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The impact of amyloid precursor protein signalling and histone deacetylase inhibition on neprilysin expression in human prostate cells

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The zinc metallopeptidase, neprilysin (NEP), is an endothelin-1 degrading enzyme whose expression is extensively downregulated in prostate cancer. The expression of NEP in neuronal cells is regulated by intramembrane proteolysis of the amyloid precursor protein (APP) through its intracellular domain (AICD) facilitating histone acetylation of the NEP promoter and gene transcription. The present study has examined whether similar mechanisms operate in prostate cell lines. The expression of APP and its processing enzymes (β- and γ-secretases) was examined in a number of prostate cell lines, and the effect of γ-secretase inhibition was explored on NEP expression and activity. The potential interaction of AICD with the NEP promoter was examined by chromatin immunoprecipitation. Our results indicated that all key components involved in APP processing were expressed in prostate cancer cell lines but suppression of AICD production using a γ-secretase inhibitor did not decrease NEP expression and activity, and no direct AICD-NEP promoter interaction could be detected. However, histone deacetylase inhibitors (valproate and trichostatin A) caused a 2- to 3-fold increase in NEP expression in PC-3 cells, and combinatorial treatment with the DNA demethylating agent, AzaC, further increased NEP expression levels. Although AICD is detectable in prostate cell lines, it does not appear to regulate NEP by AICD-mediated signalling. Apart from promoter demethylation, the data suggest that histone acetylation may facilitate partial re-activation of NEP expression in advanced prostate cancer cells. Upregulation of this tumour-suppressing protein may provide a novel therapeutic strategy in prostate cancer.

Prostate cancer (PC) is the most frequently diagnosed cancer in western males. Advanced PC in the androgen-independent, chemo-refractory state is extremely life-threatening and currently incurable. In the progression of PC to the more malignant, androgen-independent phenotype, loss of a membrane-bound zinc metallopeptidase, neprilysin (NEP), is commonly found.[1,2] NEP is an integral membrane glycoprotein and ectoenzyme which serves to inactivate circulating regulatory peptides.[3] NEP has a wide tissue expression including intestinal and kidney epithelium, brain, skeletal muscle, lung and prostate.[4–8] NEP is able to inhibit cancer cell proliferation, migration and promote cell apoptosis during chemotherapy either *via* degradation of mitogenic neuropeptides such as endothelin-1 (ET-1), or by its direct protein–protein interactions with tumour suppressor PTEN, phosphatidylinositol 3-kinase (PI3K) and ezrin/radixin/moesin (ERM) proteins.[9] In prostate cancer cell lines, there is a reciprocal relation between expression levels of the ET-1 synthetic enzyme, endothelin-converting enzyme-1 (ECE-1) and the ET-1 degrading enzyme, NEP.[2] Hence, downregulation of ECE-1[10] or upregulation of NEP[11] are proposed as viable therapeutic strategies in advanced prostate cancer.

In the prostate, NEP expression is positively regulated by androgens, *via* interaction of the activated androgen receptor with two steroid hormone response elements within the *NEP* gene.[12] However, this mechanism is lost in advanced stages of PC, which has been attributed to an initial hormone withdrawal treatment and additionally by hypermethylation of the 5′ CpG island within the NEP promoters.[13]

NEP also plays a key role in the nervous system turning off neuropeptide signalling and degrading the Alzheimer's amyloid β (Aβ) peptide.[14,15] There is accumulating evidence showing that re-expression of NEP could effectively reduce Aβ plaques in mouse brain, which could therefore contribute to prevention or delaying the onset of Alzheimer's disease.[16] In neurons, a presenilin/γ-secretase-dependent, amyloid precursor protein (APP) intracellular domain (AICD)-mediated signalling event has been reported as a novel, intrinsic

Key words: AICD, amyloid precursor protein, neprilysin, presenilin, prostate cancer, γ-secretase, valproate
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mechanism of NEP regulation.[17,18] Transcriptionally active AICD is formed by the consecutive actions of β-secretase (BACE-1) on APP followed by γ-secretase cleavage of the membrane-bound product.[19] The AICD-mediated upregulation of NEP was considered to act as a neuroprotective mechanism aiding clearance of excess Aβ in the brain.[17] In this signalling pathway, AICD, released from the plasma membrane, is transported into the nucleus and then binds to the NEP promoter as a transactivator complex with its binding partner, adaptor protein Fe65 (Fig. 1*a*).[17,20,21] Considering that Alzheimer's disease and PC are both age-related diseases and are associated with NEP loss during pathogenesis, we have explored whether such an AICD-mediated signalling mechanism is also conserved in prostate cells.

In neurons and neuroblastoma cells, chromatin remodelling plays an important role in NEP regulation, and use of chromatin modulators was shown to reactivate NEP transcription[21] and hence they have potential in the treatment of neurodegenerative disorders.[22] Increased HDAC expression and activity is commonly found in numerous human cancers, such as gastric and prostate cancers.[23,24] *Via* re-activation of a number of tumour-suppressing genes by induction of histone acetylation, treatment of cancer cells with HDAC inhibitors has been shown to decrease cell growth and invasion, provide enhanced sensitivity to chemotherapy agents and reduce epithelial-to-mesenchymal transition.[25,26] Hence, we have examined whether HDAC activity provides another causal factor leading to NEP loss in advanced PC cells.

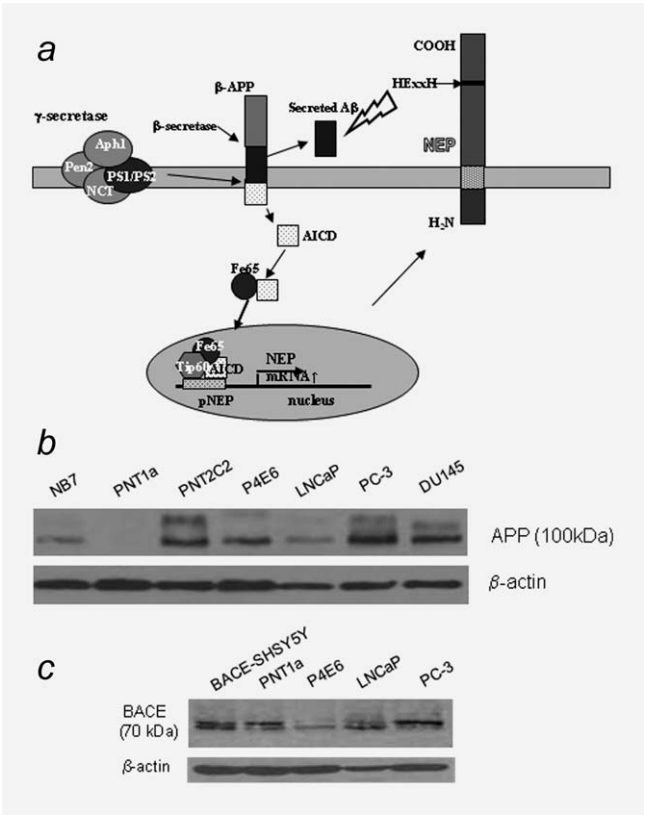

Figure 1. A model for the PS-dependent transcriptional control of NEP expression by AICD, and expression of APP and BACE-1 in human prostate cancer cell lines. (*a*) APP is cleaved sequentially by the aspartic proteinases, β-secretase and γ-secretase. This causes the production of the Aβ peptide and the APP intracellular domain (AICD). After AICD is released from the plasma membrane, it interacts with Fe65, undergoes nuclear translocation and activates transcription of NEP and other genes upon forming a transcriptional complex together with Tip60 (adapted from Pardossi-Piquard, *et al,* 2005). (*b*) Total cellular proteins (20 μg) were loaded per well. All samples were denatured by heating for 10 min at 70°C and then separated by electrophoresis on a 4–12% Bis-Tris SDS-PAGE gel. Proteins were probed by anti-APP antibody (1:2,000) at 4°C overnight and visualized by ECL. NB7 cell extracts were used as a positive control for APP. (*c*) BACE-1 levels were detected by Western blotting. Cellular proteins (30 μg per well) were loaded and separated by 3–8% Tris-acetate SDS-PAGE gel, followed by 2 hr incubation with anti-BACE antibody (1:1,000). SH-SY5Y cells overexpressing WT-BACE were used as a positive control.

