin treatment of the hippocampal neurons increased the non-amyloidogenic cleavage of APP (Fig. 7G) and caused a reciprocal decrease in both
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Aβ40 and Aβ42 (Fig. 7H and I). Thus, in both the SH-SY5Y cells and the rat hippocampal neurons, activation of the non-amyloidogenic processing of APP results in a reciprocal decrease in the amyloidogenic processing pathway, whereas in the human iPSC-derived neurons no such reciprocal relationship was observed.

Discussion

Numerous studies have validated PrP<sub>C</sub> as a key neuronal receptor for Aβ and highlighted the intrinsic role it plays in the activation of multiple downstream targets leading to neuronal impairment in AD (reviewed in (4,5)). As cleavage of PrP<sub>C</sub> by ADAM10 regulates the amount of PrP<sub>C</sub> at the cell surface (31-33), we hypothesised that modulation of ADAM10 activity, through altering cell surface PrP<sub>C</sub>, would impact on Aβ binding and toxicity. Here we show that increasing ADAM10 activity promoted the shedding of cell surface PrP<sub>C</sub> and as a result blocked the binding of Aβ to the surface of neurons and decreased their cytotoxicity as measured by activation of Fyn kinase and increase in ROS.

Initially we used the muscarinic agonist carbachol to promote the shedding of PrP<sub>C</sub> based on the observation that carbachol promotes the shedding of APP, likely through the activation of ADAM10 (28). Carbachol decreased the amount of PrP<sub>C</sub> at the cell surface which resulted in reduced binding of Aβ to the cells. Through the use of a selective ADAM10 inhibitor, we show for the first time that carbachol is acting via ADAM10 to promote the shedding of both PrP<sub>C</sub> and APP. To confirm that ADAM10 was altering Aβ binding through modulating cell surface PrP<sub>C</sub>, we used siRNA knockdown to reduce PrP<sub>C</sub> expression. This resulted in increased cell surface presentation of PrP<sub>C</sub> and a concomitant increase in Aβ binding. Although various other proteins have been reported to act as cell surface receptors for Aβ (reviewed in (4)), the effect of carbachol activation of ADAM10 on Aβ binding was mediated specifically via PrP<sub>C</sub>. This was evidenced by (i) binding being blocked using the 6D11 antibody whose epitope corresponds to the Aβ binding site on PrP<sub>C</sub> and (ii) siRNA knockdown of ADAM10 in cells lacking PrP<sub>C</sub> failing to increase Aβ binding. The Aβ used here have been well-characterised using biophysical and immunological techniques and correspond to fibrillar oligomers recognised by the conformation-specific OC antibody (11). Such OC reactive Aβ correlated with the onset and severity of AD in human brain (40) and with cognitive decline and tau aggregation and phosphorylation in a transgenic AD mouse model (41). Whether activation of ADAM10 will influence the cellular binding of other oligomeric species of Aβ will depend on whether their receptors are also susceptible to ADAM10-mediated shedding.

In addition to using carbachol to activate ADAM10, we also employed the synthetic retinoid, acitretin, which releases all-trans retinoic acid from cellular retinoic acid-binding proteins. This allows the all-trans retinoic acid to bind to retinoid acid receptor (RAR) transcription complexes which in turn bind to retinoid binding sites on the ADAM10 promoter, leading to increased ADAM10 activity (30). Acitretin has previously been reported to increase the α-secretase cleavage of APP by ADAM10 both in cellular and animal models (30). Similarly, we report that acitretin promoted the shedding of APP and PrP<sub>C</sub>, both in SH-SY5Y cells and iPSC-derived neurons, which was blocked by the ADAM10 specific inhibitor. Thus, using two independently acting activators of ADAM10 we clearly show that promoting the activity of this metalloprotease reduces the cellular binding and cytotoxicity of Aβ through modulating cell surface PrP<sub>C</sub>.

ADAM10 promotes the shedding of the ectodomain of PrP<sub>C</sub> by cleaving the protein after residue 228, close to the site of attachment of the GPI anchor (residue 231) (31). However, it has also been proposed that ADAM10 is responsible for the α-cleavage of PrP<sub>C</sub> between amino acids 110-111 just C-terminal to the Aβ binding domain (residues 95-105) (6,42), releasing the soluble N1 fragment containing the Aβ binding domain and leaving the cell associated C1 fragment (35), an action that would also be predicted to reduce Aβ binding to the cell surface. The N1 fragment has been shown to bind Aβ and suppress their toxicity in cultured murine hippocampal neurons and in an in vivo mouse model of Aβ-induced memory
dysfunction, leading to the suggestion that upregulation of N1 production could act as a cellular mechanism to protect against Aβ toxicity (43,44). Carbachol has previously been reported to increase the α-cleavage of PrPc in HEK293 cells through the action of ADAM17 (45,46). Here we show that in the SH-SY5Y cells, which express functional muscarinic receptors (29,47), that the carbachol stimulated shedding of PrPc and APP is due to activation of ADAM10, not ADAM17. Furthermore, we were unable to detect the soluble N1 fragment in the conditioned medium from the SH-SY5Y cells following carbachol treatment (data not shown). The differences between our work and that previously published (45,46) may reflect the use of different cell lines in which the relative levels of ADAM10 and ADAM17 differ and/or that in the SH-SY5Y cells the N1 fragment is rapidly metabolised. However, as activation of ADAM10 in the SH-SY5Y cells did not influence the ratio of full length PrPc to the C1 fragment, this indicates that the α-cleavage of PrPc is unaltered in our experimental model, ruling out a contribution of α-cleavage of PrPc to the mechanism by which ADAM10 activation blocks AβO binding.

