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

*Running Title* : Clusterin enhances neuronal differentiation.

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## **Abstract.**

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 prior to differentiation for 1 or 4 weeks in ACM. 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 (pNF). Maldi-TOF analysis used to determine differences in media revealed that although all three regional ACMs had cystatin C,  $\alpha$ -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 pERK, implicating its role in cell survival and differentiation. These findings further reveal differential effects of regional astrocytes on NPC behaviour and identify clusterin as an important mediator of NPC derived neuronal survival and differentiation.

## **Introduction**

Astrocytes are essential regulators of neuronal function and produce diffusible and non-diffusible neuron supporting signals, including neurotrophic factors and membrane-bound molecules (for reviews see 1, 2). 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 (3,4). In contrast, astrocytes from the mesencephalon release factors, which are more effective at promoting dopamine neuron survival than cortical or striatal astrocytes (5). These studies demonstrate that astrocytes from neurogenic regions (6) release more neurogenic factors than astrocytes from 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 7) More recently the same has been shown to be true for the developing human fetal CNS (8). These cells are capable of long-term expansion in vitro to achieve approximately 50 population doublings (9). However, as they progress in culture with time their differentiation pattern changes with the number of neurons declining and the number of astrocytes predominating (10, 11, 12).

Previous studies have shown that astrocyte condition media (ACM) increases the number of neurons from adult rodent neural stem cells (13). The aim of this present 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 NPCs. Cells were differentiated for 1 week or 1 month in control media or in regional ACM. Results show that hippocampal and midbrain ACM increase the number of TuJ1<sup>+</sup> and SMI312<sup>+</sup> neurons. Mass spectrometric 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.

## **METHODS**

### *Preparation of astrocyte-conditioned medium*

Mixed glial cultures from P0 rat pups were prepared as previously described (14, 15). Culture medium used was DMEM, 1% penicillin G/streptomycin/amphotericin and 10% Fetal Calf Serum (FCS). Medium was changed after 24h and twice weekly thereafter until the cells reached confluence (8-10 days). Microglia were removed by vigorous shaking of the cultures at 240 rpm (20 min), followed by removal of oligodendrocyte progenitor cells by shaking at 160 rpm (overnight). The remaining monolayer was treated with 20 $\mu$ M Ara-C for 72h, and washed with DMEM / 1% PSF / 1% N2. 24h later fresh DMEM / 1% PSF was added for a further 48h. 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 (3500mg/l) and L-glutamine (4mM).

### *Human neurosphere Cell Culture and Immunohistochemistry*

Human ventral mesencephalon (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 UK Department of Health guidelines (1995). Human neurospheres were expanded as previously described (9). To induce differentiation, neurospheres were plated on poly-D-lysine/laminin in DMEM, 1% N2, 1 $\mu$ g/ml Bovine serum albumin, 0.6 $\mu$ g/ml N-Acetyl-cysteine, 5nM forskolin. ACM from the cortex, hippocampus or midbrain made up 50% of the media. Half the media was replaced every 4d. 0.2  $\mu$ M BrdU was added to the medium on plating day for 7d. After 7d or 28d the cells were fixed in PFA (4%). Primary and secondary antibodies are detailed

in Suppl Table1. TUNEL cell death assays were carried out following manufacturer's guidelines (Roche).

*Imaging and quantification of Cultured Cells.*

Cultures were viewed under a Leitz DMRD fluorescent microscope attached to a Hamamatsu C4742-95 digital camera and visualised using Openlab 2.1 (Improvision, Coventry, UK). Images were taken from a minimum of 4 random fields per coverslip from 4 coverslips per culture (x20 magnification) from at least 4 independent cultures. Results were analysed by One-way ANOVA followed by Newman-keuls post-hoc test.

*MALDI Tandem Mass Spectrometric Analysis and protein identification*

Proteins in 200 µl of cell media were precipitated with acetone, resolubilised in 0.3% sodium dodecyl sulphate (SDS), reduced and alkylated with *S*-methyl methanethiosulfonate and digested with trypsin (Promega) at 37 °C overnight. The peptide mixture was zip-tipped (C18; Waters) and analysed by nanoLC-MALDI as previously described (16). Mass spectrometric analysis was achieved using a MALDI-TOF/TOF instrument (4700 Proteomics Analyzer, Applied Biosystems).

*Mass Spectrometric Data Analysis.* The MS/MS spectra were searched against all species in the National Center for Biotechnology Information (NCBI) database using GPS software (Applied Biosystems) running Mascot search algorithm (Matrix Science, Boston, MA) for peptide and protein identification. A mass tolerance of 100ppm and 0.25Da 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 (MMTS) 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 astrocytes conditioned medium.*

Following washing, Pansorbin A beads were incubated in either anti rabbit clusterin (0.1mg) or normal rabbit serum (Invitrogen) overnight at 4°C. Following x3 PBS washes; beads with absorbed IgGs were incubated with 5 ml of midbrain ACM for 6 hours at 4°C that had been collected in N2 containing medium or medium used for Mass Spec analysis (without N2). Beads were pelleted by centrifugation. ACM was collected for neuronal differentiation and beads were boiled for 5 mins 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 rpm for 20 min at 4°C.