Material and Methods

Cell culture

SH-SY5Y neuroblastoma cells were cultured in DMEM-F12 supplemented with 10% FBS (Lonza, Basel, Switzerland), 2 mM L-glutamine and 0.5% non-essential amino acids. NB7 neuroblastoma cells were cultured in RPMI-1640 containing 10% FBS and 10 mM L-glutamine. Non-malignant transformed PNT1a, PNT2-C2 and P4E6, malignant androgen-sensitive LNCaP, malignant androgen-refractory PC-3 and DU145 human prostate cell lines were grown in RPMI 1640 supplemented with 10% FBS and 2 mM L-glutamine with the following exceptions: P4E6 cells were cultured in Keratinocyte-SFM supplemented with 2% FBS, 2 mM Glutamine, 25 μg/ml bovine pituitary extract and 0.2 ng/ml recombinant epidermal growth factor, PC-3 cells were cultured in Ham's F-12 supplemented with 7% FBS and 2 mM L-glutamine. All cells were routinely maintained at 37°C with 5% CO_2.

Antibodies

Anti-APP (66-81 N-terminal) antibody was obtained from Chemicon (Temecula, CA) and used at 1:2,000 dilution for immunoblotting. Anti-ECE-1 monoclonal antibody, AEC 32-236 (generously donated by Dr. K. Tanzawa, Sankyo Research Laboratories, Tokyo) and anti-NEP monoclonal antibody, NLC-L-CD10-270 (from Novocastra, UK) were used at 1:200 and 1:100 dilutions, respectively, for immunoblotting. Anti-BACE-1 (EE17) and anti-Nicastrin antibodies were obtained from Sigma Aldrich (Gillingham, Dorset, UK) and used at 1:100 dilution for immunofluorescent (IF) microscopy, 1:1,000 dilution for immunoblotting. Anti-presenilin 1, N-terminal (1–65) and anti-presenilin 1, loop domain (263–407) polyclonal antibodies were purchased from Merck Biosciences, UK and used at 1:200 for IF microscopy, 1:2,500

for immunoblotting. Anti-Fe65 polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA) and used as 1:1,000 dilution for immunoblotting. Anti-APP C-terminal (anti-AICD) polyclonal antibody was purchased from Sigma Aldrich (Gillingham, Dorset, UK) and used at 1:1,000 dilution for IF microscopy, 1 μg per assay for chromatin immunoprecipitation (ChIP), and used at 1:2,000 for immunoblotting. Anti-IDE polyclonal antibody (Covance, Emeryville, CA) was used at 1:1,000 dilution for immunoblotting. Anti-β-actin antibody (Sigma Aldrich, UK) 1:10,000 dilution and anti-mouse/anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Novagen, UK) 1:2,000 dilution were used for immunoblotting. Alexa Fluor Molecular Probe 488 goat anti-rabbit secondary antibody (Invitrogen, Paisley, UK) was used at 1:1,000 dilution for IF microscopy.

Cell treatments

P4E6 and LNCaP cells were split to 70% confluence in T_{75} cm^2 flasks 24 hr before treatment and were then incubated with the γ-secretase inhibitor, L-685,458, for 24–48 hr. Cells cultured with the equivalent volume of DMSO were used as a vehicle control. NB7, PNT1a, PC-3 and DU145 cells were treated with 10 μM L-685,458 for 48 hr before harvesting for AICD detection. The demethylating agent 5-aza-2′-deoxycytidine (AzaC, 1 μM) and the histone deacetylase inhibitors sodium valproate (VA, 1–100 μM) or trichostatin A (TSA, 10 nM–10 μM) were added to PC-3 cells at 70–80% confluency and left for 48 hr under normal culture conditions. TSA (1 μM) treatment in combination with L-685,458 (10 μM) was also applied to PC-3 and cells were harvested 48 hr post-treatment. Cytoplasmic and nuclear proteins of harvested cells were fractionated as described previously.[27]

Western blotting

Cellular proteins were heated for 10 min at 70°C and separated by 3–8% Tris-Acetate gels with the exception that, when detecting presenilins, cellular proteins were denatured for 20 min at room temp and separated on 4–12% Bis-Tris gels with Mes buffer and, when detecting AICD, proteins were separated on 10–20% Tricine gels (Invitrogen, Paisley, UK). Following protein transfer, nitrocellulose membranes or PVDF membranes, particularly for AICD blotting, were blocked in TBST (10 mM Tris/HCl buffer, pH 7.4, with 0.05% Tween-20) containing 5% (w/v) milk powder, then incubated with specific primary antibodies for 2 hr at room temp or overnight at 4°C, followed by 1-hr incubation with secondary antibodies. Specific protein bands were visualized using enhanced chemiluminescence (ECL, Pierce, Thermoscientific, Northumberland, UK) according to the manufacturer's instructions.

Immunofluorescence

Cells were grown on sterile cover slips in six-well culture plates at 60% confluence, fixed in formaldehyde at a final concentration of 3.7% for 5 min then permeabilised using 0.1% Triton X-100 in PBS for 5 min and fixed again for 5 min at room temp. Residual formaldehyde was quenched using 50 mM NH_4Cl in PBS for 10 min. Non-specific binding sites were blocked with blocking buffer [5% (v/v) normal goat serum and 0.02% (w/v) sodium azide in PBS] for 30 min. Cells were labelled with anti-nicastrin or anti-AICD antibody (diluted in blocking buffer) for 2 hr, washed with PBS, then incubated with Alexa Fluor Molecular Probe 488 goat anti-rabbit secondary antibody (diluted in blocking buffer) for 30 min. Following counterstaining with 4′,6-diamidino-2-phenylindole (DAPI; 1:1,000 in PBS), cells were washed with PBS, and mounted on glass slides using Vectashield (Vector Laboratories, Peterborough, UK). Cells were viewed using an Olympus IX 70 inverted wide-field fluorescence microscope, and images were captured by Delta Vision.

Activity assay

Cellular proteins were extracted in the presence of EDTA-free protease inhibitor cocktail (Roche Diagnostics, Burgess Hill, Sussex, UK). The activity of NEP was assayed in 96-well plates by mixing 1 μg of cellular proteins with 50 μM substrate, Suc-AAF-AMC (Bachem, UK), 50 mM HEPES buffer with 200 mM NaCl (pH 7.2), four milli-units of Leu-aminopeptidase (Sigma, UK) in a total volume of 100 μl per assay.[21] After thorough mixing, fluorescent products were measured using a Wallac Victor 1420 multilabel plate-reader. NEP-specific activity could be suppressed completely in the presence of its selective inhibitor, phosphoramidon (10 μM).

ChIP

Adherent cells were fixed by 1% formaldehyde in PBS for 10 min at room temperature. Glycine (0.125 M) was added to the cells to stop fixation. Following thorough washing with PBS, fixed cells were scraped and pelleted by 10 min centrifugation at 2,000 rpm. Cells were suspended in 2 ml lysis buffer (10 mM Tris.Cl, pH 8.0, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 1 mM PMSF and protease inhibitor cocktail), and DNA was fragmented by 4 × 30 sec sonication at 52% power. Cell extracts were incubated overnight at 4°C with anti-AICD, normal rabbit IgG as a negative control, or acetylated H4 antibody as a positive control, and then precipitated by pre-blocked protein G-Sepharose beads for 2 hr. Beads were washed and bound compounds were eluted in 1% SDS-containing buffer as previously described.[21] Cross-links between protein and DNA were reversed by overnight incubation at 65°C in the presence of 200 μM NaCl, the proteins were then digested by proteinase K at 50°C for 1 hr, and DNA was extracted by phenol–chloroform (1:1), followed by PCR analysis using human *NEP* promoter primers.[17,21]

PCR

Precipitated DNAs were analysed either by conventional PCR using GoTaq DNA polymerase (Promega, UK) with the following amplification cycles: 94°C, 5min; 35 cycles of 94°C, 30 sec; 58°C, 30 sec; 72°C, 45 sec; final extension at 72°C, 10 min and chilled at 4°C, or by real-time PCR using iQ SYBR

Green Supermix and performed using an iCycler thermal cycler (Bio-Rad, Hemel Hempstead, UK).