AβO mediate a range of cellular actions which contribute to their neurotoxicity in AD. Binding of AβO to PrPc leads to activation of Fyn kinase (10,11) which in turn phosphorylates NMDA receptors altering their cell surface distribution (10) and directly phosphorylates tau on Tyr18 (9). AβO also induce cytotoxicity through increasing ROS (48), although to date whether this effect is mediated via their binding to PrPc has not been reported. Here we show that activation of ADAM10 blocked the AβO-dependent activation of Fyn phosphorylation mediated by PrPc. Furthermore, and for the first time, we report that in both SH-SY5Y cells and human iPSC-derived neurons AβO binding to PrPc increases cellular ROS and that this increase in ROS can be blocked by removing cell surface PrPc upon ADAM10 activation. Thus, ADAM10 activation can ameliorate the downstream cytotoxicity induced by AβO binding to PrPc and represents another potential therapeutic approach to disrupt the AβO-PrPc signalling complex in AD.

Genetic analyses of families with late-onset AD revealed two rare mutations (Q170H, R181G) in the pro-domain of ADAM10 which attenuated its α-secretase activity and shifted APP processing toward β-secretase-mediated cleavage with a 2-3 fold increase in Aβ levels, enhanced Aβ plaque load and reactive gliosis (49,50). Based on our work with human iPSC-derived neurons, it is likely that these mutations in ADAM10 will also lead to increased cell surface PrPc and enhanced AβO binding and cytotoxicity, which may contribute to the AD phenotype in individuals with such ADAM10 mutations.

The general consensus is that there is competition between the α- and β-secretases for their substrate APP. For example, in murine primary cortical neurons, ADAM10 knockdown increased sAPPβ and Aβ (51) and in human iPSC-derived neurons, inhibition of BACE1 reduced sAPPβ and Aβ and increased sAPPα (25). However, other studies have failed to observe such a reciprocal relationship (52). On the whole, though, activation of ADAM10 has been reported to result in an increase in sAPPα with a reciprocal decrease in sAPPβ and Aβ peptides in various cell and transgenic mouse models (24,30,53,54). For example, in APPV717I transgenic mice moderate neuronal overexpression of ADAM10 increased the secretion of sAPPα, reduced the formation of Aβ peptides, and prevented their deposition in plaques, while expression of mutant catalytically inactive ADAM10, which acts in a dominant negative manner, led to an increase in the number and size of plaques in the double transgenic mice (24). Stimulation of ADAM10 promoter activity with the vitamin A analogue acitretin led to an increase of mature ADAM10 protein that increased the ratio between α- and β-secretase activity in SH-SY5Y cells, while intracerebral injection of acitretin in APP/PS-1 transgenic mice led to a reduction of Aβ40 and Aβ42 (30). Thus we were somewhat surprised that although activating ADAM10 with acitretin led to a reduction in Aβ levels in the SH-SY5Y cells and in the murine hippocampal neurons, no such decrease in Aβ40, Aβ42 or sAPPβ was observed in iPSC-derived neurons from two different individuals, despite there being a similar increase in sAPPα in all three cell
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models. This result with human neurons is consistent with the result from a pilot clinical trial in which 21 mild to moderate AD patients were treated with acitretin for 4 weeks. In the AD patients acitretin caused a significant increase in CSF sAPPα when compared to a placebo control group but there was not a reciprocal significant decrease in sAPPβ and Aβ42 was unchanged between the two groups (37). Together these observations suggest that the reciprocal relationship between the non-amyloidogenic and amyloidogenic APP processing pathways may not hold true in human neurons when α-secretase cleavage is stimulated.

Regardless of whether activation of ADAM10 leads to a decrease in Aβ by shifting the balance of APP processing between the non-amyloidogenic and amyloidogenic pathways, activation of ADAM10 could be beneficial in AD by acting through other mechanisms. Activation of ADAM10 will directly increase sAPPα that has neuroprotective, neurotrophic and neurogenic properties (55-57), elevates adult neurogenesis in the hippocampus (50) and has been reported to decrease Aβ generation by directly associating with BACE1 and modulating its activity (58). In addition, as we have demonstrated here, activation of ADAM10 will increase the shedding of PrPC to reduce the cellular binding and downstream toxicity of AβO. However, as well as shedding APP and PrPC, ADAM10 also proteolytically cleaves more than 90 membrane proteins in the CNS, many of which are essential for brain development and normal physiological functions (59,60). Thus, whether activation of ADAM10 will only have beneficial effects is unclear; a situation which may be exacerbated if activation was to occur over a prolonged period of time as required to treat a chronic condition such as AD.

