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Mesenchymal stem cell conditioned medium increases glial reactivity and decreases neuronal survival in spinal cord slice cultures

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ABSTRACT

Ex vivo spinal cord slice cultures (SCSC) allow study of spinal cord circuitry, maintaining stimuli responses comparable to live animals. Previously, we have shown that mesenchymal stem/stromal cell (MSC) transplantation *in vivo* reduced inflammation and increased nerve regeneration but MSC survival was short-lived, highlighting that beneficial action may derive from the secretome. Previous *in vitro* studies of MSC conditioned medium (CM) have also shown increased neuronal growth. In this study, murine SCSC were cultured in canine MSC CM (harvested from the adipose tissue of excised inguinal fat) and cell phenotypes analysed via immunohistochemistry and confocal microscopy. SCSC in MSC CM displayed enhanced viability after propidium iodide staining. GFAP immunoreactivity was significantly increased in SCSC in MSC CM compared to controls, but with no change in proteoglycan (NG2) immunoreactivity. In contrast, culture in MSC CM significantly decreased the prevalence of βIII-tubulin immunoreactive neurites, whilst Ca^{2+} transients per cell were significantly increased. These *ex vivo* results contradict previous *in vitro* and *in vivo* reports of how MSC and their secretome may affect the microenvironment of the spinal cord after injury and highlight the importance of a careful comparison of the different experimental conditions used to assess the potential of cell therapies for the treatment of spinal cord injury.

1. Introduction

Organotypic SCSC preserve much of spinal cord structure and neuronal circuitry, whilst simultaneously allowing the manipulation *in vitro* experimentation affords [1–3]. SCSC have been used to model inflammation [4–6], microenvironmental changes [3,7,8], and potential cell therapies [9,10].

MSC injected *in vivo* into the site of spinal cord injury (SCI) release large numbers of growth factors, cytokines and immunomodulatory factors, influencing neuronal growth and angiogenesis [11–16]. Administration of bone marrow derived MSC *in vivo* showed functional improvement [13,14,17], axonal/neurite growth within the injury [13,18] and increased levels of cytokines such as BDNF, VEGF [19] IL4, IL13 [12] and NGF [20]. Similar effects and improvements have been shown following administration of MSC derived from adipose tissue [15,21,22] and umbilical cord [23,24]. In 2018, a study showed that rodent MSC CM was shown to have protective effects when injected into a rodent model of SCI [25]. There has been, however, minimal research into the effect of CM on SCSC. Early studies have shown that rat muscle cell and fibroblast CM increased neuronal growth within spinal cord explants [26,27], although more recent studies have examined how cell secretomes might influence spinal cell survival and growth after grafting experiments [28,29].

Although SCSC are useful tools in neuroscience research [30], to date no studies have specifically assessed the effects of MSC CM on murine SCSC. The aim of this study was to explore levels of cell survival, astrocyte reactivity, chondroitin sulphate proteoglycans (CSPG) and neuronal cell processes in murine SCSC following canine MSC CM treatment, providing the basis for development of a suitable *ex vivo* assay

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Abbreviations

CM	Conditioned medium
CSPG	Chondroitin sulphate proteoglycan
DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
GFAP	Glial fibrillary acidic protein
MSC	Mesenchymal Stem/Stromal Cell
NG2	Neuron-glial antigen 2
SC	Spinal cord
SCI	Spinal cord injury
SCSC	Spinal cord slice culture
PFA	Paraformaldehyde
PI	Propidium iodide
P/S	Penicillin/Streptomycin

for testing the efficacy of canine MSC CM for future veterinary clinical work. As the preparation of SCSC from dog pups is impracticable for technical and ethical issues, we decided to use murine SCSC that are widely characterized in our laboratories for the study of spinal cord circuitry *ex vivo*. Therefore, our approach of investigating canine MSC and canine MSC CM explores a new avenue in the field of veterinary medicine and has translational potential for human clinics, due to dogs having repeatedly demonstrated to be valuable translational models for human diseases [31–34].

