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Clusterin secreted by astrocytes enhances neuronal differentiation from human neural precursor cells

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Neuronal differentiation from expanded human ventral mesencephalic neural precursor cells (NPCs) is very limited. Astrocytes are known to secrete neurotrophic factors, and so in order to enhance neuronal survival from NPCs, we tested the effect of regional astrocyte-conditioned medium (ACM) from the rat cortex, hippocampus and midbrain on this process. Human NPC's were expanded in FGF-2 before differentiation for 1 or 4 weeks in ACM. The results show that ACM from the hippocampus and midbrain increase the number of neurons from expanded human NPCs, an effect that was not observed with cortical ACM. In addition, both hippocampal and midbrain ACM increased the number and length of phosphorylated neurofilaments. MALDI-TOF analysis used to determine differences in media revealed that although all three regional ACMs had cystatin C, α-2 macroglobulin, extracellular matrix glycoprotein and vimentin, only hippocampal and midbrain ACM also contained clusterin, which when immunodepleted from midbrain ACM eliminated the observed effects on neuronal differentiation. Furthermore, clusterin is a highly glycosylated protein that has no effect on cell proliferation but decreases apoptotic nuclei and causes a sustained increase in phosphorylated extracellular signal-regulated kinase, implicating its role in cell survival and differentiation. These findings further reveal differential effects of regional astrocytes on NPC behavior and identify clusterin as an important mediator of NPC-derived neuronal survival and differentiation.
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Astrocytes are essential regulators of neuronal function, and produce diffusible and non-diffusible neuron supporting signals, including neurotrophic factors and membrane-bound molecules (for review see Barres[1]). Interestingly, emerging evidence highlights that there are many regional differences in astrocytes in respect to structure and function and soluble factor production, for example, astrocytes from the hippocampus and their conditioned media can support the survival of neurons from regions other than the hippocampus.[2] In contrast, astrocytes from the mesencephalon release factors, which are more effective at promoting dopamine neuron survival than cortical or striatal astrocytes.[3] These studies show that the astrocytes from neurogenic regions[4] release more neurogenic factors than astrocytes from the non-neurogenic regions.

It is well established that stem cells can be isolated from the developing and adult rodent CNS, and expanded *in vitro* using EGF and FGF2 (for review see Gage[5]) More recently the same has been shown to be true for the developing human fetal CNS.[6] These cells are capable of long-term expansion *in vitro* to achieve ~50 population doublings.[6] However, as they progress in culture with time, their differentiation pattern changes with the number of neurons declining and the number of astrocytes predominating.[7]

Previous studies have shown that astrocyte-conditioned media (ACM) increases the number of neurons from adult rodent neural stem cells.[8] The aim of this study was to further explore the neurogenic-inducing properties of astrocytes by investigating if ACM from cortical, hippocampal or midbrain origins had any effect on neuronal differentiation of FGF2-expanded mesencephalic neural precursor cells (NPCs). Cells were differentiated for 1 week or 1 month in control media or in regional ACM. The results show that hippocampal and midbrain ACM increase the number of TuJ1$^+$ and SMI312$^+$ neurons. Mass spectrometric (MS) analysis revealed that clusterin is secreted by astrocytes from the midbrain and hippocampus, and subsequent immunodepletion experiments showed that this secreted protein is likely to be responsible for the increase in neuronal differentiation from NPCs. In addition, clusterin is a highly glycosylated protein that decreases TUNEL-positive nuclei and causes a sustained activation of ERK phosphorylation, indicating its role in cell survival and differentiation.

Results

ACM from the hippocampus and midbrain, but not cortex increase the number of neurons from human NPCs. To determine if regional ACM had an effect on the number of NPC-derived neurons, human ventral

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mesencephalon (VM) was expanded for 4 weeks in FGF2 before plating in control, cortical, hippocampal or midbrain ACM. Results demonstrate that midbrain ACM increased the number of neurons after a week and month of differentiation ($P<0.05$; Figure 1a), whereas hippocampal ACM had an effect only after a month ($P<0.05$). Interestingly cortical ACM had no effect on neurogenic potential. We next examined ACMs effects on morphology and specifically on neurofilament phosphorylation using SMI312. This antibody is specific for phospho-epitopes in neurofilaments, and was used as a marker for axonal integrity. The results show that midbrain ACM significantly increased the number of SMI312$^+$ neurons ($P<0.001$; Figures 1a and b) after a week ($P<0.01$) and a month ($P<0.01$). However, hippocampal ACM showed an increase after a month only ($P<0.05$; Figure 1a). To determine whether regional ACM had an effect on neurite length, neurite lengths per SMI312$^+$ neurons were measured. Both hippocampal and midbrain ACM significantly increased neurite length (Figure 1a). Again cortical ACM showed no effect. Cultures were also stained for the astrocyte marker GFAP and none of the ACMs had an effect on astrocyte numbers (Figure 2), revealing a neuronal specific effect for the ACM.

