Neprilysin gene expression requires binding of the amyloid precursor protein intracellular domain to its promoter: implications for Alzheimer disease

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Amyloid β -peptide (A β) accumulation leads to neurodegeneration and Alzheimer disease; however, amyloid metabolism is a dynamic process and enzymic mechanisms exist for A β removal. Considerable controversy surrounds whether the intracellular domain of the amyloid precursor protein (AICD) regulates expression of the A β -degrading metalloprotease, neprilysin (NEP). By comparing two neuroblastoma cell lines differing substantially in NEP expression, we show by chromatin immunoprecipitation (ChIP) that AICD is bound directly to the *NEP* promoter in high NEP-expresser (NB7) cells but not in low-expresser (SH-SY5Y) cells. The methylation status of the *NEP* promoter does not regulate expression in these cells, whereas the histone deacetylase inhibitors trichostatin A and valproate partly restore NEP expression and activity in SH-SY5Y cells. ChIP analysis also reveals AICD binding to the *NEP* promoter in rat primary neurons but not in HUVEC cells. Chromatin remodelling of crucial Alzheimer disease-related genes by valproate could provide a new therapeutic strategy.

Keywords: Alzheimer disease; amyloid β -peptide; histone deacetylase; neprilysin; valproate

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INTRODUCTION

The accumulation of amyloid β -peptide (A β) is a characteristic feature of Alzheimer disease and its prevention is a primary target in therapeutic strategies. Late-onset forms of Alzheimer disease might be primarily attributable to deficiencies in the clearance of A β rather than its formation (Hama & Saido, 2005). Hence, an understanding of the clearance mechanisms and their regulation could be of fundamental importance and provide new approaches to Alzheimer disease treatment. A β peptides are generated from the amyloid precursor protein (APP) through sequential proteolysis by β - and γ -secretases, which also generates the APP intracellular

domain (AICD; Kimberly *et al*, 2001). The exact function of the AICD remains unclear and controversial, but it might act as a transcriptional regulator together with the APP tail-binding protein Fe65 and the histone acetyltransferase Tip60 (HIV Tat-interacting protein 60; Cao & Südhof, 2001).

The steady-state concentration of A β is tightly regulated by perivascular mechanisms and proteolytic cleavage. Several proteases participate in brain A β metabolism *in vivo*, especially neprilysin (NEP; also known as CD10), which is a synaptic ectoenzyme the activity of which declines markedly in ageing and in Alzheimer disease (Carson & Turner, 2002; Hersh & Rodgers, 2008; Nalivaeva *et al*, 2008). Hence, the upregulation of NEP could be a new therapeutic strategy, but relatively little is known about the transcriptional regulatory mechanisms that control its expression. Recently, Pardossi-Piquard *et al* (2005) have claimed that AICD upregulates *NEP* transcription, which in turn accelerates A β degradation; however, others have questioned any significant AICD involvement in NEP regulation (Hébert *et al*, 2006; Chen & Selkoe, 2007).

The aims of this report were therefore threefold: to seek evidence of a direct interaction of AICD with the *NEP* promoters; to compare the 'chromatin signatures' of the active and repressed *NEP* genes by chromatin immunoprecipitation (ChIP); and to facilitate de-repression of *NEP* gene expression. To this end, we compared two human neuroblastoma cell lines that differ significantly in levels of *NEP* expression: SH-SY5Y and NB7 cells (Fisk *et al*, 2007). We show that AICD is bound directly to the *NEP* promoters in NB7 cells and in rat primary cortical neurons but not in SH-SY5Y or primary human umbilical vein endothelial cells (HUVEC), which also express APP (Goldgaber *et al*, 1989); that repression of *NEP* involves excess histone deacetylation, not DNA methylation, in SH-SY5Y cells; and that the *NEP* gene in SH-SY5Y cells can be partly reactivated by histone deacetylase (HDAC) inhibitors, including trichostatin A (TSA) and the widely used anti-convulsant, sodium valproate (VA).