2. Materials and methods

2.1. MSC isolation and expansion

Institutional ethical approval was obtained for this study (University of Chester: 060/16/CW/BS, May 18, 2016). MSC were isolated at the Veterinary Tissue Bank (Chirk, UK) from surgically extracted sections of dog inguinal fat pads (n = 3 donors).

Following collagenase digestion and preferential plastic adhesion (described previously [35]), cells were cultured in Dulbecco's modified Eagle medium/F-12 + GlutaMAX™ (DMEM/F-12), supplemented with 1% penicillin/streptomycin (P/S) and 10% foetal bovine serum (FBS) (all Gibco®, Life Technologies™, Paisley, UK) in a humidified atmosphere of 5% CO_2 at 37 °C. Cultures reaching 80% confluence were passaged using 0.25% trypsin-EDTA (Gibco®, Life Technologies™). Characterisation of culture-expanded cells was completed as reported previously [35] and cells were shown to exhibit an MSC phenotype, i.e., plastic adherence, an appropriate cluster of differentiation profile and tri-lineage differentiation potential [36].

2.2. Generation of MSC CM

As mouse SCSC are routinely cultured in a Neurobasal containing medium which is different from the standard MSC culture medium, MSC were conditioned using Neurobasal to generate an appropriate CM for the spinal slice assays. Briefly, MSC (passages 4–5) were seeded into a T75 culture flask at a density of 20,000 cells/cm^2 in 15 ml DMEM/F-12 and incubated overnight at 37 °C/5% CO_2 allowing cell adherence. Cells were washed once with warm sterile phosphate buffered saline (PBS); then 15 ml Neurobasal conditioning medium, consisting of Neurobasal Medium-A supplemented with 2% B27 50x, 2% 100 mM L-glutamine and 1% antibiotic/antimycotic (all Gibco®, Fisher Scientific) was added to the flask. One T75 flask produces approximately 15 ml of CM, which was harvested after 3 days incubation, filtered (0.2 μm) and stored at −80 °C in 1.5 ml aliquots. Control medium samples minus cells were prepared in tandem, following the same protocol.

2.3. SCSC preparation

Institutional ethical approval was obtained for this study (Italian Ministry of Health: authorisation number 485/2017-PR). SCSC were prepared from a total of 9 mixed gender CD1 mouse pups (postnatal day 7–11) from different litters. In each set of experiments a total of 3 pups were used to obtain between 6 and 10 slices to be put in culture. The preparation of SCSC followed a well-established protocol in the lab, as described previously [4,37] (Fig. 1). In brief, mice were euthanised and a dorsal laminectomy was performed on ice-cold cutting solution (containing in mM: 130 *N*-Methyl-D-glucamine, 10 Glucose, 24 $NaHCO_3$, 5 $MgCl_2$, 3.5 KCl, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, and pH adjusted to 7.35). Meninges were removed, and the spinal cord glued to a small agar block, dorsal side up. Transverse slices (350 μm thick) were obtained using a vibratome (Leica VT 1200, amplitude 1.7, 10 mm s^{-1}), washed and placed individually into well inserts (Millicell®, 12mm/0.4 μm, Massachusetts, USA). Inserts were placed into wells containing 250 μl high serum medium, consisting of 50% Eagle Basal Medium, 25% horse serum, 25% Hanks balanced salt solution, 0.5% glucose, 0.5% 200 mM L-glutamine, and 1% antibiotic/antimycotic (all Gibco®, Fisher Scientific) and incubated at 34 °C/5% CO_2. After 4 days of equilibration, fresh Neurobasal medium (supplemented with B27 2%, L-glutamine 2%, and antibiotics/antimycotics 1%; all Gibco®) was added to wells and slices incubated a further 3 days.

2.4. MSC CM treatment of SCSC

After seven days equilibration, medium was replaced with 250 μl MSC CM or control Neurobasal medium (n = 3 independent experiments). Culture plates were then incubated undisturbed at 34 °C/5% CO_2 for 72hr.