We have shown that activation of ADAM10 promotes the proteolytic shedding of the AβO receptor PrPC from the surface of human neuroblastoma cells and iPSC-derived neurons. This shedding of PrPC reduces the binding of AβO to cells and blocks their cytotoxicity as monitored by activation of Fyn kinase and increase in ROS. These data provide the first observation that modulating cell surface PrPC may contribute to the therapeutic potential of ADAM10 activation in AD and contribute to the neurodegeneration observed in individuals with mutations in ADAM10. In addition, we report that activation of ADAM10 does not result in a decrease in Aβ levels in the human iPSC-derived neurons, a result consistent with the results from a study following acitretin administration in humans (37), and highlighting that human iPSC-derived neurons are a valuable model system to explore the mechanisms underlying AD.

**Experimental procedures**

**Cell culture.** SH-SY5Y human neuroblastoma cells were stably transfected with the cDNA encoding murine PrPC containing the 3F4 epitope tag (human M108/M111) as described previously (61). Both untransfected (UT) and PrPC expressing SH-SY5Y cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum (FBS). NB7 human neuroblastoma cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% (v/v) FBS. Cells were maintained in a humidified incubator at 37°C in a 5% CO2, 95% air atmosphere.

**Culture and differentiation of induced pluripotent stem cells.** The iPSC lines, OX1-19 (obtained from S. Cowley, University of Oxford) (62) and SBAD03-05 (obtained from Z. Cader, StemBANCC) (63) were maintained on Matrigel (BD Biosciences) in mTeSR1 media (StemCell Technologies) containing 50 U/ml penicillin and 50 µg/ml streptomycin (Life Technologies) in a humidified incubator at 37°C in a 5% CO2, 95% air atmosphere. iPSCs were differentiated to cortical neurons as described previously (39), using dual-SMAD inhibition by 1 µM dorsomorphin and 10 µM SB431452 (Tocris). Following successful differentiation, neural progenitor cells (NPCs) were re-plated on d.35 post-induction at 250,000 cells/ well onto polyornithine and laminin-coated (Sigma) 6-well tissue culture plates and cultured until d.65 post-induction with media changes every 2-3 days. iPSC pluripotency and successful cortical neuron differentiation were confirmed using immunofluorescence microscopy with appropriate markers.

**Preparation of rat primary hippocampal neurons.** Primary neurons were prepared from...
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the hippocampi of 1-2 day-old Wistar rats and cultured as described previously (64). Neurons were cultured for 14 days with media changes every 3-4 days.

Activation and inhibition of ADAM10. To activate ADAM10, cells were incubated in OptiMEM containing GlutaMAX (Life Technologies) containing either 20 µM carbachol (Sigma) for 24 h or 20 µM acitretin (Sigma) for 48 h. To inhibit ADAM10, cells were incubated with 10 µM GI254023X (Tocris), a selective ADAM10 inhibitor, for either 24 or 48 h in OptiMEM. DMSO only treated cells were used for comparison to treated cells.

RNA interference studies. siRNA specific for human ADAM10 and a non-targeting sequence were obtained as smartpools from Dharmacon (Thermo Fisher Scientific). An additional, single siRNA sequence for human ADAM10 was also obtained for verification experiments (Ambion). SH-SY5Y (UT or PrP\textsuperscript{C} expressing) cells were seeded into T75 flasks or 24-well plates in routine culture medium and allowed to adhere overnight. The cell monolayers were washed twice with DPBS and a 25 nM (final concentration) smartpool siRNA solution was delivered as a complex with DharmaFECT-1 transfection reagent (Dharmacon) in serum-containing DMEM for 48 h. Cells were then washed twice in Dulbecco’s phosphate buffered saline (DPBS) prior to incubation in OptiMEM for 24 h.

Conditioned media, cell lysate and cell membrane preparation. Conditioned media was harvested and cell debris pelleted by centrifugation at 500 x g for 5 min. A 1ml sample of conditioned media was removed for immunoassay and stored at -20°C. Remaining conditioned media were concentrated to 100 µl in a Vivapsin 20 ml concentrator (10,000 MW membrane) by centrifugation at 1,900 x g for approximately 1 h in a bench top centrifuge at 4°C. Cells were washed in phosphate buffered saline (PBS) (Lonza), harvested and pelleted at 1400 x g for 3 min. Cells were lysed on ice for 30 min in RIPA buffer [50 mM Tris/HCl, 150 mM NaCl, 0.5% (w/v) sodium deoxycholate, 1% (v/v) Nonidet-P40, pH 8.0] containing protease and phosphatase inhibitor cocktails (Roche Diagnostics Ltd). Cell lysates were clarified by centrifugation at 12,460 x g for 10 min at 4°C and the clarified lysate stored at -20°C before use. For the preparation of membranes, cells were re-suspended in 3 ml of 50 mM HEPES, pH 7.5 and sonicated at amplitude 7 microns for 30 s using a Soniprep150. The cell suspension was then centrifuged at 2500 x g for 10 min at 4°C to pellet cell membranes and nuclei. The supernatant was then centrifuged in a Beckman Coulter Optima at 100,000 x g for 1 h at 4°C. Membranes were re-suspended in 50 mM Tris/HCl pH 7.5, 2 mM EDTA, 150 mM NaCl and 1% (w/v) CHAPSO. Protein concentration of all samples was determined by bicinchoninic acid assay.