RESULTS

NEP gene expression and histone modifications

To examine epigenetic factors regulating NEP in neuronal cell lines, we initially selected two lines that differ markedly in NEP

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expression levels. The SH-SY5Y cell line, a widely used model for studies of Alzheimer disease-related biology, expresses low levels of *NEP* messenger RNA (mRNA), protein and enzyme activity; by contrast, the NB7 cell line (Shapiro *et al*, 1993; Fisk *et al*, 2007) shows much higher levels of expression and enzyme activity (Fig 1A,B,C). This correlates with higher expression levels of both APP and Fe65 in NB7 cells (Fig 1D). A reduction of APP levels in NB7 cells by small interfering RNA (siRNA) treatment reduces expression of *NEP* mRNA (Fig 1E). As methylation of CpG islands in the *NEP* promoter region represses expression in both human prostate cancer and rat hepatocarcinoma cell lines (Usmani *et al*, 2000; Uematsu *et al*, 2006), we examined the effect of the

demethylating agent, 5-aza-2'-deoxycytidine (azaC), on *NEP* expression in SH-SY5Y and NB7 cells, and observed no upregulation of *NEP* expression in either case (Fig 1A). Similarly, bisulphite DNA conversion indicated similar levels of DNA conversion from both cell lines (data not shown); therefore, *NEP* promoter hypermethylation is not a crucial determinant of *NEP* repression in SH-SY5Y cells. Next, the acetylation status was compared between the cell lines by ChIP assay (Fig 2A). The *NEP* promoter in the NB7 cell line, but not in the SH-SY5Y cell line, was enriched with lysine acetylation of the core histones H4K8 and H4K16, which are typical chromatin marks of an active gene. By contrast, the chromatin organizing the *NEP* promoter in the SH-SY5Y cell line was marked by the presence of the histone deacetylase HDAC1, which was absent in NB7 cells.

AICD binds to the *NEP* promoters

The potential direct interaction of AICD with the *NEP* promoter was then examined by ChIP. Crosslinked and sonicated chromatin extracts from either NB7 or SH-SY5Y cells were immunoprecipitated with specific antibodies against AICD and HDAC1, with antibody to histone H3 as a positive control and IgG as a negative control. The precipitated DNA was analysed by using conventional PCR with primers spanning the *NEP* promoter region (Fig 2B). Anti-AICD IgG immunoprecipitated the *NEP* promoter region from NB7 cells but not from SH-SY5Y cells. Again, HDAC1 was associated with the chromatin of the *NEP* promoter in SH-SY5Y cells but not in NB7 cells. The size of the sheared DNA fragments was 100–400 bp, which excludes pull-down of remote DNA fragments (supplementary Fig 1A online). ChIP scanning was also performed throughout the human *NEP* gene and on the actin promoter with both AICD and HDAC1 antibodies, and the pulled-down DNA was analysed by real-time PCR using a range of primers to confirm promoter specificity (see supplementary Fig 1B online for scanning strategy and details).

Expression of the *NEP* gene can be controlled through two distinct promoters (Ishimaru & Shipp, 1995; Li *et al*, 1995) the influence of which differs between cell types, although both promoters show similar characteristics and activity in positive cell lines, and might also be active in neuronal cell types. Therefore, we compared the ability of AICD and HDAC1 antibodies to pull down both *NEP* promoters in the cell lines, and obtained identical results using conventional (Fig 2C) and real-time PCR (Fig 2D,E). AICD, but not HDAC1, interacted with both *NEP* promoters in the transcriptionally active NB7 cells but not in SH-SY5Y cells. The ChIP analysis was complemented by additional reporter construct analysis using each of the *NEP* promoters with luciferase detection. Luciferase activity driven by both *NEP* promoters was substantially higher in the NB7 cells compared with the SH-SY5Y cells (Fig 2F). Further confirmation of a transcriptional regulatory function for AICD was provided by the immunocytochemical localization of AICD in the nucleus of NB7 cells, whereas only a weak and diffuse detection of AICD was seen in SH-SY5Y cells that was predominantly cytoplasmic (Fig 2G).