Protein identification by MS. To determine the major protein components secreted by the regional astrocytes, nanoLC-MALDI-TOF/TOF-MS/MS analyses were carried out on peptides produced from tryptic digestion of proteins in the ACM. ACM was collected in a minimal phenol-free media containing high glucose and glutamine that did not contain N2 and B27 cell media supplements, which are known to contain significant amounts of potentially confounding proteins. Five main proteins were identified by mass spectrometry in all tested ACMs (Table 1). Many of these proteins are to be expected, including cystatin C, which has been observed in a previous proteomics experiment on

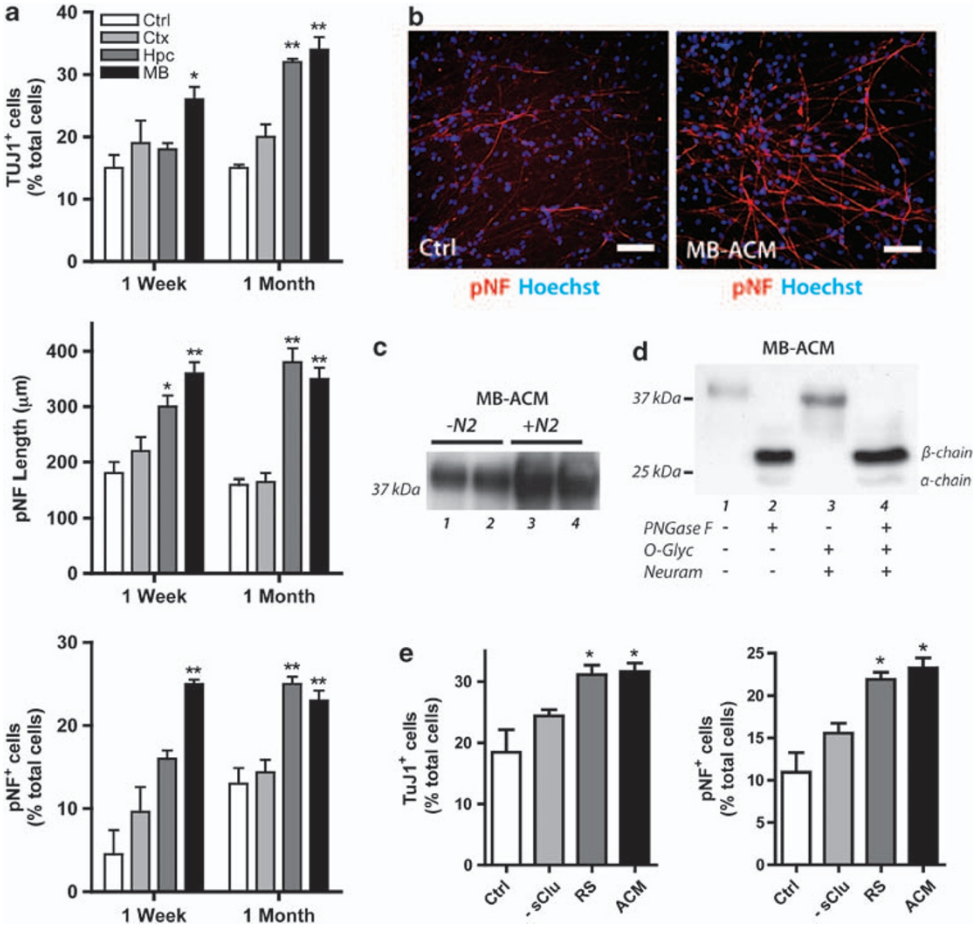

Figure 1 ACM increases neuronal differentiation from VM derived NPCs. (**a**) Midbrain ACM increases TuJ1 after 1 week, and both hippocampal and midbrain ACM increase TuJ1 after 1month of differentiation. Midbrain ACM increases phosphorylated neurofilaments$^+$ (pNF) neurons after 1 week, and both hippocampal and midbrain increase pNF after 1 month of differentiation, Both hippocampal and midbrain ACM increase neurite lengths after 1 week and 1 month. (**b**) Photomicrograph of pNF in control and cells treated with midbrain ACM after 1 month. (**c**) Western blot showing clusterin immunodepletion from midbrain ACM. Lanes 1, 2 ACM without N2, lanes 3, 4 ACM with N2, $n=2$ separate samples. (**d**) Secreted clusterin is a highly glycosylated heterodimeric protein: lane 1, untreated MB-ACM. Lane 2 MB-ACM treated with PNGase to remove *N*-linked oligosaccharides. Lane 3, MB-ACM treated with *O*-glycosidase + neuramidase to eliminate *O*-linked oligosaccharides. Lane 4 complete deglycosylation of clusterin. The two