Deglycosylation of cell lysate samples. All deglycosylation solutions were purchased from Prozyme. Cell lysates were made up to 100 µg protein in 150 µl volume, and boiled for 5 min in 20% (v/v) 5 x N-glycanase reaction buffer and 4% denaturation solution. After cooling, 4% detergent solution was added to each tube. To one of the tubes, 0.5% (v/v) N-glycanase (200 mU) was added and incubated at 37°C for 16 h.

Immunoblotting. Samples were made up in dissociation buffer [1x dissociation buffer (100 mM Tris-HCl, 2% (w/v) sodium dodecyl sulphate, 10% (v/v) glycerol, 100 mM dithiothreitol, 0.02% (w/v) bromophenol blue, pH 6.8] and heated at 95°C for 5 min. Proteins were resolved by SDS-PAGE on 7-17% acrylamide Tris-glycine gels and then transferred to Hybond PVDF membranes (GE Healthcare). Following electrotransfer, the membranes were blocked for 1 h in PBS with 0.1% Tween 20 (PBST) and 5% (w/v) non-fat milk and then incubated with primary antibody overnight at 4°C. Antibodies were probed using the following primary antibodies: anti-APP (22C11, Millipore), SAF32 (anti-PrP N-terminus, Cayman Chemical), 8H4 (anti-PrP residues 175-185), anti-ADAM10 antibody (Abcam), 6E10 (anti-A\textbeta\textsubscript{1-17}, Merck Biosciences), AC15 (anti-\textbeta-actin) and synapsin 1 (Sigma), 2B3 (anti-human sAPP\textalpha, Immuno-Biological Laboratories), anti-phospho-Src
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family kinase (Tyr-416, Cell Signalling Technology). Primary antibodies were detected by incubation with horseradish peroxidase-conjugated secondary antibody, both in PBST containing 2% BSA. Bound horseradish peroxidase conjugates were visualized using the ECL® detection system with a Syngene Gbox XT4 (Syngene). Densitometric analysis was performed using Genetools analysis software (Syngene).

ImageStream imaging cytometry. SH-SY5Y cells expressing PrPC were incubated in OptiMEM containing GlutaMAX and either 20 µM carbachol for 24 h or 20 µM acitretin for 48 h. DMSO only treated cells were used for comparison to treated cells. Cells were collected in PBS without metals and recovered by centrifugation (300xg for 5 min). All subsequent procedures were carried out at 4°C. Cells were resuspended in blocking buffer (10% donkey serum in PBS containing metals) at 2 x 10^6 cells per ml and incubated in primary antibody, SAF32, for 1 h before washing twice with PBS and recovering cells by centrifugation. Cells were then resuspended in blocking buffer and incubated with donkey anti-mouse AlexaFluor® 488 (Invitrogen) for 30 min in the dark. Cells were washed twice in PBS, fixed in 3% (v/v) paraformaldehyde and then finally resuspended in PBS before analysis using the ImageStreamX MkII imaging cytometer (Amnis). Brightfield, fluorescence and dark field scatter images were collected at 40x magnification for 3000 cells over 6 biological repeats. Cells were identified by the area and aspect ratio parameters. In focus cells were identified as having a Gradient RMS measure of >40. AlexaFluor® 488 emission was generated by a 488nm laser set to 100mW in the INSPIRE software (Amnis). Data was analysed in IDEAS software (Amnis) and exported to Flowjo v10 (Tree Star, Inc) to generate overlay histograms.

Aβ oligomer preparation. Synthetic biotin-Aβ_{1-42} containing a 6-carbon linker between the biotin moiety and the N-terminus of Aβ was purchased from AnaSpec (San Jose, USA). AβO were prepared as described previously (11). Briefly, Aβ peptide was dissolved in 1,1,1,3,3,3-hexafluoropropan-2-ol to break down any aggregated material, dried under a stream of N₂ gas and stored at -80°C. Peptide films of biotin-Aβ_{1-42} were dissolved in dimethyl sulfoxide to 1 mM and then resuspended in Ham’s F12 medium (Lonza) to a final monomer concentration of 100 µM and incubated for 18-24 h at room temperature. The preparation was then centrifuged at 14 000 x g for 20 min to pellet out any fibrillar material and the supernatant retained as the oligomer preparation.

Fluorescence microscopy. SH-SY5Y cells were cultured in growth medium to ~60% confluence on glass coverslips before required incubations (addition of carbachol, acitretin or GI254023X, as described) or ADAM10 siRNA treatments were carried out. For AβO binding experiments, cells were incubated with 500 nM AβO (total peptide concentration) diluted in OptiMEM for 30 min at 37°C. Where indicated, a 20 min pre-incubation ± 10 µg/ml 6D11 antibody (Covance, BioLegend) was carried out before incubation with AβOs. Post-incubation, cells were fixed with 4% (v/v) paraformaldehyde and blocked overnight at 4°C in DPBS with 5% (v/v) fish skin gelatine. Where required cells were permeabilised in 0.1% Triton-X100 for 10 min at room temperature before blocking. Coverslips were subsequently incubated for 2 h in the same buffer containing primary antibody, washed and then incubated with the corresponding fluorescently-labelled secondary antibody. PrPC was detected using SAF32 antibody followed by donkey anti-mouse AlexaFluor® 488, MAP2 was detected with anti-MAP2 (Millipore) followed by goat anti-chicken AlexaFlour 647 Cy5-IgG (Invitrogen) and biotin-Aβ_{1-42} for detection of AβO using Texas Red-conjugated streptavidin (Invitrogen). Nuclei were counterstained by washing briefly in DAPI stain and coverslips were mounted onto glass slides using Fluoromount-G (Southern Biotech). Cells were visualised using a DeltaVision Optical Restoration Microscopy System (Applied Precision). Data were collected from twelve, 0.5 µm-thick optical sections and three-dimensional data sets were deconvolved using the softWoRx program (Applied Precision). Images analysed were individual z-sections taken from the middle of the data stack, representing a section through the centre of the cell. The number of cells...
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analysed is indicated in individual figure legends. Fluorescence around the cell membrane was quantified using ImageJ software as described previously (65). This was plotted as pixel intensity versus distance around the cell using Microsoft Excel, and then the percentage of cell surface with detectable staining was calculated from multiple images.