2.5. Cell viability in SCSC

As we have previously demonstrated that an initial phase of cell death follows the preparation of SCSC [29] and we wanted to rule out any possible detrimental effect of CM onto cell survival *ex vivo*, we devised a series of experiments to ascertain the degree of cell death at the end of the culture period. To do so, following culture in MSC CM or control medium (n = 3), 10 μl of 1.5 mM propidium iodide (PI) solution was added to slices and incubated at 34 °C/5% CO_2 for 10 min. SCSC were then washed twice each in PBS (pH 7.4–7.6) and dH_2O, before fixation in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4 for 1 h at room temperature. Washes were repeated prior to montage in anti-fade fluorescence-free mounting solution. Slides were visualised and imaged under 20x magnification via widefield fluorescence microscopy (Leica DM6000, Leica Microsystems, Wetzlar, Germany). Using ImageJ software, the "Count Particles" function counted PI-stained nuclei of area sizes between 7 and 40 mm^2. Values were expressed as number of PI-stained nuclei per mm^2.

2.6. Immunohistochemistry and confocal microscopy

Following culture in MSC CM or control medium (n = 3), slices were fixed for 1 h at room temperature in 4% PFA, followed by repeated washes with PBS, then incubated with 250 μl blocking buffer (0.3% Triton-X, 2.5% normal horse serum in PBS) for 1 h at room temperature, continuously shaking. Slices were then incubated overnight at +4 °C with 250 μl blocking buffer containing the primary antibodies diluted at optimal titre (below) or in blocking buffer alone. Rabbit polyclonal primary antibodies (obtained from Abcam) were anti-βIII tubulin (1/1000) (ab18207), anti-GFAP (Glial fibrillary acidic protein) (1/1000) (ab7260), and anti-NG2 (Neuron-glial antigen 2) (1/1000) (ab129051). Slices were subsequently washed repeatedly with PBS and incubated with horse anti-rabbit Dylite 488 (1/250) (DI-1088, VectorLabs) in PBS for 3 h at room temperature. After further washes, slices were mounted