Results

Presence of APP, β-secretase and γ-secretase components in prostate cell lines

Expression of APP and its two key cleavage machineries in amyloid production, the β- and γ-secretases, which are involved in AICD release, is a pre-requisite of PS-dependent, AICD-mediated gene transcriptional control.[19,27] Hence, we first examined the presence of APP and β-secretase (BACE-1) in prostate cells. Western blotting results showed that APP proteins were expressed in all examined prostate cell lines, being extremely high in PNT2C2 and PC-3 cells but rare in PNT1a cells (Fig. 1*b*). Compared to APP expression in the neuroblastoma cell line, NB7, APP in prostate cells was expressed at equivalently high or even greater levels. Results from Western blotting revealed that endogenous BACE-1 protein was detected in all PNT1a, LNCaP and PC-3 cells with levels comparable to those in SH-SY5Y cells overexpressing BACE-1 but showed a lower level in P4E6 cells (Fig. 1*c*).

Nicastrin (NCT) and presenilins (PS1/PS2) are two main components of a functional γ-secretase. As shown in Figure 2*a*, expression of NCT was detected in both LNCaP and P4E6 cell lines and localised at the plasma membrane. The active form of PS1, existing as an N-terminal fragment (NTF, 35 kDa) and a separate but interacting C-terminal fragment (CTF, 18–20 kDa), was shown to be present in human prostate cell lines at a level similar to that in the neuroblastoma SH-SY5Y cell line (Fig. 2*b*). PS1 levels fluctuated throughout the tested cell lines but no clear correlation with NEP or ECE-1 expression levels could be seen (Fig. 2*b*). However, the levels of the ET-1 synthetic enzyme, ECE-1, and the ET-1 degrading enzyme, NEP, showed a reciprocal relationship in expression levels between the cell lines consistent with a previous report (Fig. 2*b*).[2] Densitometry data indicated that PS1-NTF and PS1-CTF levels did not differ substantially between any non-cancerous and carcinoma cell lines (Fig. 2*c*). These results imply that a change of active PS1 levels is not a common event occurring in prostate cells under cancerous conditions and may not be a causative factor responsible for NEP loss in PC cells.

Nuclear accumulation of AICD is a pre-requisite for its transactivating activity. To examine the existence of a potential AICD-mediated regulatory mechanism for NEP in prostate cells, subcellular AICD localisation was examined by both immunofluorescence (Fig. 3*a*) and cellular fractionation followed by Western blotting (Fig. 3*b*). As a cell line possessing an established AICD-mediated NEP regulatory mechanism, NB7 cells showed strong AICD staining within cell nuclei, corresponding to AFT nuclear transcription complexes,[28] and in perinuclear regions. In contrast, AICD staining in LNCaP cells was weak and was apparently excluded from nuclei. In PNT1a cells, AICD staining was detectable in the nuclear region, but was more diffuse than in NB7 cells. It is noteworthy that AICD was, however, readily detectable in PC-3 cells despite no NEP expression in this cell line, and showed a heterogeneous staining pattern indicating both nuclear AICD staining (indicated by red arrows) and cytoplasmic accumulation of AICD.

To confirm the immunofluorescence data, AICD presence was examined in nuclear and cytosolic fractions prepared from prostate cell extracts by immunoblotting, using techniques previously established with neuronal cell lines.[27] As shown in Figure 3*b*, AICD (6.5 kDa) was detectable by immunoblotting in both cytoplasmic and nuclear fractions of prostate cell extracts. Nuclear AICD was readily detected in PC-3 and DU145 cells by immunoblotting but was much less prominent in non-malignant PNT1a cells, consistent with weak APP expression in this cell line as shown in Figure 1*b*. After treatment of cells with the γ-secretase inhibitor, L-685,458 (10 μM), AICD was sharply decreased in all these cell lines, including the neuronal NB7 as shown previously.[21] These results established the existence of a γ-secretase activity-dependent AICD production in prostate cells. However, nuclear presence of AICD did not necessarily correlate with NEP expression, *e.g.* in PC-3 cells, implying other transcriptional regulatory processes predominate, at least in relation to the *NEP* gene.

Does AICD interact with the NEP promoter in prostate cells?

The direct binding of AICD to the NEP promoter was examined by ChIP assay in three NEP-expressing prostate cell lines: PNT1a, P4E6 and LNCaP. Unlike a strong NEP signal detected by ChIP in NB7 cells (Fig. 4*a*), a neuroblastoma cell line having high NEP expression and direct binding of AICD to its promoter,[21] no signal was observed in the prostate cell line PNT1a *via* conventional PCR analysis following anti-AICD antibody precipitation (Fig. 4*a*). Antibody to histone H4 was used as a positive control and IgG as a negative control. Similar experiments were performed with P4E6 and LNCaP prostate cell extracts and the immunoprecipitated NEP in these samples was then quantified by real-time PCR. While AICD clearly bound to the NEP promoter in NB7 cells (Fig. 4*a*), there was no detectable AICD-NEP promoter binding with the P4E6 or LNCaP extracts, although the H4 control was positive (Fig. 4*b*).

Effect of γ-secretase inhibition on NEP activity in P4E6 and LNCaP cells

Consistent with the ChIP results, treatment of P4E6 and LNCaP cells with the γ-secretase inhibitor L-685,458 (1–10 μM; 24–48 hr) did not result in any reduction of NEP activity (Table 1). No significant change in either NEP or ECE-1 protein levels was detected by Western blotting in these cells upon L-685,458 treatment (data not shown). These results imply that the regulation of NEP transcription is independent of γ-secretase activity and hence AICD production in these cells, which is most likely due to an absence of AICD–*NEP* promoter interaction.