For the fluorescence microscopy of iPSCs and iPSC-derived cortical neurons, cells were grown on laminin-coated coverslips, fixed with 4% (v/v) paraformaldehyde and blocked for 4 h at room temperature in DPBS with 10% (v/v) donkey serum. Where required cells were permeabilised in 0.2% Triton-X100 for 4 min at room temperature before blocking. Coverslips were then incubated with iPSC-specific markers (PAX6, Oct4, SSEA-4 and nanog; Abcam) to check pluripotency and neuronal markers (Satb2, Tbr1 and MAP2; Abcam) to confirm differentiation. Each primary antibody was detected with the corresponding secondary antibody, AlexaFlour 488, 594 or 647. DAPI stain was applied and coverslips were mounted using Prolong gold containing DAPI (Southern Biotech). Cells were visualised using an EVOS FL Cell Imaging System (Thermo Fischer Scientific).

qPCR. SH-SY5Y cells expressing PrP<sup>C</sup> were incubated in OptiMEM containing GlutaMAX and 20 µM acitretin for 48 h. DMSO only treated cells were used for comparison to treated cells. Cells were harvested and RNA was extracted using the RNeasy plus kit (QIAgen) according to the manufacturer’s instructions. cDNA was synthesised using 1µg of prepared RNA using the iScript cDNA synthesis kit (BioRad) according to the manufacturer’s instructions. The mRNA expression level of ADAM10 was analysed by real time qPCR using the SYBR green method (Applied Biosystems) with the sense and anti-sense primers previously reported (66). Samples were analysed in triplicate on a Quantstudio 3 (Life Technologies) and relative expression was calculated using ribosomal qPCR as the control.

Multiplex immunoassay. Aβ40 and Aβ42 were measured using the V-PLEX Aβ peptide panel 1 (6E10) assay (Meso Scale Discovery (MSD), #K15200E). sAPPα and sAPPβ were measured using the sAPPα/sAPPβ multiplex assay kit (MSD, #K15120E) according to the manufacturer’s instructions. Assay plates were blocked and conditioned cell medium samples and standards buffered with 500 mM HEPES, pH 7.4 to a final concentration of 50 mM were loaded in duplicate. Following washing, and secondary antibody incubation, assays were read using the Meso Quickplex SQ 120 and analysed using MSD Workbench 4.0 software. The protein concentration of the conditioned medium was determined by bicinchoninic acid assay and sAPPα, sAPPβ and Aβ levels corrected for total protein concentration.

ROS assay. For the detection of ROS in cells, H<sub>2</sub>O<sub>2</sub> production was measured with the ROS-Glo™ H<sub>2</sub>O<sub>2</sub> assay system (Promega). SH-SY5Y cells expressing PrP<sup>C</sup> were seeded onto black walled, clear bottom 96-well plates and allowed to adhere overnight. Cells were then incubated ± 20 µM acitretin diluted in OptiMEM for 48 h before incubation with or without AβO (500 nM) in the presence of 10 µM menadione (Sigma) and the H<sub>2</sub>O<sub>2</sub> substrate at 37°C for 90 min. ROS-Glo detection solution and signal enhancer were then added and, after 20 min incubation at room temperature, luminescence was measured with a Synergy HT Bio-Tek fluorimeter using Gen5 software. For experiments with cortical neurons the above method was used with the following modifications: cells were plated at d.35 and cultured until d.65. Following acitretin incubation, 1% BSA was added to cell cultures for 10 min to block the non-specific binding of AβO to the laminin coating on the plates prior to incubation with 2.5µM AβOs.