*Deglycosylation of conditioned media*

18 ul of ACM from Midbrain and Hippocampus were 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; 40000 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 1x 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% Tween20 (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, Inc) and HRP-labelled secondary antibody anti-rabbit IgG (1:3000; DAKO; in blocking buffer) protein bands were visualized with ECL plus chemiluminescence developing agent (Amersham ECL plus, GE Healthcare, UK).

#### *Semi-automated Image Acquisition and Analysis*

15000 cells were plated on poly-D-lysine coated 96-well plates. 24h later cells were stimulated with 10nM clusterin (Prospecbio) for between 5 min and 6 hours. 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 (Suppl T2). Image acquisition and analysis was performed on an IN Cell Analyzer workstation (GE Healthcare) using a Multi Target Analysis algorithm to quantify fluorescence intensity in nuclear and cytoplasmic compartments (in total and ppERK1/2 image channels).

## Results

*ACM from the hippocampus and midbrain, but not cortex increase the number of neurons from human NPCs.*

In order to determine if regional ACM had an effect on the number of NPC derived neurons, human VM was expanded for 4 weeks in FGF2 prior to 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 ( $P < 0.05$ ; Fig 1A), while 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. Results show that midbrain ACM significantly increased the number of SMI312+ neurons ( $P < 0.001$ ; Fig 1A, 1B) after a week ( $P < 0.01$ ) and a month ( $P < 0.01$ ). However, hippocampal ACM showed an increase after a month only ( $P < 0.05$ ; Fig 1A). In order to determine if 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 mass spectroscopy*

In order 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 ACMs tested (Table 1). Many of these proteins are to be expected, including cystatin C, which has been observed in a previous proteomics experiment on astrocytic secretions (17). However, clusterin, which is a secreted sulphated 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. In order to exclude any effect of the minimal media used for the conditioning, this was done in ACM (containing N2) as well as in ACM without N2 as was used for mass spectrometric analysis. Results show that there was a similar level of clusterin in normal media compared to media used for mass spectrometric analyses (Fig 1C) with a rabbit serum control negative for clusterin (data not shown). **We next wanted to determine if clusterin in our midbrain and hippocampal ACM was glycosylated. The two subunits of secreted clusterin can be resolved after treatment with endo-glycosidases. Fig 1D and Fig S1 show that secreted clusterin is highly glycosylated with more than 30% of its molecular weight corresponding to both O-linked and N-linked oligosaccharides.** In order 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. Results show that upon removal of clusterin (-sClu) there was a significant decrease in the number of TuJ1+ neurons and also pNF (Fig 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 if the protein clusterin alone could increase neuronal number to a similar extent to midbrain ACM. Clusterin was added at a concentration of 10nM and found to significantly increase the number of TuJ1+ neurons (Fig 3A, D). In order to determine if this was via an increase in cell survival or an increase in proliferation, TUNEL staining and BrdU analysis were performed. Results show that 10nM clusterin decreased the number of apoptotic nuclei compared to untreated cells (Fig 3B, D) but did not significantly affect cell proliferation (Fig 3C, suppl Fig 2). Interestingly, 10nM 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 10nM clusterin caused a sustained increase in ERK phosphorylation indicating its potential to regulate cellular differentiation and survival (Fig 3E, F).

*Clusterin increases neuronal differentiation from cortical NPCs*

In order 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 10nM). Clusterin at 10nM (but not at 5nM) increased the number of TuJ1+ neurons (Fig 4A,D), had no effect on cell proliferation (Fig 4B) but decreased TUNEL+ nuclei (Fig 4C, 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 phosphorylated neurofilaments and has previously been shown to be axon specific (18). Phosphorylation of neurofilaments during development is fundamental to maturation and stabilisation of the neuronal cytoskeleton, as well as an increase in axonal diameter (19). We show that hippocampal and midbrain ACM also increase the neurite length of phosphorylated neurofilament 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 due to the heterogeneity observed in their profile of membrane receptors, ion channels and gap junction coupling (20, 21, 22). It is well known that astrocytes can produce soluble factors that influence function in the CNS; in fact their main functions 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 (13, 23) as well as mouse embryonic stem cells (24).

Maldi-TOF analysis revealed a number of proteins were present in all ACMs tested. Cystatin C is a low molecular weight protein (13kDa), which is expressed in the brain of rat, monkey and man and is localised predominately in astrocytes but also in some neurons (25). 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 (26). In addition, its combined delivery with FGF2 *in vivo* to the adult dentate gyrus resulted in increased neurogenesis in this brain region (26).