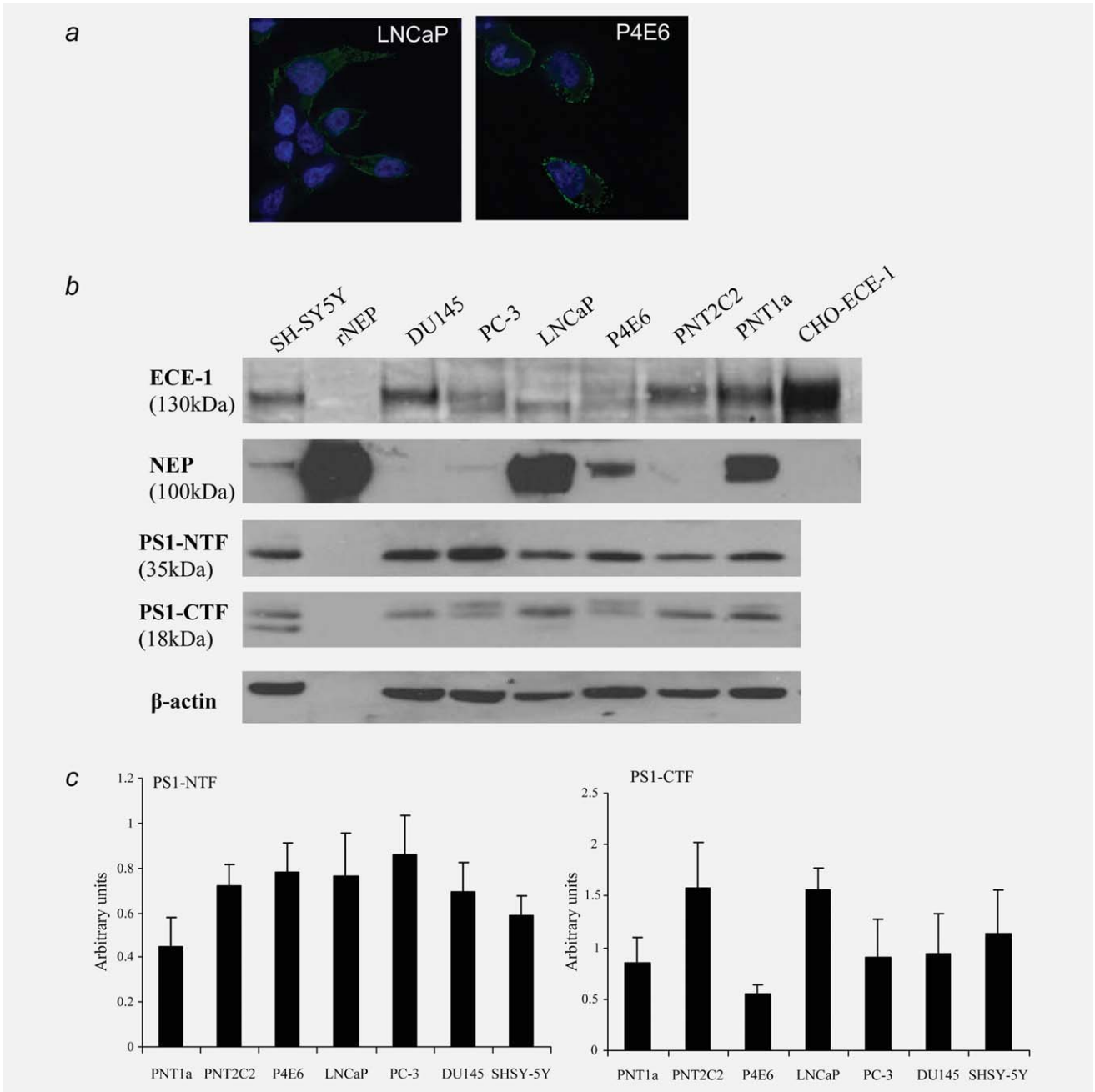

Figure 2. Expression of Nicastrin, ECE-1, NEP and PS1 N-/C-terminal fragments in human prostate cancer cell lines. (*a*) Nicastrin (NCT) expression and localization in prostate LNCaP and P4E6 cell lines was visualized by immunofluorescence microscopy. Cells were fixed using formaldehyde, and permeabilised using 0.1% Triton X-100. Non-specific binding sites were blocked in 5% normal goat serum and the cells were incubated in 1:100 anti-NCT primary antibody. Cells were then incubated with 1:1,000 goat anti-rabbit secondary antibody, and also stained with DAPI. (*b*) Western blotting image showing ECE-1, NEP and PS1 N-terminal fragments and C-terminal fragments (PS1-NTF/CTF) in a panel of human prostate cell lines. Neuroblastoma SH-SY5Y cells were used as a positive control for PS1. Cellular proteins were treated with DTT at room temperature for 20 min. Each sample (20 μg) was loaded and separated by 4–12% Bis-Tris SDS-PAGE gel electrophoresis using MES buffer. Specific proteins were probed with anti-PS1-NTF/CTF antibodies (1:2,500), then visualised by ECL. (*c*) Densitometry data showing relative PS1-NTF/ CTF levels among samples, normalised to β-actin. All data are presented as mean ± SD from three independent experiments.

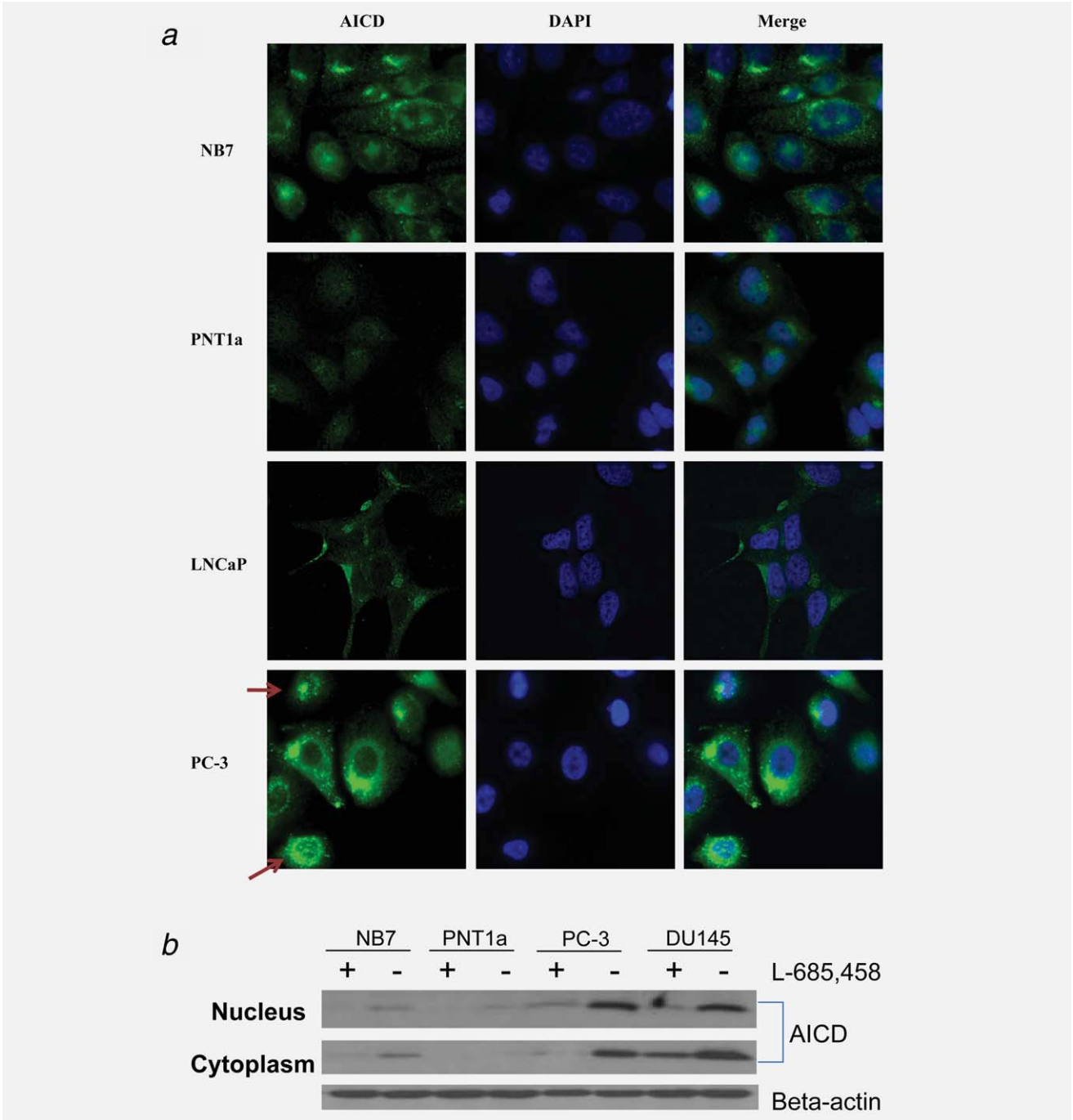

Figure 3. Analysis of AICD subcellular localisation. (*a*) Comparison of AICD subcellular localisation in prostate PNT1a, LNCaP, PC-3 cells and neuroblastoma NB7 cells by immunofluorescent microscopy. Cells were fixed and permeabilised as described in Methods. Upon blocking in 5% normal goat serum, cells were probed with 1:1,000 anti-AICD primary antibody, then incubated with 1:1,000 goat anti-rabbit secondary antibody. Cell nuclei were stained with 1:1,000 DAPI. Images were examined using an Olympus IX 70 inverted wide-field fluorescence microscope, and captured using Delta Vision. (*b*) AICD levels in cytoplasmic and nuclear fractions were examined in both prostate and neuroblastoma cells by Western blotting. Cytoplasmic and nuclear proteins were fractionated as described previously.[27] Proteins were denatured at 70°C for 10 min and then separated on 10–20% Tricine gels, followed by electrotransfer to PVDF membranes. AICD (6 kDa) was visualized as routine by ECL. Cells were also treated with 10 μM L-685,458 for 48 hr and then harvested for cytoplasmic/nuclear fractionation as above, followed by AICD detection. NB7 cells were used as a positive control for AICD.[27]