The production of AICD requires the action of γ -secretase on the APP carboxy-terminal domain. The effect of a potent γ -secretase inhibitor, L-685,458 (Shearman *et al*, 2000), on *NEP* expression and promoter occupancy was therefore investigated using conventional and real-time PCR and ChIP analysis. Treatment of NB7 cells with 10 μ M L685,458 for 48 h substantially

Fig 1 | Comparative analysis of *NEP*, APP and Fe65 expression in SH-SY5Y and NB7 cells. *NEP* expression is substantially higher in NB7 cells compared with SH-SY5Y cells at the level of (A) mRNA by conventional PCR, (B) protein immunoblotting (20 μ g cell lysate) and (C) enzyme activity (mean of three experiments, each assayed in triplicate for enzyme activity). AzaC does not affect *NEP* mRNA expression in either cell line (A). (D) Immunoblotting of cell extracts (50 μ g protein) with antibodies against human APP and Fe65. (E) Effect of APP gene silencing by APP siRNA on *NEP* mRNA expression in NB7 and SH-SY5Y cells, assessed by real-time PCR (siRNA treatment, see Methods), compared with effects of GAPDH or a scrambled siRNA (mean of three experiments). APP, amyloid precursor protein; azaC, 5-aza-2'-deoxycytidine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger RNA; *NEP*, neprilysin; siRNA, small-interfering RNA.

Fig 2 | Chromatin immunoprecipitation analysis of the *NEP* promoters in SH-SY5Y and NB7 cells. (A,B) ChIP and conventional DNA analysis shows that the *NEP* promoter 2 in NB7, but not in SH-SY5Y cells, has enriched lysine acetylation of histone H4 in positions K8 and K16, and is marked by AICD, whereas the SH-SY5Y *NEP* promoter 2 is marked by HDAC1. ChIP with antibody to H3 was used as a positive control in (B) and IgG as a negative control. (C) ChIP analysis of the *NEP* promoters 1 and 2 in NB7 and SH-SY5Y cells with antibodies to AICD and HDAC1. (D,E) ChIP followed by real-time PCR analysis with (D) anti-AICD and (E) anti-HDAC1 of the *NEP* promoters 1 and 2 in NB7 and SH-SY5Y cells (mean of five experiments). (F) Relative luciferase luminescence from NB7 or SH-SY5Y cells transfected with either *NEP* promoters 1- or 2-luciferase constructs (mean of three experiments). (G) Immunocytochemical detection of AICD. Localization of AICD was observed in the nuclei of NB7 cells (upper panel, a), whereas only predominantly cytoplasmic and weak detection of AICD was observed in SH-SY5Y cells (lower panel, d). Cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; b,e); captured images were digitally merged and are shown in (c,f). AICD, amyloid precursor protein intracellular domain; ChIP, chromatin immunoprecipitation; HDAC1, histone deacetylase 1; NEP, neprilysin.

reduced NEP expression (Fig 3A,B,C), and anti-AICD IgG pulled down the *NEP* promoter region only in cells untreated with L685,458 (Fig 3D,E). L685,458 treatment reduced AICD and increased HDAC1 promoter binding in NB7 cells but not in SH-SY5Y cells (Fig 3E). L685,458 treatment or siRNA knock-down

of APP in NB7 cells reduced histone acetylation marks (H4K16, H4K8) on the *NEP* promoter (supplementary Fig 2 online).

We then sought confirmation of AICD or HDAC interaction with the *NEP* promoters in primary cells. For this purpose, ChIP analysis was also applied to preparations of rat primary cortical

Fig 3 | Chromatin immunoprecipitation analysis of AICD and HDAC1 binding to the NEP promoters in primary cells, and effects of the γ -secretase inhibitor L685,458 in NB7 and SH-SY5Y cells. (A) NB7 and SH-SY5Y cells were treated with (+) or without (–) L685,458 (10 μ M, 48 h), and NEP expression was compared using conventional PCR. (B) Gel densitometry relative to β -actin for NB7 cells from (A) is presented graphically (mean of three experiments; * P < 0.05). (C) Real-time PCR of RNA extracts from NB7 and SH-SY5Y cells incubated with or without L685,458 (10 μ M, 48 h; mean of five experiments). (D) ChIP with anti-AICD followed by conventional DNA analysis of NB7 cells treated with or without L685,458 (ChIP with IgG used as a negative control). (E) ChIP with anti-AICD or anti-HDAC1 followed by real-time PCR DNA analysis with primers to NEP promoter 2 in NB7 and SH-SY5Y cells treated with or without L685,458 (mean of three experiments). (F,G) AICD and HDAC1 ChIP followed by real-time PCR analysis with primers to the NEP promoters 1 and 2, and a coding region of the NEP gene, in (F) rat primary cortical neurons and (G) HUVEC cells (mean of three experiments). AICD, amyloid precursor protein intracellular domain; ChIP, chromatin immunoprecipitation; HDAC1, histone deacetylase 1; NEP, neprilysin.