Moreover, cystatin C has been shown to have a role in neuroprotection in vivo, for example, Olsson and colleagues demonstrated that focal ischaemia resulted in significantly greater brain infarcts in a cystatin C gene knockout mouse (27). Furthermore, cystatin C has been shown to prevent the degeneration of rat substantia nigra dopamine neurons both in vitro and in vivo (28). Taken together these results demonstrate the importance of this protein in cell survival. Another 2 proteins found in ACM,  $\alpha$ -2 macroglobulin and extracellular matrix 2 have been implicated in neurite outgrowth.  $\alpha$ -2 macroglobulin is a major serum glycoprotein found in adult and fetal brain cells (29) including astrocytes (30). It has been shown to bind neurotrophic factors, such as NGF and NT3, which are secreted by astrocytes (31). We have previously shown that human NPC's express the trk C receptor (32), so binding of  $\alpha$ -2 macroglobulin to NT3 secreted by astrocytes may be playing a role in the neurite outgrowth seen with hippocampal and midbrain ACM. Additionally, extracellular matrix glycoprotein, falls into the family of neurite promoting factors, so this too may be playing a role in neurite outgrowth (33).

The other major protein that was found in midbrain and hippocampal ACM is clusterin. This is a major secretory glycoprotein of 75-80kDa (34), which to date has been implicated in several diverse physiological functions (35, 36). It has been shown to have greater expression in the brain than in other tissues (37). Its expression is highest in astrocytes, while its expression in neurons shows regional differences both in rat and human brain (38). Here we show that it has no effect on the proliferation of NPCs but it does promote their differentiation by sustaining ERK phosphorylation (for review see 39) and decreasing apoptotic nuclei. Interestingly, an increase in pERK has been associated with increased cell survival (for review see 40), 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 hours after its addition to astrocyte cultures (41). Indeed, several reports have shown that clusterin is a sensitive marker for different kinds of degeneration and neuronal injury (42). It is thought to play a neuroprotective role in Alzheimers disease (AD), because neurons expressing clusterin have enhanced resistance to neuronal death (43). Two recent large genome wide association studies demonstrated that clusterin is an important susceptibility gene for late onset AD (44, 45). Indeed, clusterin has been found in the regions of the brain most affected in AD, the hippocampus and entorhinal cortex (for review see 46). However, clusterin is rarely found in neurons containing neurofibrillary tangles (43). In addition, the presence of ApoE4/4 allele significantly decreased the amount of clusterin in the frontal lobe in AD patients (47). Together these observations suggest that the increased expression of clusterin in AD may represent a protective effect. In further support of its pro and antiapoptotic effects, studies using *clu*<sup>-/-</sup> mice, demonstrate that autoimmune myocardial damage is increased (48) and clusterin has neuroprotective properties in permanent focal cerebral ischaemia (49, 50). 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 (51), 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 52) and HIF1 $\alpha$  (for review see 53) 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.

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## Legends

**Figure 1. ACM increases neuronal differentiation from VM derived NSCs.** A, Midbrain ACM increases TuJ1 after 1 week and both hippocampal and midbrain ACM increase TuJ1 after one month of differentiation. Midbrain ACM increases phosphorylated neurofilaments<sup>+</sup> (pNF) neurons after 1 week and both hippocampal and midbrain increase pNF after one 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, NSCs 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 to rabbit serum control or ACM. \*P<0.05, \*\*P<0.01 versus control and CTX ACM.

**Table 1.** Proteins identified in the conditioned medium of cultured astrocytes from various brain regions. The numbers of peptides observed by MALDI-TOF/TOF tandem mass spectrometry and the percentage of amino acid sequence covered for each protein is indicated.

**Figure 2. Clusterin or regional ACM does not influence GFAP positive cells**

A, Photomicrographs of GFAP, untreated and treated with clusterin, B, regional ACM does not significantly increase the number of astrocytes after 1 or 4 weeks differentiation. Scale bar = 50  $\mu$ m

**Figure 3. Clusterin promotes neuronal differentiation from VM derived HNSC's 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  $\mu$ m. E, Photomicrographs of pERK at the indicated times. Scale bar = 100  $\mu$ m. F, 10nM clusterin increase pERK (squares) but not total ERK, (triangles).