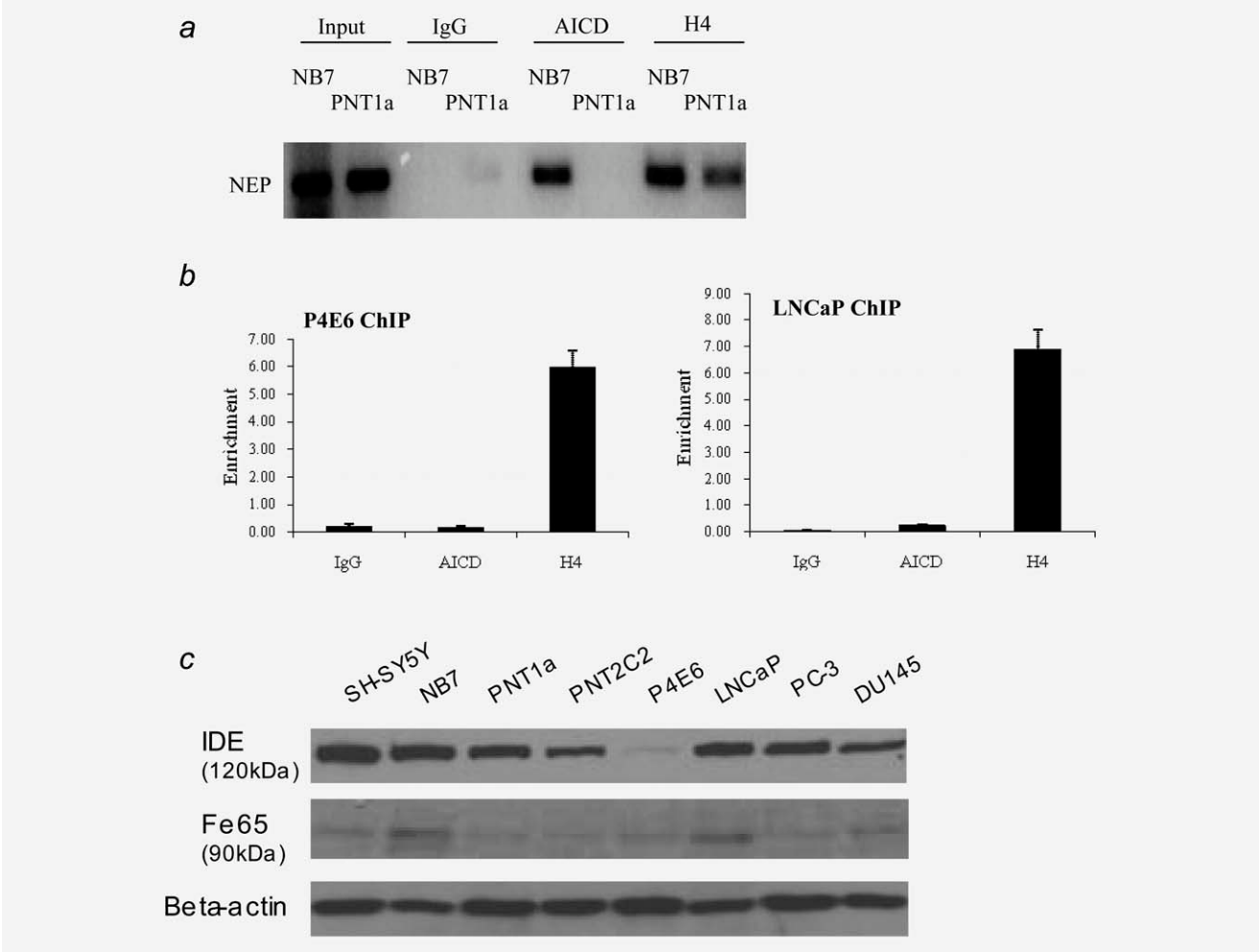

Figure 4. ChIP analysis of potential interaction between AICD and the NEP promoter, and examination of Fe65 and IDE expression in prostate cells. (*a*, *b*) ChIP assay examining the interaction between AICD and the NEP promoter sequence as described previously.[21,27] Neuroblastoma NB7 cells were used as a positive control for AICD binding to the NEP promoter. Three prostate cell lines (PNT1a, P4E6 and LNCaP) expressing NEP were compared for pull-down of the NEP promoter by anti-AICD antibody. DNA was extracted from cells, fragmented by 4 × 30 sec sonication at 52% power, and then precipitated by anti-acetylated H4 (positive control), anti-AICD or normal rabbit IgG (negative control) antibodies. Precipitated DNA was analysed by both (*a*) conventional and (*b*) real-time PCR. (*c*) Expression of adaptor protein Fe65 and AICD-degrading enzyme IDE were detected in a panel of prostate cell lines by Western blotting. Extracts of neuroblastoma cells (SH-SY5Y and NB7) were used as positive controls. Cellular proteins (30 μg) were loaded and separated by SDS-PAGE electrophoresis, then probed with 1:1,000 anti-Fe65 and 1:1,000 IDE antibodies.

AICD stability and Fe65 expression in prostate cell lines

AICD is a labile molecule and insulin-degrading enzyme (insulysin; IDE) is the main enzyme responsible for AICD degradation. Moreover, binding of AICD with adaptor protein Fe65 is required for AICD nuclear translocation and the subsequent formation of an active transcription complex (AICD/Fe65/Tip60) in the cell nucleus.[20] Hence, to explore possible reasons for the apparent absence of AICD-NEP promoter interaction in prostate cells, IDE and Fe65 expression were compared among prostate cell lines. IDE and Fe65 levels did not differ significantly among the cell lines examined (Fig. 4*c*), apart from lower IDE expression in P4E6 cells. These levels also did not correlate with AICD (Figs. 3*a* and 3*b*) or NEP (Fig. 2*b*) levels. Notably, there was no less IDE expression shown in prostate cancer PC-3 and DU145 cells, which maintain AICD at a much higher level than the others. Also, Fe65 expression in LNCaP cells was shown to be comparable to that in NB7 cells (Fig. 4*c*), although there was no obvious AICD nuclear translocation detected in this prostate cell line (Fig. 3*a*).

Histone deacetylase inhibitors are able to upregulate NEP transcription in metastatic PC cells

CpG hypermethylation of *NEP* in PC-3 and DU145 cells is thought to be a key factor blocking *NEP* transcription.[13] It has long been recognized that methylated DNA can recruit

Table 1. Effects of the γ-secretase inhibitor, L-685,458, on NEP activity in LNCaP and P4E6 prostate cells

		NEP-specific activity [nmol/min/mg protein]	
Treatment	Samples	LNCaP	P4E6
24 hr	Untreated	152 ± 3.2	21 ± 1.1
	DMSO	146 ± 3.2	21 ± 0.6
	L-685,458 1 μM	142 ± 2.1	22 ± 0.4
	L-685,458 5 μM	158 ± 8.7	21 ± 0.4
	L-685,458 10 μM	154 ± 7.5	21 ± 0.4
48 hr	Untreated	142 ± 10.4	22 ± 0.8
	DMSO	151 ± 7.7	22 ± 0.9
	L-685,458 1 μM	153 ± 3.7	21 ± 1.3
	L-685,458 5 μM	153 ± 6.9	24 ± 2.3
	L-685,458 10 μM	151 ± 5.4	22 ± 0.4

Cells were treated with L-685,458 at the stated concentrations or with vehicle (DMSO) for 24 or 48 hr before harvesting and measurement of NEP activity by fluorometry. Data represent the mean of three experiments ± SD.

HDAC and, conversely, that HDAC can reinforce DNA methylation.[29,30] Hence, we examined whether HDAC inhibition could increase NEP expression in PC cells. The HDAC inhibitor VA was applied to PC-3 cells and its NEP transcription and activity were then examined. It was found that VA at a concentration of 100 μM could significantly increase NEP mRNA levels to ∼230% of that in the untreated cells. Although there was a trend to upregulate NEP transcription, lower concentrations (1–10 μM) of VA were shown to be insufficient to induce a significant change in *NEP* mRNA levels (Fig. 5*a*). Consistently, an increase in NEP at the protein level and its enzymatic activity after 48-hr treatment with 100 μM VA was also revealed by Western blotting and fluorometric activity assay (Figs. 5*b* and 5*c*), showing a 2.9-fold and 1.7-fold increase in NEP protein and activity, respectively.

As VA is a drug with multiple actions apart from its HDAC inhibitory effect, TSA, a more specific HDAC inhibitor, has also been examined and showed a similar inductive effect on NEP transcription in PC-3 cells (Fig. 5*d*). TSA at concentrations higher than 100 nM was shown to increase NEP transcription significantly and the maximal effect occurred at a concentration of 1 μM, resulting in a 3.2-fold increase in NEP mRNA levels (Fig. 5*d*) and 1.7-fold increase in activity at 48-hr post-treatment (Fig. 5*e*).