Statistical analysis. Data were analysed as stated in the figure legends, and n numbers are specified. For statistical analysis, data were analysed using GraphPad Prism 7.00. A normal distribution was assumed for all cell data as mean values were recorded from a population of cells (based on the assumption that cells from a clonal population will respond in a similar manner) and therefore parametric analyses were performed. For comparison between two data
sets, an independent t-test was applied with Welch’s correction (equal standard deviations not assumed). For the rat primary hippocampal neuron data a Mann Whitney U test was used to compare between groups as a normal distribution could not be assumed or determined from the sample size. For multiple comparison a one-way ANOVA with Tukey’s post-hoc correction for pairwise comparisons was used. Data are shown as mean ± SEM and p<0.05 was considered significant. Levels of significance are defined in the figure legends.
Acknowledgements: We gratefully acknowledge the financial support of Alzheimer’s Research UK (PG2013-12), the Medical Research Council (MC_PC_16033, MR/N013255/1, MR/M024997/1, MC EX MR/N50192X/1), the Dr Donald Dean Fund in Dementia Research, and the University of Manchester. The Bioimaging Facility microscopes used in this study were purchased with grants from BBSRC, Wellcome and the University of Manchester Strategic Fund. The ImagestreamX MkII used in this study was purchased with funding from the Medical Research Council and accessed through the University of Manchester Flow Cytometry Core Facility. None of the funding bodies had any role in the design of the study, the collection, analysis or interpretation of the data, or in the writing of the manuscript.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Authors’ contributions: HHJG, KABK and NMH designed the study. HHJG, NJC, HAR, KF, ACJ, GJH, JB and KABK performed the research. SAC, SC and MZC provided reagents. HHJG, NJC, KF, GJH, KABK and NMH analysed the data. HHJG, KABK and NMH wrote the paper. All authors read and approved the final manuscript.
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FOOTNOTES
List of abbreviations: AβO, amyloid-β oligomers; AD, Alzheimer’s disease; APP, amyloid precursor protein; ADAM10, a disintegrin and metalloprotease 10; BACE1, β-site APP cleaving enzyme 1; iPSC, induced-pluripotent stem cell; LTP, long term potentiation; NMDA, N-methyl-D-aspartate; PrPC, cellular prion protein; ROS, reactive oxygen species; sAPPα, soluble APP fragment produced by α-secretase cleavage; sAPPβ, soluble sAPPβ produced by β-secretase cleavage.

Figure legends
Figure 1. Promoting the shedding of PrPC decreases AβO binding in an ADAM10 dependent manner. (A) Immunoblots of sPrPC and sAPPα in concentrated conditioned media, and of ADAM10, PrPC, APP and actin in cell lysates, from SH-SY5Y cells expressing PrPC incubated ± carbachol (Cch, 20µM) and ± GI254023X (GI, 10µM) in OptiMEM for 24h at 37°C. (B) sPrPC, (C) sAPPα and (D)ADAM10 immunoblots were quantified and are represented as a percentage of control (n=4). (E) ImageStream imaging cytometry analysis showing negative (N), mid-(M) and high-(H) levels of cell surface PrPC expression in SH-SY5Y cells expressing PrPC incubated without (blue) and with (red) carbachol (Cch, 20µM). (F) Immunofluorescence microscopy images showing staining of PrPC (green) and Aβ-biotin (red) in non-permeabilised SH-SY5Y cells expressing PrPC incubated ± carbachol (20µM) in OptiMEM for 3h at 37°C followed by incubation with AβO (500nM) for 30 min at room temperature. Scale bar = 5µm. (G) Quantification of PrPC cell surface expression (n=3) and AβO cell surface binding (n=3). (H) Immunoblots of PrPC in cell lysates, prepared from SH-SY5Y cells expressing PrPC incubated ± carbachol (20µM), and treated without or with N-glycanase for 16 h at 37°C. (I) Quantification of deglycosylated full-length (FL) and C1 PrPC species, expressed as ratio of FL:C1 (n=4). Statistical analyses: one-way ANOVA with Tukey’s post-hoc correction for multiple-comparison data, or independent t-test with Welch’s correction for two-sample comparison. Data shown as mean ± SEM, n.s. not significant, *p<0.05, **p<0.01, ****p<0.0001.

Figure 2. Knockdown of ADAM10 reduces the shedding of PrPC and increases AβO binding. (A) Immunoblots of ADAM10 (pro- (p) and mature (m) forms), PrPC, APP and actin in cell lysates and of sPrPC and sAPPα in concentrated conditioned media from SH-SY5Y cells expressing PrPC incubated with either siRNA targeted against ADAM10 (+) (Dharmacon, smartpool) or a non-targeting (-) siRNA control for 48 h followed by incubation with OptiMEM for a further 24 h. (B) ADAM10, (C) PrPC and sPrPC and (D) APP and sAPPα immunoblots were quantified and are represented as a percentage of control (n=4-6). (E) Immunofluorescence microscopy images showing staining of PrPC (green) and Aβ-biotin (red) in non-permeabilised SH-SY5Y cells expressing PrPC following siRNA treatment targeted against ADAM10 and incubation in the absence or presence of the PrPC antibody, 6D11, for 20 min at 37°C followed by AβO incubation (500nM) for 30 min at room temperature. Scale bar = 5µm. (F) Quantification of PrPC cell surface staining (n=5) and (G) AβO binding to cells (n=5). Statistical analyses: one-way ANOVA with Tukey’s post-hoc correction for multiple-comparison data, or independent t-test with Welch’s correction for two-sample comparison. Data shown as mean ± SEM, n.s. not significant, * p<0.05, **p<0.01, ****p<0.0001.