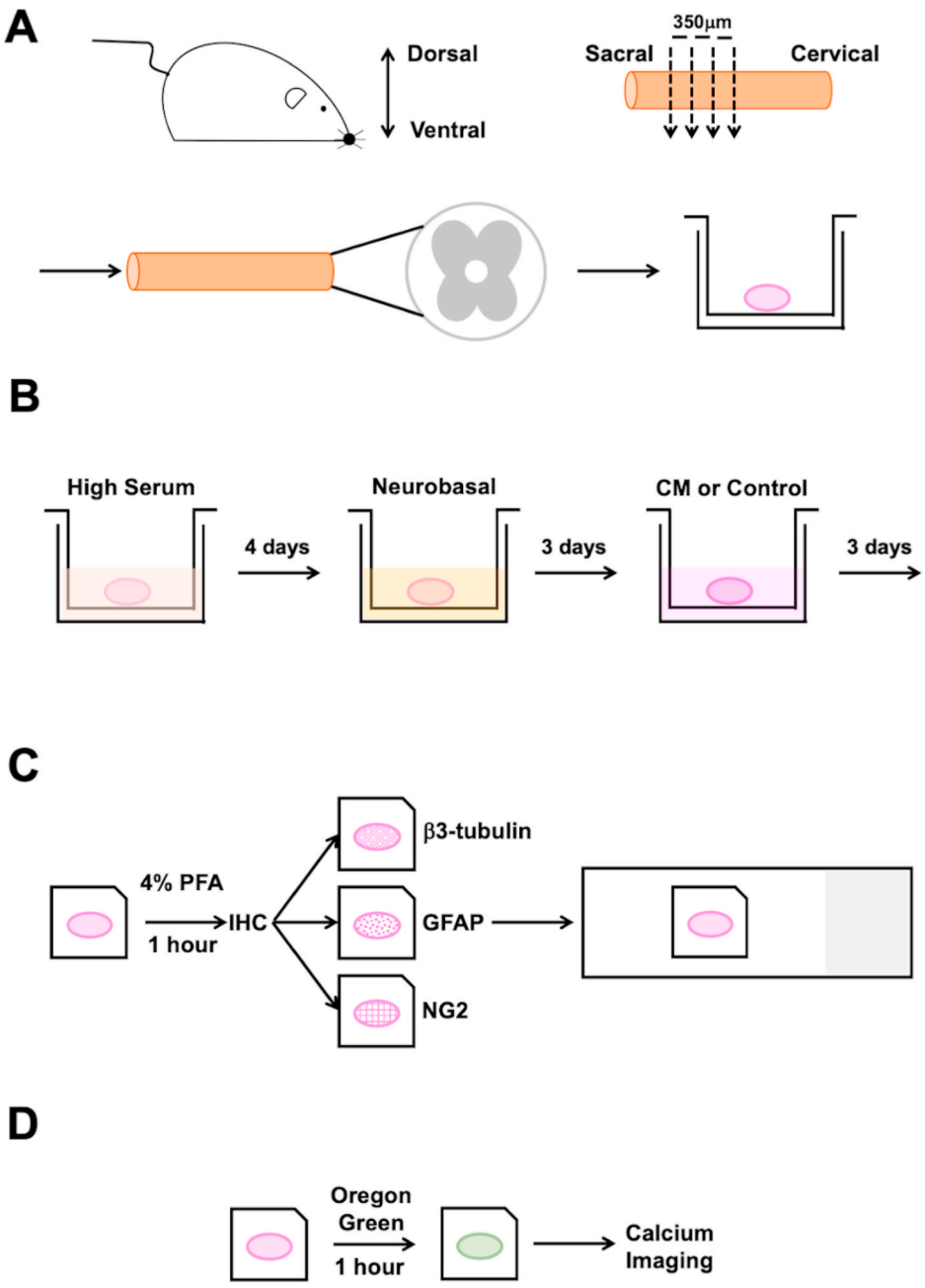

Fig. 1. Schematic representation of experimental design: treating SCSC with MSC CM versus control medium. A) Mice were dissected to remove the spinal cord and the lumbar region sliced at 350 μm intervals. Slices were washed and placed individually in well inserts. B) SCSC were equilibrated in high serum and Neurobasal media prior to treating with either MSC CM or control medium. C) After 3 days of treatment with MSC CM or control medium, SCSC were fixed and immunohistochemistry performed before mounting on slides or D) incubated with Oregon Green 488 BAPTA-1AM prior to calcium imaging analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

using an anti-fade fluorescence-free mounting solution containing DAPI (VectorLabs). Confocal microscopy was used to visualise and image immunoreactivity (Leica TCS SP5) and the ImageJ neurite tracer software [38,39] was used for image analysis of fluorescence intensity and cell process lengths.

2.7. Calcium imaging

Single cell calcium imaging, a method correlating the functional data based on calcium shifts operated by different intracellular and extracellular mechanisms integrated with their cell phenotypes, is a widely recognized mean to assess the response of neuronal and glial cells to local stimuli and has been used extensively *in vitro* (isolated cells), *ex vivo* (brain or spinal cord slices) and *in vivo* (two-photon microscopy) [40]. The method involves the use of a cell-permeable calcium indicator or genetically encoded calcium indicator that, once entered the cytosol or synthesized by the cell, responds to variations of the intracellular calcium concentration by changing the intensity of fluorescent emission or the ratio of fluorescent emission at two different wavelengths [41]. Here we used the cell permeant indicator Oregon Green™ that was loaded into SCSC as previously described [42]. Briefly, following treatment with MSC CM or control medium, SCSC were incubated with 250 μl loading solution for 1 h at 34 °C/5% CO_2. The loading solution consisted of 1 μl Oregon Green™/DMSO mix (50 μg Oregon Green™ 488 BAPTA ((1,2-bis(o-aminophenoxy)ethane-N,N,N′,N’-tetraacetic acid)-1AM in 4 μl DMSO) and 2 μl 20% pluronic F-127 acid (in DMSO)) in 1 ml Neurobasal medium. Slices were washed and mounted onto a confocal microscope (Leica TCS SP5) stage equipped with a buffer transfer system continually perfusing the preparation with oxygenated artificial cerebral spinal fluid (125 mM NaCl, 2.5 mM KCl, 25 mM $NaHCO_3$, 1 mM $NaHPO_4$, 25 mM glucose, 1 mM $MgCl_2$, 2 mM $CaCl_2$ in dH_2O). Digitised time-lapse images were collected under 40x water immersion objective, (excitation wavelength 488 nm, emission wavelength 495–530 nm) at 204 ms frame intervals totalling 1000 frames.