Combinatorial treatment with an HDAC inhibitor and a demethylating agent is able to increase NEP expression additively in metastatic PC cells

To examine whether the combinatorial use of HDAC inhibitor and demethylating agent could exert a synergistic effect on NEP re-expression, PC-3 cells were treated with AzaC and VA/TSA simultaneously under normal culture conditions. As shown in Figure 5*f*, 1 μM AzaC alone was shown to increase NEP mRNA to ∼300% of its level in the untreated cells. This effect could be further enhanced to 440 and 510% in the combinatorial treatments with 100 μM VA or 1 μM TSA, respectively. In contrast, there was no additional effect detected in a combinatorial treatment with AzaC and 100 nM TSA on top of the effect by AzaC alone. By fluorometric assay, the NEP activity was found to be increased 1.6-fold after treatment with 1 μM AzaC alone, and this was further enhanced to 2-fold and 3.5-fold, respectively, when 100 μM VA or 1 μM TSA was applied in combination with AzaC (Fig. 5*g*).

TSA-induced NEP upregulation is independent of AICD generation

As nuclear AICD levels were detectable in PC-3 cells (Figs. 3*a* and 3*b*), we examined whether AICD plays any role in NEP reactivation after HDAC inhibition. To address this, the effect of γ-secretase inhibitor on cells treated with TSA was tested. As shown in Figure 5*h*, NEP protein expression was induced in PC-3 cells after treatment with TSA (1 μM) alone but this effect was not reversed by treatment with L-685,458. This suggests that the HDAC inhibitor-induced upregulation of NEP is not caused by an AICD effect in these cells.

Discussion

A common feature among two age-related diseases, PC and Alzheimer's disease, is the loss of expression of the zinc peptidase NEP, important for degradation of the mitogenic ET-1 peptide in the prostate and of amyloid-β peptide in the brain. Upregulation of NEP expression may therefore be a viable therapeutic strategy in both diseases. The amyloid precursor protein (APP) intracellular domain (AICD) is a product of APP processing *via* β-secretase followed by γ-secretase cleavage at the ε-site, and this γ-secretase-mediated regulated intramembrane proteolysis (RIP) is catalysed by a protein complex involving nicastrin and presenilin.[31] AICD is very labile and can be further degraded in the cytoplasm by the IDE or the proteasome.[32,33] Due to its small size (∼6 kDa) and rapid degradation, this molecule was not identified until 13 years after the discovery of the APP protein.[34] However, as a molecule that was hypothesized to contribute to the pathophysiology of Alzheimer's disease, AICD has attracted increasing research interest in the past few years, and the understanding of its cellular functions has been quickly expanded. The involvement of AICD in different cellular pathways including gene transcription, apoptosis, development, synaptic plasticity and cytoskeletal dynamics has been suggested,[35] and AICD is proposed to act in a Notch intracellular domain (NICD)-like manner.[20,28] Several putative target genes have been implicated such as genes encoding glycogen synthase kinase-3β (GSK-3β, an enzyme playing an important role in the pathogenesis of Alzheimer's disease, Huntington's disease and Type II diabetes),[36] metastasis suppressor KAI1,[37] NEP,[17] tumour suppressor protein p53[38,39]

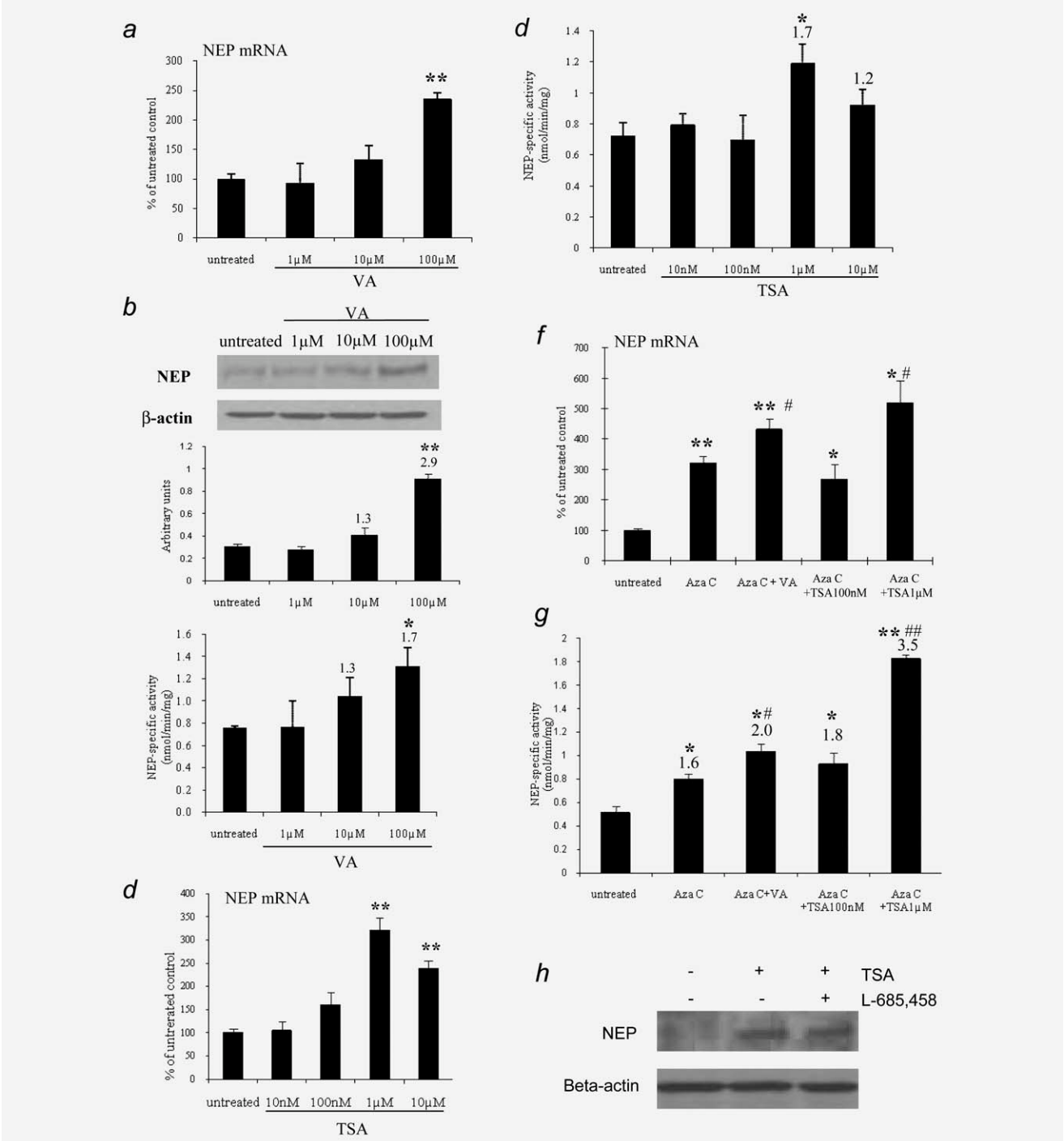

Figure 5. Effect of the histone deacetylase inhibitors, valproate (VA) and trichostatin A (TSA), and their combinatorial effect with AzaC or L-685,458 on NEP gene expression in prostate cancer PC-3 cells. (*a–c*) Cells were incubated with valproate at a final concentration of 1–100 μM for 48 hr under normal culture conditions. NEP mRNA (*a*), protein (*b*) levels and enzyme activity (*c*) were examined by real-time PCR, Western blotting and fluorometric activity assay, respectively. (*d–e*) Cells were incubated with TSA at a final concentration of 10 nM–10 μM for 48 hr under normal culture conditions. NEP mRNA (*d*) and enzyme activity (*e*) were examined. (*f–g*) Cells were either incubated with AzaC (1 μM) alone or in combination with VA (100 μM) or TSA (as indicated) for 48 hr under normal culture conditions. NEP mRNA (*f*) and enzyme activity (*g*) were examined. Significance of difference was tested by unpaired student's *t*-test, and $*p < 0.05$, $**p < 0.001$ were considered to be statistically significant. * indicates the significance compared with untreated control, while # indicates the significance compared with AzaC treated sample. (*h*) Cells were incubated with either TSA (1 μM) alone or in combination with L-685,458 (10 μM) for 48 hr under normal culture conditions. NEP protein levels were detected by Western blotting.

and, most recently, aquaporin-1.[40] Although the ability of AICD to regulate gene transcription has been controversial and intensively debated,[41–44] this regulatory mechanism has now been unequivocally demonstrated in neuronal cells.[17,19,21,27,40] However, its role in other cell types, including prostate cells, remains largely unexplored.