Figure 3. Knockdown of ADAM10 with an independent siRNA sequence reduces the shedding of PrPC and increases AβO binding. (A) Immunoblots of ADAM10 (pro- (p) and mature (m) forms), PrPC, APP and actin in cell lysates and of sPrPC and sAPPα in concentrated conditioned media from SH-SY5Y cells expressing PrPC incubated with either siRNA targeted against ADAM10 (+) (Ambion, single siRNA) or a non-targeting (-) siRNA control for 48 h followed by incubation with OptiMEM for a further 24 h. (B) ADAM10, (C) PrPC and sPrPC and (D) APP and sAPPα immunoblots were quantified and are represented as a percentage of control (n=3). (E) Immunofluorescence microscopy images showing staining of PrPC (green) and Aβ-biotin (red) in non-permeabilised SH-SY5Y cells expressing PrPC
following siRNA treatment targeted against ADAM10 and incubation in the absence or presence of the PrP<sup>C</sup> antibody, 6D11, for 20 min at 37°C followed by AβO incubation (500nM) for 30 min at room temperature. Scale bar = 5µm. (F) Quantification of PrP<sup>C</sup> cell surface staining (n=5) and (G) AβO binding to cells (n=5). Statistical analyses: one-way ANOVA with Tukey’s post-hoc correction for multiple-comparison data, or independent t-test with Welch’s correction for two-sample comparison. Data shown as mean ± SEM, n.s. not significant, * p<0.05, **p<0.01, ***p<0.001.

Figure 4. ADAM10 fails to increase AβO binding in cells lacking PrP<sup>C</sup>. Immunoblot of ADAM10 and actin in cell lysates and of sAPPα in concentrated conditioned media from untransfected SH-SY5Y cells, which lack endogenous PrP<sup>C</sup>, incubated with either siRNA targeted against ADAM10 (+) or a non-targeting (-) siRNA control for 48 h followed by incubation with OptiMEM for a further 24h. ADAM10 and sAPPα immunoblots were quantified and are represented as a percentage of control (n=3). (C) Immunofluorescence microscopy images showing staining of Aβ-biotin (red) in non-permeabilised untransfected SH-SY5Y cells following siRNA treatment targeted against ADAM10 and incubation with AβO (500nM) for 30 min at room temperature. Scale bar = 5µm. (D) Quantification of AβO binding to cells (n=5). Statistical analyses: independent t-test with Welch’s correction for two-sample comparison. Data shown as mean ± SEM, **p<0.01, ****p<0.0001.

Figure 5. Acitretin increases PrP<sup>C</sup> shedding and decreases AβO binding and toxicity in an ADAM10-dependent manner. (A) Immunoblots of sPrP<sup>C</sup> and sAPPα in concentrated conditioned media and of ADAM10, PrP<sup>C</sup>, APP and actin in cell lysates from SH-SY5Y cells expressing PrP<sup>C</sup> incubated ± GI254023X (GI, 10µM) and ± acitretin (Acit) (20µM) diluted in OptiMEM for 48h at 37°C. (B) sPrP<sup>C</sup>, (C) sAPPα and (D) ADAM10 immunoblots were quantified and are represented as a percentage of control (n=4). (E) Relative expression of ADAM10 mRNA in SH-SY5Y cells expressing PrP<sup>C</sup> incubated ± acitretin (Acit, 20µM) (n=6). (F) ImageStream imaging cytometry analysis showing negative (N), mid-(M) and high-(H) levels of cell surface PrP<sup>C</sup> expression in SH-SY5Y cells expressing PrP<sup>C</sup> incubated without (blue) and with (red) acitretin (Acit, 20µM). (G) Immunofluorescence microscopy images showing staining for PrP<sup>C</sup> (green) and Aβ-biotin (red) in non-permeabilised SH-SY5Y cells expressing PrP<sup>C</sup> incubated ± acitretin (20µM) in OptiMEM for 48h at 37°C followed by incubation with AβO (500nM) for 30 min at room temperature. Scale bar = 5µm. (H) Quantification of PrP<sup>C</sup> cell surface staining (n=5) and AβO-binding to cells (n=5). Statistical analyses: independent t-test with Welch’s correction for two-sample comparison. Data shown as mean ± SEM, n.s. not significant, * p<0.05, **p<0.01, ***p<0.001.

Figure 6. Activation of ADAM10 by acitretin decreases surface PrP<sup>C</sup> and decreases AβO toxicity in iPSC-derived neurons. (A) Immunofluorescence microscopy images showing staining for the pluripotency markers Pax6, Oct4, SSEA-4 and Nanog in permeabilised OX1-19 iPSCs. Scale bar = 200µm. (B) Immunofluorescence microscopy images showing staining for the neuronal markers Satb2, Tbr1, and MAP2 in OX1-19 iPSCs differentiated to cortical neurons at day 60. Scale bar = 100µm. (C) Immunoblots of APP, ADAM10, PrP<sup>C</sup> and actin in cell lysates from OX1-19 iPSCs differentiated to neurons at day 65. (D) sAPPα was quantified, using the mesoscale discovery (MSD) system, in conditioned media from day 65 OX1-19 cortical neurons incubated ± GI254023X (GI, 10µM) and ± acitretin (20µM) in OptiMEM for 48h at 37°C (n=6). (E) Immunofluorescence microscopy images
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showing staining for PrPC (green) in day 50 OX1-19 cortical neurons incubated ± acitretin (20µM) in OptiMEM for 48h at 37°C. Scale bar = 5µm. (F) Quantification of PrPC cell surface staining on non-permeabilised MAP2 stained neurites, expressed as a percentage of control (n=3). (G) ROS were measured using the ROS-Glo assay in day 65 OX1-19 cortical neurons ± acitretin (20µM) in OptiMEM for 48h at 37°C. Cells were incubated with 1% BSA for 10 min to block the non-specific binding of AβO and then treated with or without AβO (2.5µM) for 90 min at 37°C. Luminescence was measured and is represented as a percentage of control (n=4-7). Statistical analyses: one-way ANOVA with Tukey post-hoc correction for multiple-comparison data, or independent t-test with Welch’s correction for two-sample comparison. Data shown as mean ± SEM, n.s. not significant, * p<0.05, **p<0.01, ****p<0.0001.