Gain, offset and pinhole were constant throughout imaging. SCSC activity was unchanged when challenged with 60 mM KCl, making it difficult to unequivocally identify calcium oscillating cells as neurons [43]; therefore, the term 'active cells' has been used thereafter.

2.8. Statistical analysis

Statistical analysis was completed using IBM SPSS statistics software. Each independent experiment (n = 3) contained a minimum of 3 spinal cord slices per dish incubated with MSC CM or control medium. Data sets were tested for normal distribution using Shapiro Wilk while parameter relationships and group differences were tested for significance using Student *t*-tests (normally distributed data) or Mann-Whitney U tests (non-normally distributed data). Data have been shown as means and standard errors of the means (s.e.m.) or standard deviations (s.d.; for pooled data only) as indicated in figure legends. Significance was accepted at $p \leq 0.05$ (5% level).

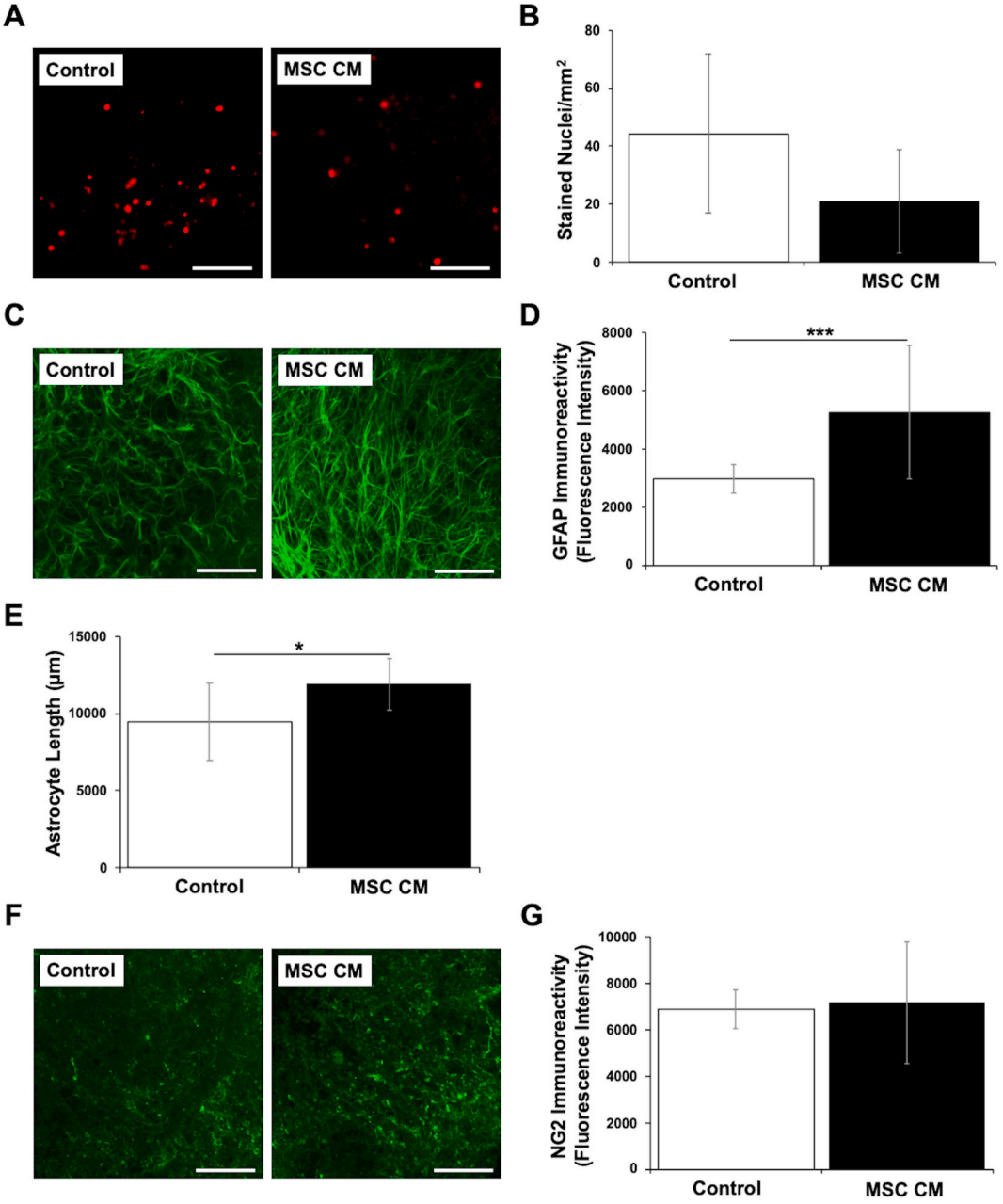

Fig. 2. MSC CM preserved cell survival and promoted astrocytic hypertrophy within SCSC. (A) Representative fluorescent images of SCSC in control medium (left) and MSC CM (right) after staining with PI. (B) Analysis revealed increased dead cell numbers per mm^2 in control medium compared to MSC CM. Data are means ± s.e.m. (*t*-Test; n = 3 donors) $p = 0.329$. Scale bar = 50 µm. (C) Representative fluorescent images of astrocytic hypertrophy visualised by GFAP antibody, of SCSC treated with control medium and MSC CM. Greater intensity of staining was observed from SCSC treated with MSC CM. (D) Data analysis revealed a significant increase in GFAP fluorescent intensity and (E) a moderate increase in the length of astrocyte processes in SCSC treated with MSC CM. Data are means ± s.d. (Mann-Whitney