subunits of secreted clusterin can be resolved after treatment with endoglycosidases. (**e**) NPCs differentiated for a month under control conditions, ACM immunodepleted of clusterin (−sClu), with rat anti-clusterin–IgG substituted for rabbit serum during immunodepletion (RS) and with ACM. There is a reduction in the number of TuJ1- and pNF-positive neurons when clusterin is immunodepleted from the media (−sClu) compared with rabbit serum control or ACM. *$P<0.05$, **$P<0.01$ *versus* Ctrl and CTX ACM

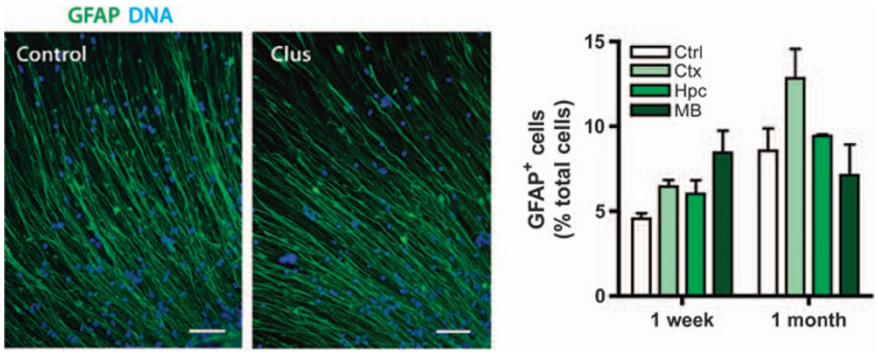

Figure 2 Clusterin or regional ACM do not influence the number of astrocytes. Photomicrographs of GFAP, untreated (Control) and treated with 10 nM clusterin (Clus). Scale bar = 50 μm

Table 1 Proteins identified in the conditioned medium of cultured astrocytes from various brain regions

			Midbrain		Hippocampus		Cortex	
Protein	**Accession number**	**Mw (kDa)**	**Peptides**	**Coverage (%)**	**Peptides**	**Coverage (%)**	**Peptides**	**Coverage (%)**
α-2-Macrogobulin	6 978 425	164	10	8.7	6	6	3	3
Clusterin	17 985 951	51	4	11	4	11	—	—
Cystatin C	226 712	13	3	39	3	30	3	39
Extracellular matrix protein 2	6 978 789	70.5	2	6	—	—	4	10
Vimentin	1 353 212	53.7	2	6	8	22	11	27

The number of peptides observed by MALDI-TOF/TOF tandem mass spectrometry and the percentage of amino acid sequence covered for each protein are indicated

astrocytic secretions.[9] However, clusterin, which is a secreted sulfated glycoprotein, was one of the major proteins found in midbrain and hippocampal ACM. Interestingly, the MALDI-TOF/TOF-MS/MS database search software was not able to identify clusterin in the cortical ACM, suggesting that this protein, if present at all, is secreted at very low levels.