neurons that actively express NEP, and to HUVEC cells that express only extremely low levels of NEP. The AICD, but not the HDAC1, antibody was able to pull down the NEP promoter regions from primary neurons (Fig 3F), but the converse was true of HUVEC, which showed as much as a 15-fold enrichment in HDAC1 at the NEP promoters (Fig 3G).

As HDAC binding to the NEP promoter correlated with the repression of gene expression, the effect of VA—a widely used anticonvulsant and HDAC inhibitor (Göttlicher *et al*, 2001)—was also examined on NEP expression. Treatment of cells with 10 μ M VA for 48 h had no significant effect on NEP expression in NB7 cells, but upregulated NEP mRNA levels in SH-SY5Y cells (Fig 4A). A structurally distinct HDAC inhibitor, TSA, also upregulated NEP expression, and the increased expression of NEP mRNA detected using real-time PCR in both TSA- and VA-treated SH-SY5Y cells, but not in NB7 cells (Fig 4B), was matched by a significant increase in NEP enzyme activity in SH-SY5Y cells (Fig 4C) but not NB7 cells (Fig 4D). The increase in AICD binding to the NEP promoter in SH-SY5Y cells after TSA treatment was matched by a corresponding decrease in HDAC1 binding (Fig 4E).

DISCUSSION

Since Cao & Südhof (2001) first reported that the release of AICD from APP could stimulate reporter gene activation, much controversy has surrounded the validity of this mechanism. Opposing conclusions on whether NEP expression is regulated by APP processing to AICD were reported by Pardossi-Piquard *et al* (2005) and Chen & Selkoe (2007). Hébert *et al* (2006) also failed to detect significant stimulation of NEP expression through AICD, yet Eisele *et al* (2007) showed that the tyrosine kinase inhibitor, Gleevec, increased the levels of AICD and NEP, and lowered that of cellular A β . We adopted an independent approach using ChIP to establish, for the first time to our knowledge, histone acetylation as a crucial factor in the direct regulation of NEP expression by AICD. Chromatin remodelling, through increased histone acetylation, is associated with the recovery of learning and memory in a transgenic mouse model, which can be induced by environmental enrichment (Fischer *et al*, 2007). Environmental enrichment also upregulates NEP expression, and reduces A β levels and amyloid deposition in transgenic mice, although this was not correlated with any specific chromatin changes (Lazarov *et al*, 2005).

Fig 4 | Effect of the HDAC inhibitors VA and TSA on NEP expression and enzyme activity. (A) SH-SY5Y and NB7 cells were incubated without (control) or with VA (10 μ M, 48 h), and NEP mRNA expression was assessed using conventional PCR. (B) Effects of TSA (100 nM) and VA (10 μ M) treatment for 48 h on NEP mRNA levels in SH-SY5Y and NB7 cells, as assessed using real-time PCR (mean of five experiments). (C,D) Effects of TSA and VA on NEP activity in (C) SH-SY5Y and (D) NB7 cells (mean of three experiments, each assayed in triplicate for enzyme activity). (E) ChIP analysis with anti-AICD and anti-HDAC1, followed by real-time PCR of pulled-down DNA with primers to the *NEP* promoter 2 in NB7 and SH-SY5Y cells treated with TSA (100 nM, 48 h) compared with control (mean of three experiments; * P < 0.05). AICD, amyloid precursor protein intracellular domain; HDAC, histone deacetylase; mRNA, messenger RNA; NEP, neprilysin; TSA, trichostatin A; VA, sodium valproate.