The present study has, for the first time, shown that APP, together with its processing enzymes BACE-1 and PS1, as well as nicastrin, Fe65 and IDE protein, have levels of expression in both normal and malignant human prostate cells at levels comparable to the model neuroblastoma lines, SH-SY5Y and NB7. AICD was also detectable in some prostate cell lines in both nuclear and cytosolic compartments. These findings suggest that APP or its metabolites may have potential cellular effects in the maintenance of prostate cell physiology which merit further investigation. APP itself can promote tumour growth[45] but BACE-1, nicastrin, Fe65 and AICD have not previously been examined in prostate cells. Treatment of prostate cells with L-685,458, a γ-secretase inhibitor that directly binds to PS1 with high potency,[46] did not cause a reduction in NEP expression, which argues against a direct role of the AICD metabolite of APP in the regulation of NEP transcription in prostate cells but does not exclude such a role in relation to other prostate-expressed genes. The overall cellular expression levels of NEP reflect both constitutive and regulated components that can vary and be cell specific. For example, in fibroblasts, AICD-regulated NEP activity is much higher than in blastocysts.[17] The lack of effect of AICD in prostate cells could just reflect the fact that, in these cells, most of the NEP expression is constitutive or regulated more powerfully by other means (positively by androgens and negatively by DNA hypermethylation).

In addition, the effect of HDAC inhibition on NEP transcription has been examined in prostate cancer cells, in which hypermethylation was previously shown to suppress transcription.[13] Despite the view that HDAC inhibition could only upregulate the expression of non-methylated genes but played little role in highly methylated genes,[47] there is increasing evidence that HDAC inhibitors can induce CpG demethylation *via* chromatin acetylation.[48,49] It has been found that the HDAC inhibitors, TSA and VA, could trigger DNA demethylation in a gene-specific manner, with preference in some but not all methylated tumour-suppressor genes, such as MMP2 and E-cadherin.[48,49] Our present study has established that the HDAC inhibitors VA (100 μM) and TSA (1 μM) can enhance NEP expression in PC-3 cells, at the levels of mRNA, protein and enzymic activity. Previous studies, *via* Southern blotting analysis for promoter modification, revealed a hypermethylation pattern within the 5′ CpG island of NEP promoters in all tested PC cell lines and tissues,[13] suggesting a major contribution of CpG methylation in PC-linked NEP suppression, which was presumed to be a key, but not sole, regulatory factor in NEP expression under cancerous conditions. In this context, the combined use of the demethylating agent AzaC with HDAC inhibitors VA and TSA on PC-3 cells is now shown to provide a significantly larger inductive effect on NEP transcription. Furthermore, the additional application of a γ-secretase inhibitor did not abolish the effect of an HDAC inhibitor on NEP upregulation, which excluded the possibility that the HDAC inhibitors were able to induce NEP in PC cells *via* AICD-mediated histone acetylation. Whether the presence of detectable nuclear AICD levels in a variety of prostate cancer lines reflects a role in regulating transcription of other genes is unclear but merits further consideration.

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References

1. Papandreou CN, Usmani B, Geng Y, Bogenrieder T, Freeman R, Wilk S, Finstad CL, Reuter VE, Powell CT, Scheinberg D, Magill C, Scher HI, et al. Neutral endopeptidase 24.11 loss in metastatic human prostate cancer contribute to androgen-independent progression. *Nat Med* 1998;4:50–7.
2. Usmani BA, Harden B, Maitland N J, Turner AJ. Differential expression of neutral endopeptidase-24.11 (neprilysin) and endothelin-converting enzyme in human prostate cancer cell lines. *Clin Sci* 2002;103 (Suppl 48):314S–7S.
3. Turner AJ, Tanzawa K. Mammalian membrane metallopeptidases: NEP, ECE, KELL, and PEX. *FASEB J* 1997;11:355–64.
4. Gee NS, Matsas R, Kenny AJ. A monoclonal antibody to kidney endopeptidase-24.11. Its application in immunoadsorbent purification of the enzyme and immunofluorescent microscopy of kidney and intestine. *Biochem J* 1983;214:377–86.
5. Matsas R, Kenny AJ, Turner AJ. An immunohistochemical study of endopeptidase-24.11 ("enkephalinase") in the pig nervous system. *Neurosci* 1986;18: 991–1012.
6. Broccolini A, Gidaro T, Morosetti R, Gliubizzi C, Servidei T, Pescatori M, Tonali PA, Ricci E, Mirabella M. Neprilysin participates in skeletal muscle regeneration and is accumulated in abnormal muscle fibres of inclusion body myositis. *J Neurochem* 2006;96: 777–89.
7. Cohen AJ, Bunn PA, Franklin W, Magill-Solc C, Hartmann C, Helfrich B, Gilman L, Folkvord J, Helm K, Miller YE. Neutral endopeptidase: variable expression in human lung, inactivation in lung cancer, and modulation of peptide-induced calcium flux. *Cancer Res* 1996;56:831–9.
8. Albrecht M, Gillen S, Wilhelm B, Doroszewicz J, Aumuller G. Expression, localization and activity of neutral endopeptidase in cultured cells of benign prostatic hyperplasia and prostate cancer. *J Urol* 2002;168:336–42.
9. Sumitomo M, Shen R, Nanus DM. Involvement of neutral endopeptidase in neoplastic progression. *Biochim Biophys Acta* 2005;1751:52–9.
10. Hong Y, Macnab S, Lambert LA, Turner AJ, Whitehouse A, Usmani BA. Herpesvirus saimiri (HVS)-based endothelin converting enzyme-1 (ECE-1) shRNA expression decreases prostate cancer cell invasiion and migration. *Int J Cancer*, in press.
11. Horiguchi A, Zheng R, Goodman Jr OB, Shen R, Guan H, Hersh LB, Nanus DM.