Clusterin increases neuronal differentiation from VM-derived neurospheres. To determine whether clusterin was responsible for increased neuronal differentiation, immunodepletion studies were undertaken on midbrain ACM. To exclude any effect of the minimal media used for the conditioning, this was carried out in ACM (containing N2) as well as in ACM without N2 as was used for MS analysis. Results show that there was a similar level of clusterin in normal media compared with media used for MS analyses (Figure 1c) with a rabbit serum control negative for clusterin (data not shown). We next wanted to determine whether clusterin in our midbrain and hippocampal ACM was glycosylated. The two subunits of secreted clusterin can be resolved after treatment with endoglycosidases. Figure 1d and Supplementary Figure S1 show that secreted clusterin is highly glycosylated with >30% of its molecular weight corresponding to both *O*- and *N*-linked oligosaccharides. To determine the effect of clusterin on neuronal differentiation, neurospheres were differentiated in media, which had either had clusterin immunodepleted or still had it present. The results show that on removal of clusterin (−sClu), there was a significant decrease in the number of TuJ1$^+$ neurons, and also phosphorylated neurofilament (pNF; Figure 1e). The rabbit serum control did not have any effect and neuronal differentiation was not significantly different from untreated ACM. This demonstrates that secreted clusterin is having a trophic effect on neuronal differentiation.

We next wanted to determine whether the protein clusterin alone could increase neuronal number to a similar extent to midbrain ACM. Clusterin was added at a concentration of 10 nM and found to significantly increase the number of TuJ1$^+$ neurons (Figures 3a and d). To determine whether this was via an increase in cell survival or an increase in proliferation, TUNEL staining and BrdU analysis were performed. Results show that 10 nM clusterin decreased the number of apoptotic nuclei compared with untreated cells (Figures 3b and d), but did not significantly affect cell proliferation (Figure 3c, Supplementary Figure 2). Interestingly, 10 nM clusterin reduced the number of TUNEL$^+$ nuclei to a similar extent to midbrain ACM, suggesting this protein is present at a similar concentration in midbrain ACM. Furthermore, 10 nM clusterin caused a sustained increase in ERK phosphorylation, indicating its potential to regulate cellular differentiation and survival (Figure 3e and f).

Clusterin increases neuronal differentiation from cortical NPCs. To determine if clusterin could increase neuronal differentiation from another brain region, cortically derived NPCs expanded under identical conditions as described for VM cultures, were treated with clusterin (5 or 10 nM). Clusterin at 10 nM (but not at 5 nM) increased the number of TuJ1$^+$ neurons (Figures 4a and d), had no effect