**Figure 4. Clusterin increases neuronal differentiation from cortical derived**

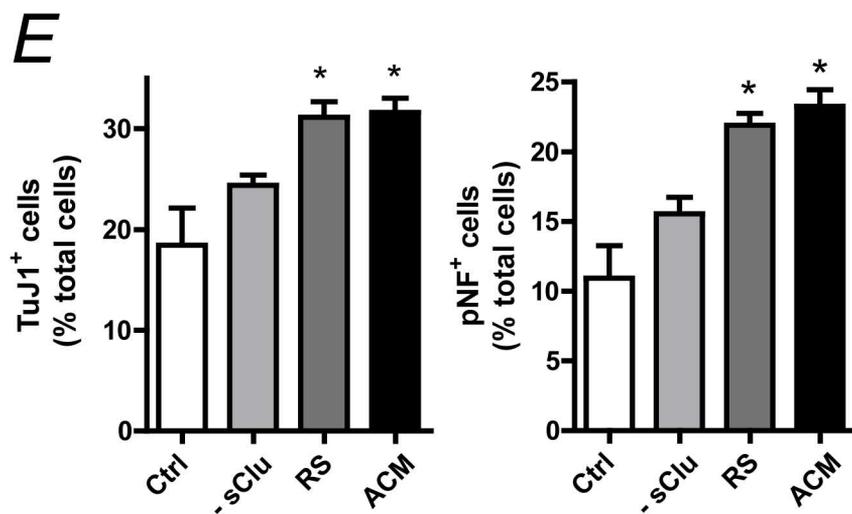
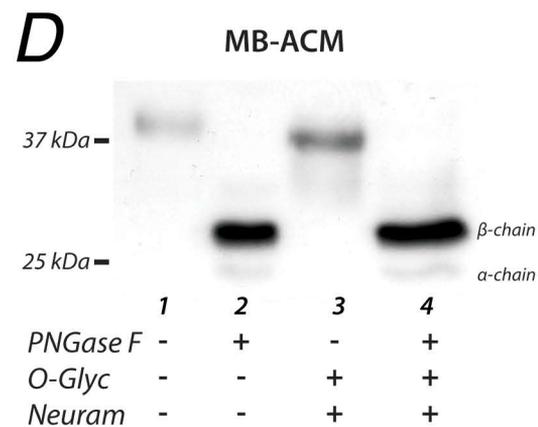
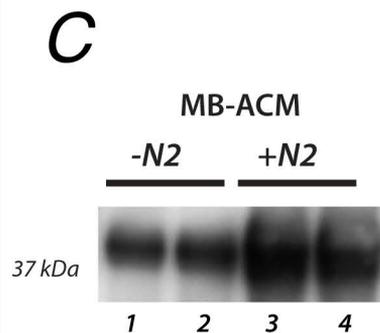
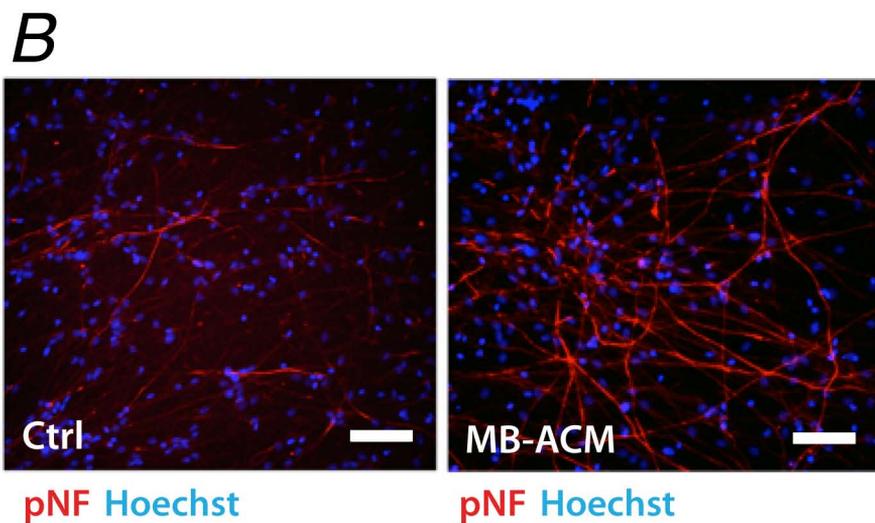
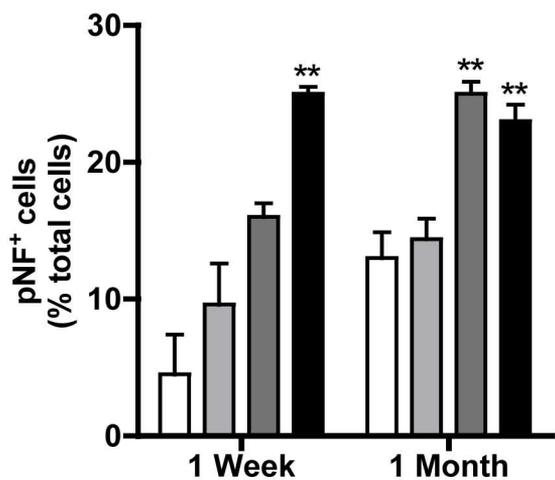
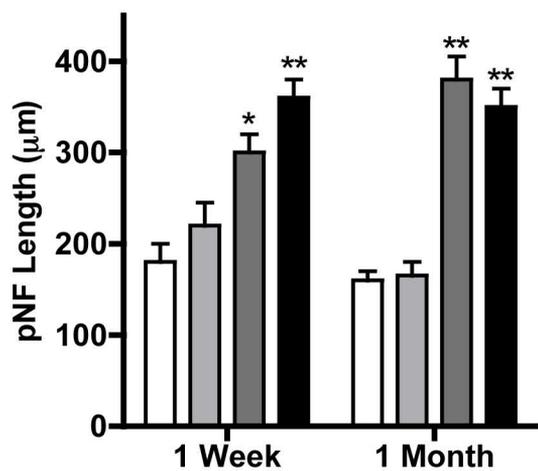
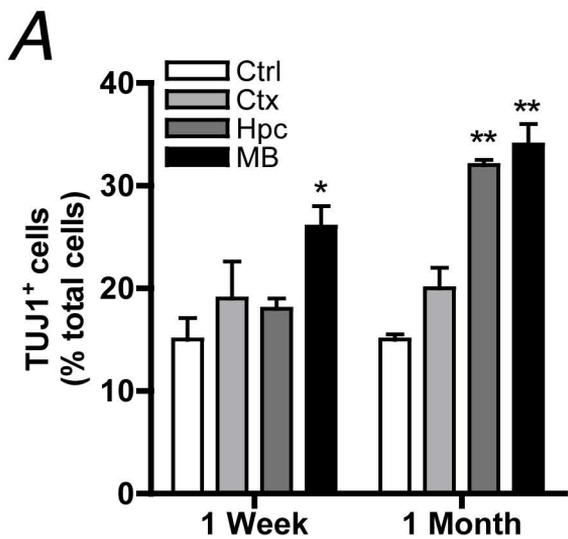
**HNPC's by increasing cell survival.** A, 10nM clusterin increases the number of TuJ1+ neurons, B, clusterin does not increase proliferation, C, clusterin decreases TUNEL+ nuclei after 1 week, D, Photomicrographs of TuJ1, TUNEL+ nuclei in untreated cultures. Scale bar = 50  $\mu$ m. \*P<0.05, \*\*P<0.01 versus ctrl.