Lentiviral vector neutral endopeptidase gene transfer suppresses prostate cancer tumor growth. *Cancer Gene Ther* 2007;14: 583–9.
12. Shen R, Sumitomo M, Dai J, Hardy DO, Navarro D, Usmani B, Papandreou CN, Hersh LB, Shipp MA, Freedman LP, Nanus DM. Identification and characterization of two androgen response regions in the human neutral endopeptidase gene. *Mol Cell Endocrinol* 2000;170:131–42.
13. Usmani BA, Shen R, Janeczko M, Papandreou CN, Lee WH, Nelson WG, Nelson JB, Nanus DM. Methylation of the neutral endopeptidase gene promoter in human prostate cancers. *Clin Cancer Res* 2000;6:1664–70.
14. Matsas R, Fulcher IS, Kenny AJ, Turner AJ. Substance P and [Leu]enkephalin are hydrolyzed by an enzyme in pig caudate synaptic membranes that is identical with the endopeptidase of kidney microvilli. *Proc Natl Acad Sci USA* 1983;80:3111–5.
15. Nalivaeva NN, Fisk LR, Belyaev ND, Turner AJ. Amyloid-degrading enzymes as therapeutic targets in Alzheimer's disease. *Curr Alzheimer Res* 2008;5:212–24.
16. El-Amouri SS, Zhu H, Yu J, Marr R, Verma IM, Kindy MS. Neprilysin: an enzyme candidate to slow the progression of Alzheimer's disease. *Neurobiology* 2008; 172:1342–54.
17. Pardossi-Piquard R, Petit A, Kawarai T, Sunyach C, Costa CA, Vincent B, Ring S, D'Adamio L, Shen J, Muller U, Hyslop PS, Checler F. Presenilin-dependent transcriptional control of the Aβ-degrading enzyme Neprilysin by intracellular domains of βAPP and APLP. *Neuron* 2005;46: 541–54.
18. Pardossi-Piquard R, Dunys J, Yu G, St George-Hyslop P, Alves da Costa C, Checler F. Neprilysin activity and expression are controlled by nicastrin. *J Neurochem* 2006;97:1052–6.
19. Goodger ZV, Rajendran L, Trutzel A, Kohli BM, Nitsch RM, Konietzko U. Nuclear signaling by the APP intracellular domain occurs predominantly through the amyloidogenic processing pathway. *J Cell Sci* 2009;122:3703–14.
20. Kimberly WT, Zheng JB, Guenette S, Selkoe DJ. The intracellular domain of the β-amyloid precursor protein is stabilized by Fe65 and translocates to the nucleus in Notch-like manner. *J Biol Chem* 2001;276: 40288–92.
21. Belyaev ND, Nalivaeva NN, Makova NZ, Turner AJ. Neprilysin gene expression requires binding of the amyloid precursor protein intracellular domain to its promoter: implications for Alzheimer disease. *EMBO Rep* 2009;10:94–100.
22. Nalivaeva NN, Belyaev ND, Turner AJ. Sodium valproate: an old drug with new roles. *Trends Pharmacol Sci* 2009;30: 509–14.
23. Song J, Noh JH, Lee JH, Eun JW, Ahn YM, Kim SY, Lee SH, Park WS, Yoo NJ, Lee JY, Nam SW. Increased expression of histone deacetylase 2 is found in human gastric cancer. *APMIS* 2005;113:264–8.
24. Halkidou K, Gaughan L, Cook S, Leung HY, Neal DE, Robson CN. Upregulation and nuclear recruitment of HDAC1 in hormone refractory prostate cancer. *Prostate* 2004;59:177–89.
25. Catalano MG, Poli R, Pugliese M, Fortunati N, Boccuzzi G. Valproic acid enhances tubulin acetylation and apoptotic activity of paclitaxel on anaplastic thyroid cancer cell lines. *Endocr Relat Cancer* 2007; 14:839–45.
26. Annicotte JS, Iankova I, Miard S, Fritz V, Sarruf D, Abella A, Berthe ML, Noel D, Pillon A, Iborra F, Dubus P, Maudelonde T, Culine S, Fajas L. Peroxisome proliferation-activated receptor gamma regualtes E-cadherin expression and inhibits growth and invasion of prostate cancer. *Mol Cell Biol* 2006;26:7561–74.
27. Belyaev ND, Kellett KA, Beckett C, Makova NZ, Revett TJ, Nalivaeva NN, Hooper NM, Turner AJ. The transcriptionally active amyloid precursor protein (APP) intracellular domain is preferentially produced from the 695 isoform of APP in a {beta}-secretase-dependent pathway. *J Biol Chem* 2010;285: 41443–54.
28. Konietzko U, Goodger ZV, Meyer M, Kohli BM, Bosset J, Lahiri DK, Nitsch RM. Co-localization of the amyloid precurosr protein and Notch intracellular domains in nuclear transcription factories. *Neurobiol Aging* 2010;10:58–73.
29. Nan X, Ng HH, Johnson Ca, Laherty CD, Turner BM, Eisenman RN, Bird A. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* 1998; 393:386–9.
30. Fuks F, Burgers WA, Godin N, Kasai M, Kouzarides T. DNMT3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription. *EMBO J* 2001;20:2536–44.
31. Edbauer D, Winkler E, Regula JT, Pesold B, Steiner H, Haass C. Reconstitution of gamma-secretase activity. *Nat Cell Biol* 2003;5:486–8.
32. Edbauer D, Willem M, Lammich S, Steiner H, Haass C. Insulin-degrading enzyme rapidly removes the beta-amyloid precursor protein intracellular domain (AICD). *J Biol Chem* 2002;277:13389–93.
33. Nunan J, Shearman MS, Checler F, Cappai R, Evin G, Beyreuther K, Masters CL, Small DH. The C-terminal fragment of the Alzheimer's disease amyloid protein precursor is degraded by a proteasome-dependent mechanism distinct from gamma-secretase. *Eur J Biochem* 2001;268: 5329–36.
34. McLendon C, Xin T, Ziani-Cherif C, Murphy MP, Findlay KA, Lewis PA, Pinnix I, Sambamurti K, Wang R, Faug A, Golde TE. Cell-free assays for gamma-secretase activity. *FASEB J* 2000;14:2383–6.
35. Muller T, Meyer HE, Eqensperger R, Marcus K. The amyloid precurosr protein intracellular domain (AICD) as modulator of gene expression, apoptosis, and cytoskeletal dynamics-relevance for Alzheimer's disease. *Prog Neurobiol* 2008; 85:393–406.
36. Eldar-Finkelman H. Glycogen synthase kinase 3: an emerging therapeutic target. *Trends Mol Med* 2002;8:126–32.
37. Baek SH, Ohgi KA, Rose DW, Koo EH, Glass CK, Rosenfeld MG. Exchange of N-CoR corepressor and Tip60 coactivator complexes links gene expression by NF-kappaB and beta-amyloid precursor protein. *Cell* 2002;110:55–67.
38. Alves da Costa C, Sunyach C, Pardossi-Piquard R, Sévalle J, Vincent B, Boyer N, Kawarai T, Girardot N, St George-Hyslop P, Checler F. Presenilin-dependent gamma-secretase-mediated control of p53-associated cell death in Alzheimer's disease. *J Neurosci* 2006;26:6377–85.
39. Checler F, Sunyach C, Pardossi-Piquard R, Sevalle J, Vincent B, Kawarai T, Girardot N, St George-Hyslop P, da Costa CA. The gamma/epsilon-secretase-derived APP intracellular domain fragments regulate p53. *Curr Alzheimer Res* 2007;4: 423–6.
40. Huysseune S, Kienlen-Campard P, Hebert S, Tasiaux B, Leroy K, Devuyst O, Brion JP, De Strooper B, Octave JN. Epigenetic control of aquaporin 1 expression by the amyloid precursor protein. *FASEB J* 2009; 23:4158–67.
41. Hebert SS, Serneels L, Tolia A, Craessaerts K, Derks C, Filippov MA, Muller U, De Strooper B. Regulated intramembrane proteolysis of amyloid precursor protein and regulation of expression of putative target genes. *EMBO Rep* 2006;7:739–45.
42. Chen AC, Selkoe DJ. Response to: Pardossi-Piquard et al., "Presenilin-dependent transcriptional control of the Abeta-degrading enzyme neprilysin by intracellular domain of betaAPP and APLP." *Neuron* 2007;46:541–54.
43. Pardossi-Piquard R, Dunys J, Kawarai T, Sunyach C, Alves da Costa C, Vincent B, Sevalle J, Pimplikar S, St George-Hyslop P, Checler F. Response to correspondance: Pardossi-Piquard et al., "Presenilin-dependent transcriptional control of the Abeta-degrading enzyme neprilysin by intracellular domain of betaAPP and

APLP." Neuron 46, 541–554. *Neuron* 2007; 53:483–6.
44. Waldron E, Isbert S, Kern A, Jaeger S, Martin AM, Hebert SS, Behl C, Weggen S, De Strooper B, Pietrzik CU. Increased AICD generation does not result in increased nuclear translocation or activation of target gene transcription. *Exp Cell Res* 2008;314:2419–33.
45. Takayama K, Tsutsumi S, Suzuki T, Horie-Inoue K, Ikeda K, Kaneshiro K, Fujimura T, Kumagai J, Urano T, Sakaki Y, Shirahige K, Sasano H, et al. Amyloid precursor protein is a primary androgen target gene that promotes prostate cancer growth. *Cancer Res* 2009;69:137–42.
46. Shearman MS, Beher D, Clarke EE, Lewis HD, Harrison T, Hunt P, Nadin A, Smith AL, Stevenson G, Castro JL. L-685,458, an aspartyl protease transition state mimic, is a potent inhibitor of amyloid beta-protein precursor gamma-secretase activity. *Biochemistry* 2000;39: 8698–704.
47. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB. Synergy of demthylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat Genet* 1999;21: 103–7.
48. Ou JN, Torrisani J, Unterberger A, Provencal N, Shikimi K, Karimi M, Ekstrom TJ, Szyf M. Histone deacetylase inhibitor Trichostatin A induces global and gene-specific DNA demethylation in human cancer cell lines. *Biochem Pharmacol* 2007;73:1297–307.
49. Milutinovic S, D'Alessio AC, Detich N, Szyf M. Valproate induces widespread epigenetic reprogramming which involves demethylation of specific genes. *Carcinogenesis* 2007;28:560–71.