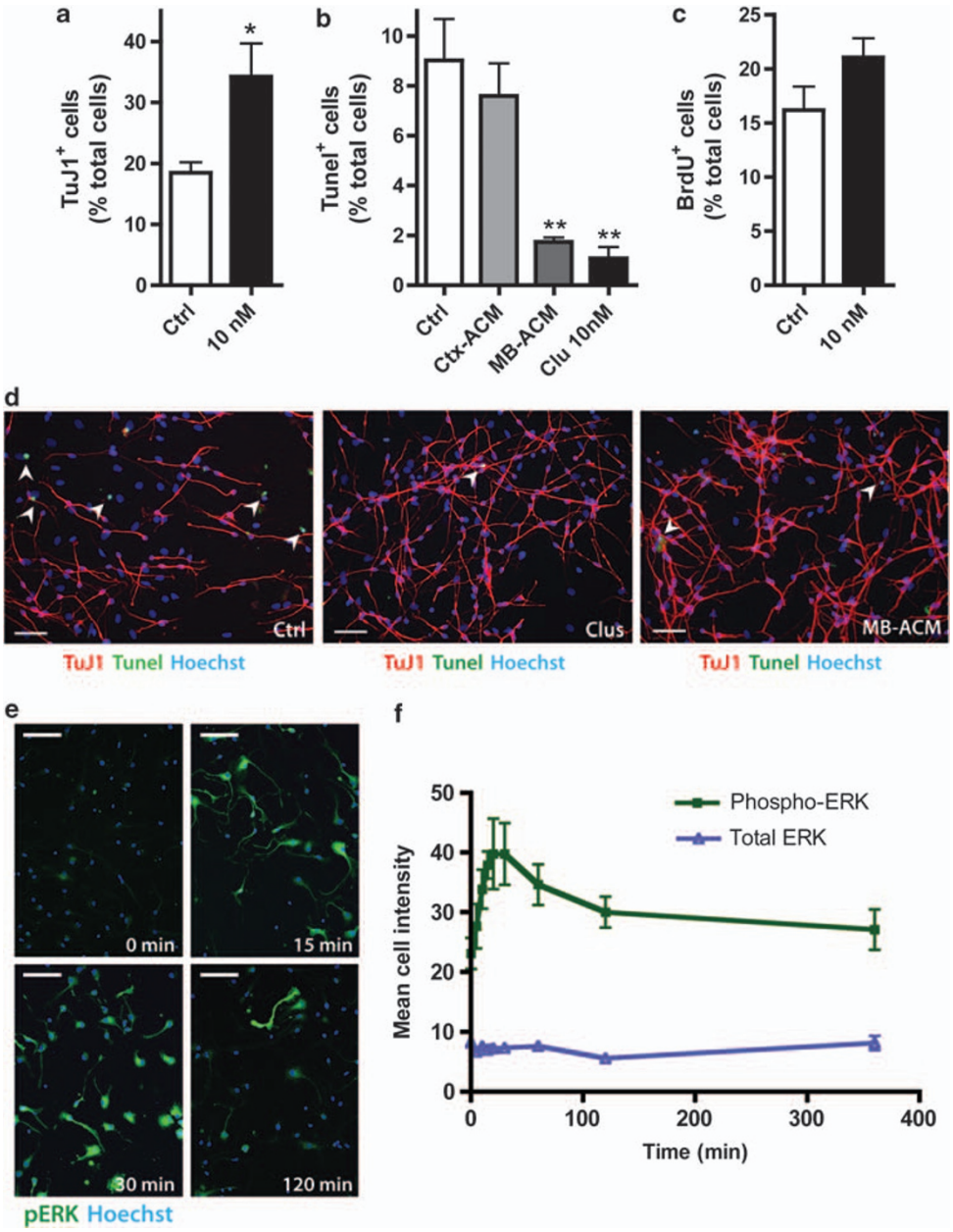

Figure 3 Clusterin promotes neuronal differentiation from VM derived hNPCs via increasing cell survival. (**a**) Clusterin increases the number of TuJ1$^+$ neurons, (**b**) clusterin decreases TUNEL$^+$ nuclei to a greater extent than CTX-ACM and to a similar extent to midbrain ACM, (**c**) clusterin does not increase proliferation. (**d**) Photomicrograph of TUNEL$^+$ nuclei. Scale bar = 50 μm. (**e**) Photomicrographs of pERK at the indicated times. Scale bar = 100 μm. (**f**) 10 nM clusterin increases pERK (squares) but not total ERK, (triangles). $^*P<0.05$, $^{**}P<0.01$ *versus* Ctrl

on cell proliferation (Figure 4b) but decreased TUNEL$^+$ nuclei (Figures 4c and d), similar to that reported above for midbrain NPCs. This demonstrates that the effects of clusterin are not region specific, although its secretion is.

Discussion

ACM derived from the hippocampus and midbrain increases neuronal differentiation from expanded human NPCs, as assessed by TuJ1 and SMI312. SMI312 identifies nNFs and has previously been shown to be axon specific.[10] Phosphorylation of neurofilaments during development is fundamental to maturation and stabilization of the neuronal cytoskeleton, as well as an increase in axonal diameter.[11] We show that hippocampal and midbrain ACM also increase the neurite length of pNF-positive neurons, indicating their role in neuronal maturation. Cortical ACM had no such effect. Most astrocytes are known to have similar morphological features, however, they manifest functional differences in various CNS regions, probably because of the heterogeneity observed in their profile of membrane receptors, ion channels and gap junction coupling (for review see Wilkin *et al.*[12]). It is well known that astrocytes can produce soluble factors that influence function in the CNS; in fact their main functions