The loss of NEP also triggers androgen-independent prostate cancer progression driven by peptide mitogens, as NEP is an androgen-dependent gene (Papandreou *et al*, 1998). Hence, NEP de-repression is a desirable objective in two distinct diseases of ageing: Alzheimer disease and prostate cancer. However, unlike in prostate cancer cell lines (Usmani *et al*, 2000), we have shown that DNA methylation of the *NEP* promoter does not mediate its transcriptional repression in the neuronal lines studied. Methylation is also not responsible for NEP repression in hepatobiliary cells in Alagille syndrome (Byrne *et al*, 2007). In SH-SY5Y cells, but not NB7 cells, the chromatin of the *NEP* promoter was associated with HDAC1 and lacked typical histone acetylation marks (H4K16, H4K8). Conversely, ChIP assays confirmed the presence of AICD on the *NEP* promoters on NB7 cells. A γ -secretase inhibitor blocked AICD production in NB7 cells and inhibited NEP expression. Hence, neuronal NEP expression is determined, in part, by the chromatin acetylation status of its promoter, and AICD participates in transcriptional activation by direct binding to the *NEP* promoter. Rat primary cortical neurons were also enriched in AICD but not HDAC1 on the *NEP* promoters, whereas the converse applied with HUVEC.

Although much attention has focused on the potential function of HDAC inhibitors in cancer therapy, recently there have been encouraging data on their application in neurodegenerative diseases (Morrison *et al*, 2007). Here, two different HDAC inhibitors could partly restore *NEP* transcription and enzyme activity in SH-SY5Y cells, but could not activate NEP expression further in NB7 cells. It is clear that several cell-specific pathways regulate the control of NEP expression and that the identification

of other such mechanisms could provide new therapeutic strategies in neurodegeneration or prostate cancer.

HDAC inhibitors also induce expression of the urokinase plasminogen activator and hence that of plasmin (Pulukuri *et al*, 2007)—another amyloid-degrading enzyme (Nalivaeva *et al*, 2008)—and promoting the plasmin proteolytic cascade has been shown to enhance the clearance of A β from the brain (Jacobsen *et al*, 2008). The relative safety and efficacy of the HDAC inhibitor, VA, has been established over 30 years of clinical use in the treatment of epilepsy (Peterson & Naunton, 2005), as well as in the behavioural complications of dementias (Salzman, 2001). VA can also enhance neuronal survival after induction of apoptosis *in vivo* and *in vitro* (Li *et al*, 2008). Hence, VA, or other HDAC inhibitors, might be important in Alzheimer disease treatment if they can also facilitate removal of the A β peptide through reactivation of amyloid-degrading enzymes or other neuroprotective genes.

METHODS

Cell culture. The SH-SY5Y cell line was donated by Dr JL Biedler (Sloan-Kettering Institute, NY, USA) and the NB7 cell line by Dr V Kidd (St Jude Children's Research Hospital, Memphis, TN, USA). Primary cortical neuronal cultures were prepared from 1- to 3-day-old Wistar rats, as described previously, and purity was checked by immunostaining with anti-Tuj1 (Fisk *et al*, 2007). HUVEC cells were provided by Clare Ulyatt and Dr A Whyteside (University of Leeds) and used not later than after 3 to 4 passages. SH-SY5Y cells were cultured in DMEM F-12 media with 10% (v/v) foetal bovine serum, 50 U/ml penicillin, 50 μ g/ml streptomycin and 2 mM glutamine. NB7 and primary neuronal cells were

cultured as described by Fisk *et al* (2007). Treatment with azaC (100 µg/ml), TSA (100 nM), L-685,458 (10 µM) or VA (10 µM) was for 48 h.

Gene expression analysis. Cell RNA was prepared using the RNeasy extraction kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. RNA was treated with DNaseI (Invitrogen, Paisley, UK) and cDNA was prepared using the iScript cDNA kit (Bio-Rad, Hemel Hempstead, UK). cDNA was amplified using conventional PCR or real-time PCR as described by Zuccato *et al* (2007). DNA amplified by conventional PCR was analysed in 2% agarose gels containing ethidium bromide (1 µg/ml) and visualized on the Molecular Imager Gel Doc XR System with Quantity One 4.6.1 program (Bio-Rad, Hercules, CA, USA). Image densitometry was performed using the Aida Array Analyzer 4.15 software. Real-time PCR was performed in an iCycler Thermal Cycler with the MulticolourPCR detection system (Bio-Rad, USA) using SYBR Green (Bio-Rad, UK) incorporation, and expression was reported relative to actin mRNA. Details of the primer sequences used for PCR are listed in supplementary Table online.