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**Conflict of Interest statement.**

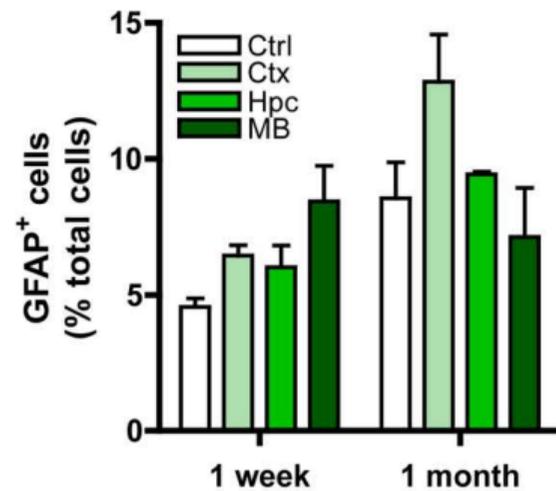
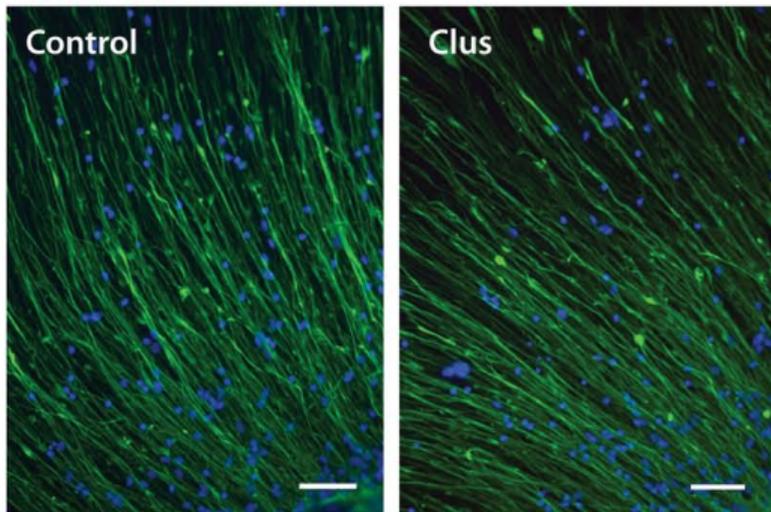
The authors declare no conflict of Interest.