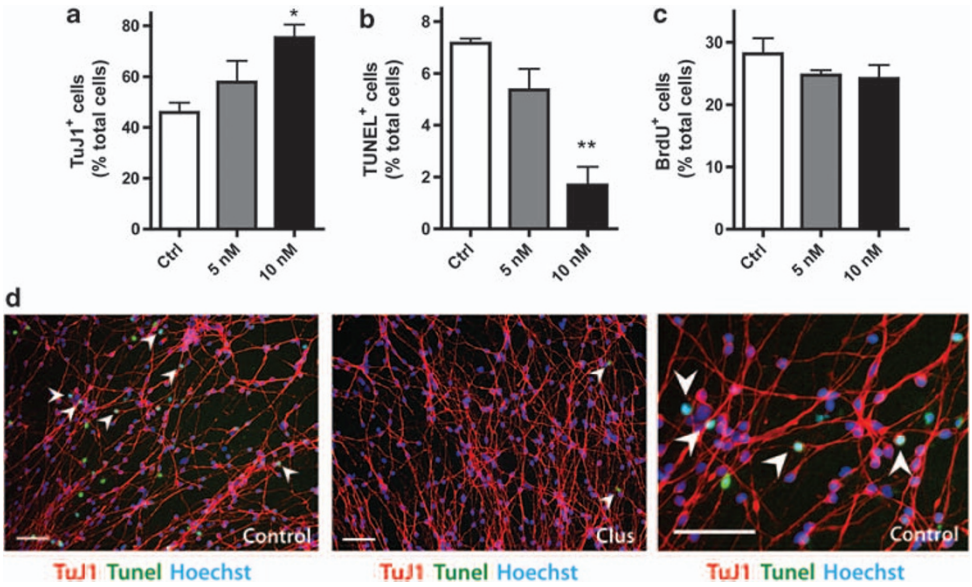

Figure 4 Clusterin increases neuronal differentiation from cortical-derived hNPCs by increasing cell survival. (**a**) Clusterin (10 nM) increases the number of TuJ1$^+$ neurons, (**b**) clusterin does not increase proliferation, (**c**) clusterin decreases TUNEL$^+$ nuclei after 1 week, (**d**) photomicrographs of TuJ1, TUNEL$^+$ nuclei in untreated cultures. Scale bar = 50 μm. *$P<0.05$, **$P<0.01$ *versus* Ctrl

were thought to be structuring the brain and supplying neurons with trophic support. However, it has recently been shown that astrocytes can promote neurogenesis from both adult neural and skin-derived stem cells,[8,13] as well as mouse embryonic stem cells.[14]

MALDI-TOF analysis revealed a number of proteins were present in all ACMs tested. Cystatin C is a low-molecular weight protein (13 kDa), which is expressed in the brain of rat, monkey and man, and is localized predominately in astrocytes but also in some neurons.[15] Interestingly, it has been purified from the conditioned medium of adult hippocampal neural stem cell cultures, and has been shown to be required for the mitogenic activity of FGF2.[16] In addition, its combined delivery with FGF2 *in vivo* to the adult dentate gyrus resulted in increased neurogenesis in this brain region.[16] Moreover, cystatin C has been shown to have a role in neuroprotection *in vivo*, for example, Olsson *et al.*[17] demonstrated that focal ischemia resulted in significantly greater brain infarcts in a cystatin C gene-knockout mouse. Furthermore, cystatin C has been shown to prevent the degeneration of rat substantia nigra dopamine neurons both *in vitro* and *in vivo*.[18] Taken together, these results demonstrate the importance of this protein in cell survival. Another two proteins found in ACM, α-2 macroglobulin and extracellular matrix 2 have been implicated in neurite outgrowth. The α-2 macroglobulin is a major serum glycoprotein found in adult and fetal brain cells[19] including astrocytes.[20] It has been shown to bind neurotrophic factors, such as NGF and NT3, which are secreted by astrocytes.[21] We have previously shown that human NPC's express the trk C-receptor,[22] so binding of α-2 macroglobulin to NT3 secreted by astrocytes may be having a role in the neurite outgrowth seen with hippocampal and midbrain ACM. In addition, extracellular matrix glycoprotein, falls into the family of neurite promoting factors, so this too may be having a role in neurite outgrowth.[23]