Figure 7. The effect of acitretin on Aβ production is cell-type dependent. (A) sAPPβ, (B) Aβ40 and (C) Aβ42 were quantified by MSD analysis in conditioned media from SH-SY5Y cells expressing PrPC incubated ± acitretin (20µM) in OptiMEM for 48 h at 37°C (n=3). (D) sAPPβ, (E) Aβ40 and (F) Aβ42 were quantified by MSD analysis in conditioned media from day 65 cortical neurons incubated ± acitretin (20µM) in OptiMEM for 48 h at 37°C (n=6 differentiations of two iPSC lines, OX1-19 and SBAD). (G) Immunoblot and quantification of sAPPα in concentrated conditioned media and quantification of (H) Aβ40 and (I) Aβ42 by MSD analysis in conditioned media from rat primary hippocampal neurons incubated ± acitretin (20µM) in OptiMEM for 48h at 37°C (n=3). Data are expressed as percentage of control. Statistical analyses: Mann Whitney U test for n=3 and independent t-test with Welch’s correction for n>3. Data shown as mean ± SEM, n.s. not significant, * p<0.05, **p<0.01, ***p<0.001.
**Figure 1**

(A) Western blot analysis showing the expression levels of sPrP, sAPPα, ADAM10, PrP, and APP as well as actin as a loading control. The blots show the protein bands at different molecular weights.

(B) Bar graph representing the relative expression levels of sPrP and sAPPα. The data indicates a significant increase in sPrP and sAPPα expression upon treatment with Cch.

(C) Bar graph showing the relative expression levels of APP. The treatment with Cch results in a significant increase in APP expression.

(D) Bar graph depicting the expression levels of ADAM10. Cch treatment leads to a significant increase in ADAM10 expression compared to the control.

(F) Immunofluorescence images of cells treated with Cch and control conditions. The images show the nuclear (blue) and cellular (green) staining, highlighting the effects of Cch on cellular morphology.

(H) Western blot analysis showing the expression levels of FL and C1 before and after treatment with N-Glycanase. The blots indicate a decrease in FL expression and an increase in C1 expression after N-Glycanase treatment.

(I) Bar graph representing the ratio of FL/C1 PrP. The ratio remains relatively unchanged after treatment with Cch.
Figure 3

A. Western blot analysis showing the expression levels of PrP\textsuperscript{c}, APP, actin, sPrP\textsuperscript{c}, and sAPP\textsubscript{α} with or without ADAM10 siRNA treatment. 

B. Graph showing the percentage of ADAM10 expression with or without ADAM10 siRNA treatment.

C. Graph showing the percentage of PrP\textsuperscript{c} expression with or without ADAM10 siRNA treatment.

D. Graph showing the percentage of sPrP\textsuperscript{c} expression with or without ADAM10 siRNA treatment.

E. Immunofluorescence images showing PrP\textsuperscript{c} surface staining with or without ADAM10 siRNA treatment.

F. Graph showing the percentage of PrP\textsuperscript{c} surface staining with or without ADAM10 siRNA treatment and 6D11 antibody.

G. Graph showing the percentage of Ab\textsubscript{40} surface staining with or without ADAM10 siRNA treatment and 6D11 antibody.
Figure 5

**A**

Western blot analysis showing the expression levels of various proteins. 

**B,C**

Graphs showing the percentage of sPrP and sAPPα in the control and treated groups.

**D**

Graph showing the percentage of ADAM10 in the control and treated groups.

**E**

Graph showing the relative mRNA expression of ADAM10.

**F**

Bar graph representing the relative PrP expression in control and Acit-treated groups.

**G**

Immunofluorescence images showing surface staining of PrPα and α-PrP.

**H**

Graph illustrating the surface staining percentage control and treated groups.

**I**

Western blot for pSFK and Fyn in the presence of Aβ and Acit.

**J,K**

Bar graphs showing the percentage of pSFK/Fyn and ROS in the control and treated groups.
Figure 6
Figure 7
Proteolytic shedding of the prion protein via activation of metallopeptidase ADAM10 reduces cellular binding and toxicity of amyloid-β oligomers

Heledd H Jarosz-Griffiths, Nicola J Corbett, Helen A Rowland, Kate Fisher, Alys C Jones, Jennifer Baron, Gareth J Howell, Sally A Cowley, Satyan Chintawar, M. Zameel Cader, Katherine A.B. Kellett and Nigel M. Hooper

J. Biol. Chem. published online March 14, 2019

Access the most updated version of this article at doi: 10.1074/jbc.RA118.005364

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