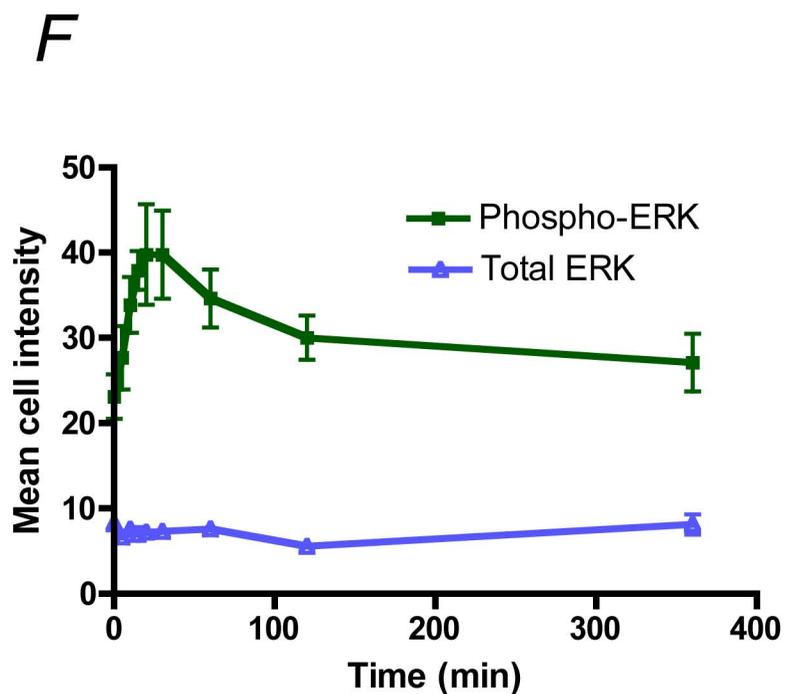
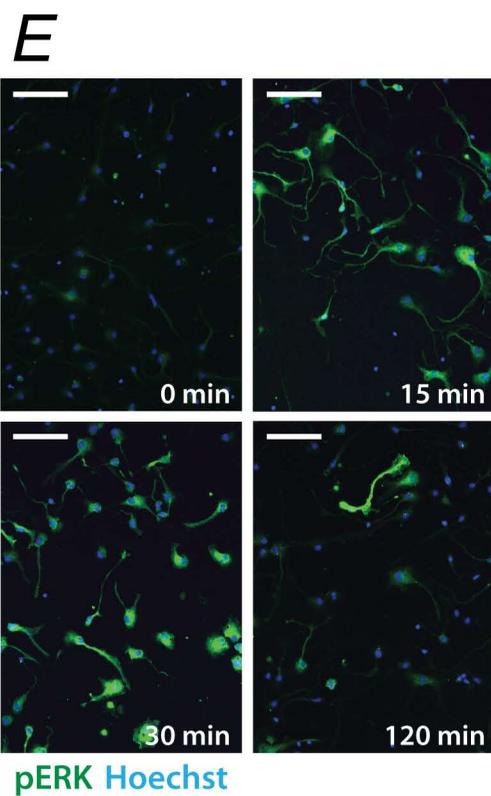
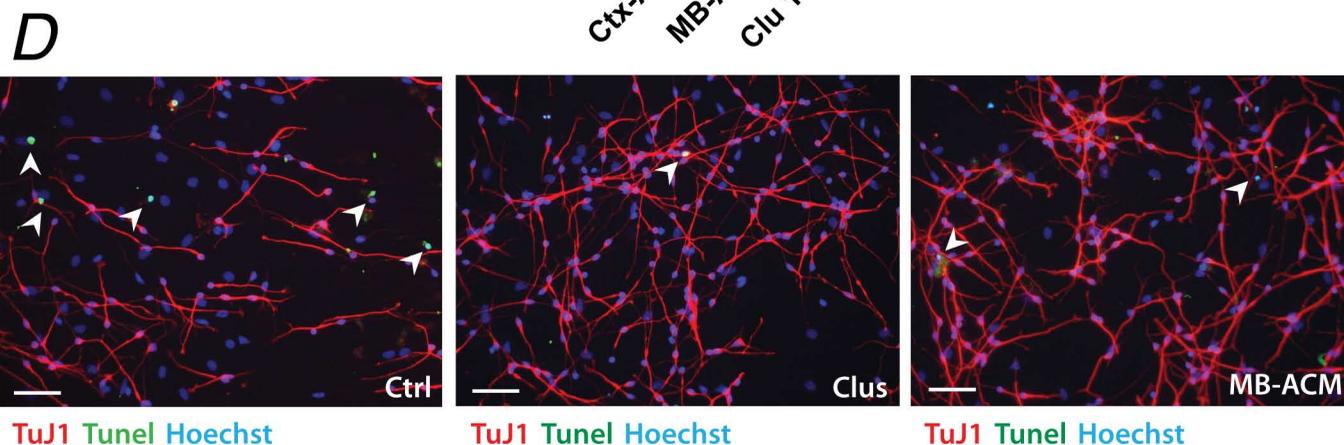
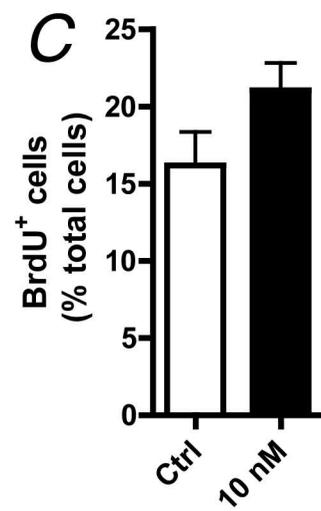
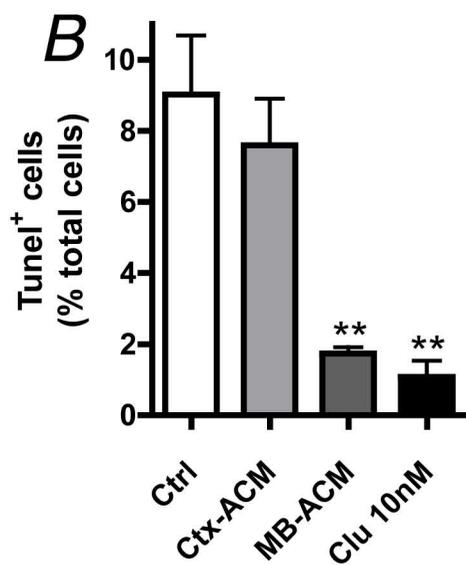
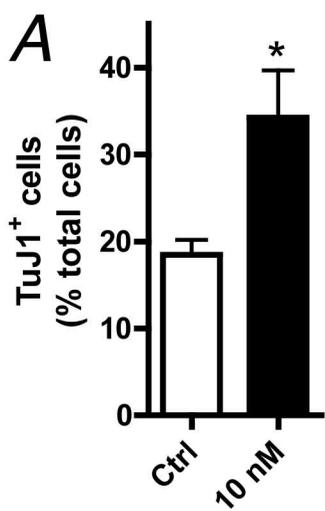