The other major protein that was found in midbrain and hippocampal ACM is clusterin. This is a major secretory glycoprotein of 75–80 kDa, which to date has been implicated in several diverse physiological functions (for review see Rosenberg and Silkensen[24]). It has been shown to have greater expression in the brain than in other tissues, and its expression is highest in astrocytes, whereas its expression in neurons shows regional differences both in rat and human brain.[25] In this study, we show that it has no effect on the proliferation of NPCs, but it does promote their differentiation by sustaining extracellular signal-regulated kinase (ERK) phosphorylation and decreasing apoptotic nuclei. Interestingly, an increase in phosphorylated ERK (pERK) has been associated with increased cell survival (for review see Nishida and Gotoh[26]), so together our data on sustained pERK and decrease in TUNEL-positive nuclei suggests clusterin is promoting both cell survival and differentiation. Interestingly, clusterin has previously been shown to have both pro- and anti-apoptotic potential. In the lipopolysaccharide model of glial activation, a process which occurs following injury, clusterin levels are increased 24 h after its addition to astrocyte cultures.[27] Indeed, several reports have shown that clusterin is a sensitive marker for different kinds of degeneration and neuronal injury.[28] It is thought to have a neuroprotective role in Alzheimers disease (AD), because neurons expressing clusterin have enhanced resistance to neuronal death.[29] Two recent large genome-wide association studies demonstrated that clusterin is an important susceptibility gene for late onset AD.[30,31] Indeed, clusterin has been found in the regions of the brain most affected in AD, the hippocampus and entorhinal cortex (for review see Nuutinen *et al.*[32]). However, clusterin is rarely found in neurons containing neurofibrillary tangles.[29] In addition, the presence of ApoE4/4 allele significantly decreased the amount of clusterin in the frontal lobe in AD patients.[33] Together these observations suggest

that the increased expression of clusterin in AD may represent a protective effect. In further support of its pro- and anti-apoptotic effects, studies using clu−/− mice, demonstrate that autoimmune myocardial damage is increased[34] and clusterin has neuroprotective properties in permanent focal cerebral ischemia.[35] In contrast, in the same knockout strain the absence of clusterin reduces cell death in neonatal hypoxia–ischaemia via a caspase-3-independent brain injury pathway,[36] suggesting it functions to exacerbate neuronal damage under these circumstances. Interestingly, there are a number of other molecules such as nitric oxide (for review see Choi *et al.*[37]) and HIF1a (for review see Piret *et al.*[38]) that serve to promote or inhibit cell death depending on the cellular context and clusterin may fit into this same category.

In conclusion, we have shown that clusterin secreted from midbrain astrocytes, increases neurogenesis and neurite outgrowth from expanded human NPCs. This protein may have implications for repair in the CNS.

Materials and Methods

Preparation of astrocyte-conditioned medium. Mixed glial cultures from P0 rat pups were prepared as previously described.[39] Culture medium used was Dulbecco's Modified Eagle's Medium (DMEM), 1% penicillin G/streptomycin/amphotericin and 10% fetal calf serum. Medium was changed after 24 h and twice weekly thereafter until the cells reached confluence (8–10 days). Microglia were removed by vigorous shaking of the cultures at 240 r.p.m. (20 min), followed by removal of oligodendrocyte progenitor cells by shaking at 160 r.p.m. (overnight). The remaining monolayer was treated with 20 μM Ara-C for 72 h, and washed with DMEM/1% PSF/1% N2. At 24 h later, fresh DMEM/1% PSF was added for a further 48 h. Media was then filtered, aliquoted and stored at −20°C. To enable the ACM to be analysed on the mass spectrometer, DMEM was replaced with phenol-free DMEM and supplemented with glucose (3500 mg/l) and L-glutamine (4 mM).

Human neurosphere cell culture and immunohistochemistry. Human VM or cortex (8–10 weeks post conception: $n=6$) was collected following routine terminations of pregnancies. The methods of collection conformed to arrangements recommended by the Polkinghorne Committee (1989) and the United Kingdom Department of Health guidelines (1995). Human neurospheres were expanded as previously described.[6] To induce differentiation, neurospheres were plated on poly-D-lysine/laminin in DMEM, 1% N2, 1 μg/ml bovine serum albumin, 0.6 μg/ml *N*-acetyl-cysteine, 5 nM forskolin. ACM from the cortex, hippocampus or midbrain made up 50% of the media. Half the media was replaced every 4 days. BrdU (0.2 μM) was added to the medium on plating day for 7 days. After 7 days or 28 days, the cells were fixed in PFA (4%). Primary and secondary antibodies are detailed in Supplementary Table 1. TUNEL cell death assays were carried out following manufacturer's guidelines (Roche, Burgess Hill, UK).

Imaging and quantification of cultured cells. Cultures were viewed under a Leitz DMRD fluorescent microscope attached to a Hamamatsu C4742-95 digital camera and visualized using Openlab 2.1 (Improvision, Coventry, UK). Images were taken from a minimum of four random fields per coverslip from four coverslips per culture ($\times$ 20 magnification) from at least four independent cultures. Results were analysed by one-way ANOVA followed by Newman–keuls *post hoc* test.