APP mRNA silencing (siRNA). NB7 or SH-SY5Y cells were grown to approximately 40% confluence in antibiotic-free growth medium and transfected with siRNA duplexes (Ambion, s1501) targeting the 3'-coding exon of the human APP gene:

sense sequence: GACUGAACAUAGCACAUGAAt

antisense: UUCAUGUGCAUGUUCAGUCtg

Transfection with Lipofectamine 2000 (Invitrogen) was performed according to the manufacturer's protocol. Cells were transfected with 100 nM duplexes targeted to human APP, siRNA targeted to human GAPDH or control duplexes (Ambion, Warrington, UK) targeted to a scrambled sequence. Control flasks were subjected to mock transfection with double-distilled H₂O. After 24 h transfection, RNA was isolated and NEP expression analysed by real-time PCR.

Reporter gene analysis. NEP promoter 1- and 2-luciferase constructs (a generous gift from Dr F. Checler, University of Nice, France) were transfected using Lipofectamine 2000 into NB7 or SH-SY5Y cells together with the thymidine-kinase *Renilla* construct with the pGL2 vector as control. After transfection (48 h), luciferase and *renilla* luminescence was measured by the Promega Dual-Luciferase Reporter Assay System (Promega UK Ltd, Southampton, UK) according to the manufacturer's protocol using as luminometer the MicroLumat plus LB96 V (Berthold, Harpenden, UK). The data represent the ratio of Luciferase to *Renilla* luminescence in the experimental cell extracts normalized to control cell extracts.

ChIP analysis. Chromatin immunoprecipitation was performed as described previously (Zuccato *et al*, 2007). NB7 and SH-SY5Y cells were fixed, extracts were sonicated and primary antibodies were applied following treatment with Protein G Sepharose, decrosslinking and DNA extraction and analysis by conventional or real-time PCR. Real-time PCR data are represented as the fold of enrichment of DNA pulled down with the specific antibody over that immunoprecipitated with IgG. Antibodies used in ChIP experiments were as follows: anti-AICD (BR188, Lefranc-Jullien *et al*, 2006), a generous gift from Dr M. Goedert (Cambridge, UK); anti-HDAC1, anti-H3 and IgG were from Abcam (Cambridge, UK). Antibodies to predominant sites of H4 acetylation were prepared by N. Belyaev (White *et al*, 1999).

Western blotting. Cell lysates were prepared and subjected to electrophoresis and immunoblotting as described by Fisk *et al*

(2007). Recombinant human NEP was a gift from Dr D. Nanus (Cornell University, NY, USA). Antibodies to NEP were obtained from Novocastra Laboratories (Newcastle, UK), to APP from Chemicon (Temecula, CA, USA), to Fe65 from Santa Cruz Biotechnologies Ltd (Santa Cruz, CA, USA) and to actin from Sigma (Poole, UK).

Immunostaining. NB7 or SH-SY5Y cells were grown on coverslips, fixed with formaldehyde at 2% final concentration, permeabilized and incubated for 2 h at 20 °C with rabbit anti-AICD at a dilution of 1:100 in 0.1% Triton X-100/PBS, followed by treatment with FITC-conjugated secondary antibodies (Sigma) for 1 h at a dilution of 1:200 in PBS-Triton X-100. After cell nuclei were stained with 4,6-diamidino-2-phenylindole, cells were observed using an Olympus IX70 deconvolution microscope and images were captured using Delta Vision from Applied Precision (Issaquah, WA, USA).

Fluorometric assay of NEP activity. NEP activity was assayed with a fluorogenic substrate Suc-Ala-Ala-Phe-7-AMC (50 µM; Bachem, St Helens, UK) in the presence or absence of the inhibitor, thiorphan (Sigma; 10 µM), as described previously (Fisk *et al*, 2007).

Statistical analysis. All results are given as mean ± s.e.m. from *n* experiments. Student's *t*-tests were used to ascertain the statistical significance with a threshold of *P* < 0.05.

Supplementary information is available at *EMBO reports* online (<http://www.emboereports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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