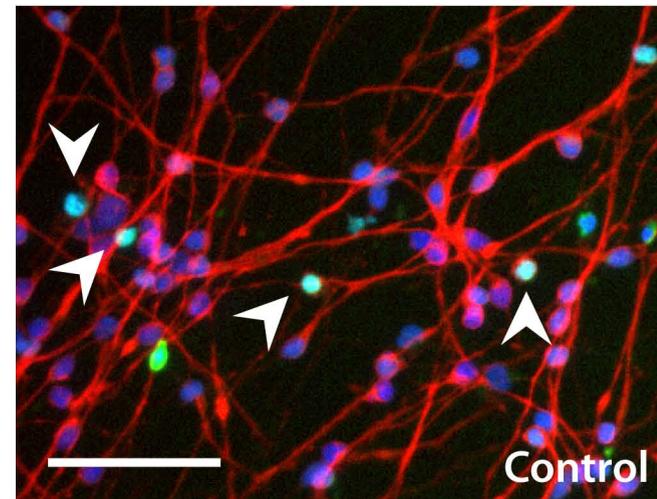
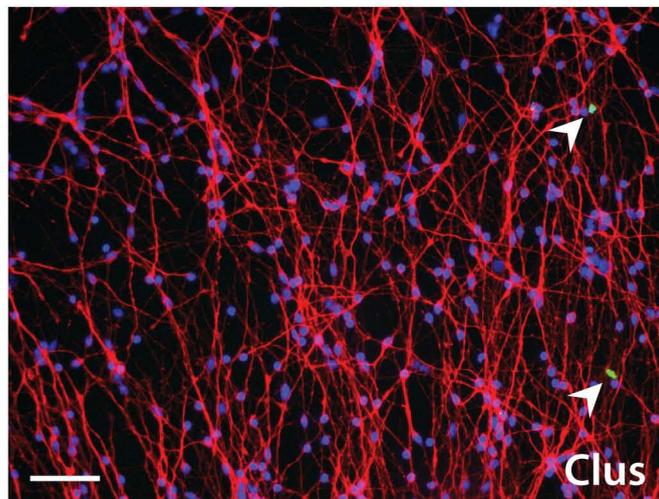
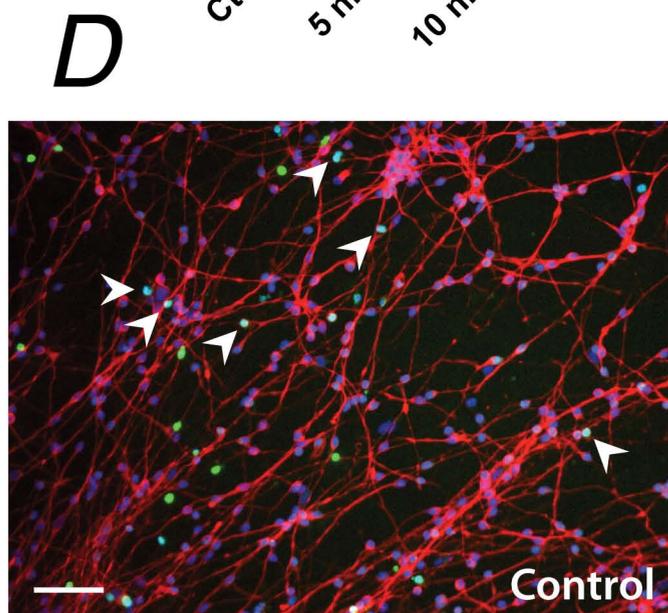
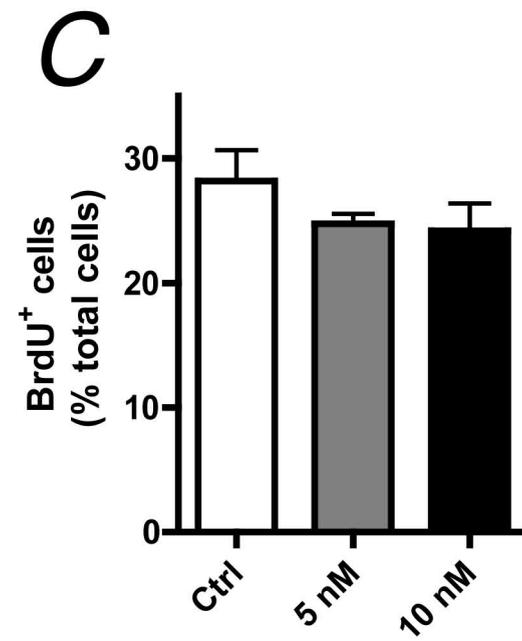
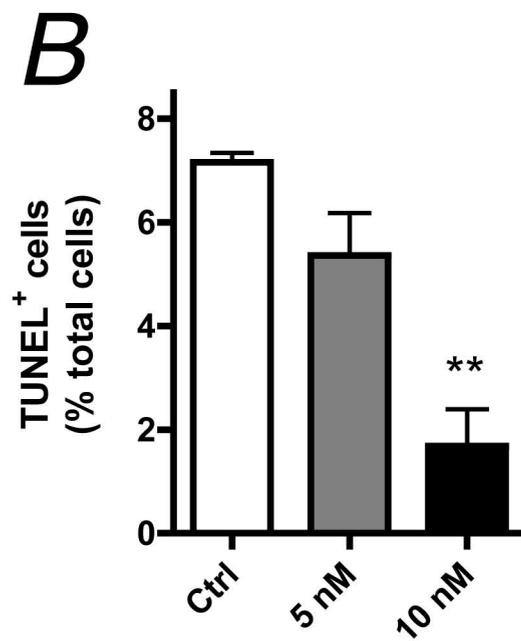
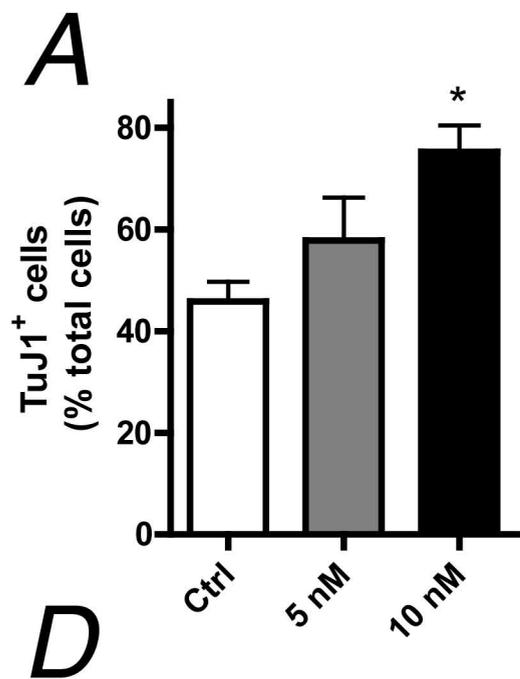
Protein	Accession Number	Mw (kDa)	Midbrain		Hippocampus		Cortex	
			Peptides	Coverage	Peptides	Coverage	Peptides	Coverage
<i>α-2-macrogobulin</i>	6978425	164	10	8.7%	6	6%	3	3%
<i>clusterin</i>	17985951	51	4	11%	4	11%	-	-
<i>cystatin C</i>	226712	13	3	39%	3	30%	3	39%
<i>extracellular matrix protein 2</i>	6978789	70.5	2	6%	-	-	4	10%
<i>vimentin</i>	1353212	53.7	2	6%	8	22%	11	27%

**Table 1**

## GFAP DNA







TuJ1 Tunel Hoechst

TuJ1 Tunel Hoechst

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