MALDI tandem MS analysis and protein identification. Proteins in 200 μl of cell media were precipitated with acetone, resolubilized in 0.3% SDS, reduced and alkylated with *S*-methyl methanethiosulfonate and digested with trypsin (Promega, Southampton, UK) at 37°C overnight. The peptide mixture was zip-tipped (C18; Waters) and analysed by nanoLC-MALDI as previously described.[40] MS analysis was achieved using a MALDI-TOF/TOF instrument (4700 Proteomics Analyzer, Applied Biosystems, Warrington, UK).

MS data analysis. The MS/MS spectra were searched against all species in the National Center for Biotechnology Information database using GPS software (Applied Biosystems) running Mascot search algorithm (Matrix Science, Boston, MA, USA) for peptide and protein identification. A mass tolerance of 100 p.p.m. and 0.25 Da was used for precursors and fragment ions, respectively. The data searches were performed with the following criteria: variable modifications with methionine oxidation, *S*-methyl methanethiosulfonate derivation of cysteine and N-terminal pyroglutamate. Two missed cleavages were allowed. At least 95% confidence interval threshold was used for peptide identification and manual validation was also performed for each MS/MS spectrum.

Immunodepletion of clusterin from midbrain ACM. Following washing, Pansorbin A beads were incubated in either anti rabbit clusterin (0.1 mg) or normal rabbit serum (Invitrogen, Renfrew, UK) overnight at 4°C. Following $\times$ 3 PBS washes; beads with absorbed IgGs were incubated with 5 ml of midbrain ACM for 6 h at 4°C that had been collected in N2-containing medium or medium used for MS analysis (without N2). Beads were pelleted by centrifugation. ACM was collected for neuronal differentiation and beads were boiled for 5 min in lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EGTA) containing a cocktail of protease inhibitors (Complete, Mini, Roche) and centrifugation at 14 000 r.p.m. for 20 min at 4°C.

Deglycosylation of conditioned media. A volume of 18 μl of ACM from midbrain and hippocampus was incubated for 10 min at 100°C with 2 μl of denaturing buffer (NEB). Denatured ACMs were treated with 500 U of peptide: *N*-glycosidase F (PNGase F, NEB) to remove *N*-linked oligosaccharides; 40 000 U of endo-α-*N*-acetylgalactosaminidase (*O*-glycosidase, NEB) and 50 U of neuramidase (NEB) to eliminate *O*-linked oligosaccharides. Reactions were carried out in 1% NP40 and 1 $\times$ G7 reaction buffer at 37°C overnight. Deglycosylated extracts were run in 12% SDS–Tris–glycine gels and probed for clusterin.

SDS gel electrophoresis and western blot analysis. After electrophoretical separation of protein extracts in 10% SDS–Tris–glycine gels, proteins were blotted onto 0.45 μm PVDF membranes. Membranes were blocked with 5% non-fat dry milk and 0.05% Tween 20 (Sigma-Aldrich, Munich, Germany) in TBS-buffer (BioRad, Munich, Germany). After incubation with primary antibody (anti-Rat clusterin, 1: 100 in blocking buffer; rabbit polyclonal antibody BioVendor Laboratory Medicine, Brno, Czech Republic) and HRP-labelled secondary antibody anti-rabbit IgG (1 : 3000; Dako, Ely, UK; in blocking buffer) protein bands were visualized with ECL plus chemiluminescence developing agent (Amersham ECL plus, GE Healthcare, Little Chalfont, UK).

Semi-automated image acquisition and analysis. In all, 15 000 cells were plated on poly-D-lysine coated 96-well plates. After 24 h, cells were stimulated with 10 nM clusterin (Prospecbio, Rehovot, Israel) for between 5 min and 6 h. Cells were fixed with 4% PFA and stained with anti-p44/42 MAP kinase and anti-phospho p44/42 MAP kinase overnight. Appropriate secondary antibodies were used (Supplementary Table 1). Image acquisition and analysis were performed on an IN Cell Analyzer workstation (GE Healthcare) using a multitarget analysis algorithm to quantify fluorescence intensity in nuclear and cytoplasmic compartments (in total and ppERK1/2 image channels).

Conflict of interest

The authors declare no conflict of interest.

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