U, *t*-Test; n = 3 donors) $^*p \leq 0.05$, $^{***}p \leq 0.001$. Scale bar = 100 µm. (F) Representative fluorescent images of CSPG levels visualised by NG2 antibody, of SCSC treated with control medium and MSC CM. Intensity of staining is marginal between conditions. (G) Data analysis revealed a slight, but non-significant increase in NG2 fluorescent intensity between SCSC treated with control medium and MSC CM. Data are means ± s.d. (Mann-Whitney U; n = 3 donors) $p = 0.355$. Scale bar = 100 µm.

3. Results

3.1. Culturing SCSC within MSC CM enhanced GFAP astrocytic immunostaining but did not affect CSPG levels in glia

There was increased cell viability in SCSC cultured in MSC CM compared to SCSC in control medium, although this difference was non-significant. The number of PI-stained nuclei was 17 ± 11 (per mm^2) in SCSC within MSC CM, compared to 39 ± 17 (per mm^2) in SCSC within control medium (Fig. 2A and B).

MSC CM increased astrocytic reactivity as delineated by GFAP immunoreactivity in SCSC compared to control medium (Fig. 2C). Both observed levels of GFAP staining (Fig. 2D) and astrocytic process total length (Fig. 2E) were significantly increased in SCSC cultured in MSC CM compared with control medium ($p \leq 0.005$ and $p \leq 0.05$, respectively), although there were no marked increases in CSPG levels (NG2 immunostaining).

3.2. Culturing SCSC within MSC CM reduced the prevalence of neuronal cell processes but increased cellular signalling activity

SCSC cultured within MSC CM showed a significant decrease in total neurite length (Fig. 3A) compared to control medium, determined by βIII-tubulin immunopositivity ($p \leq 0.005$) (Fig. 3B). The length of βIII-tubulin immunopositive neurites in MSC CM and control medium were 2458 μm ± 1026 μm and 5722 μm ± 1472 μm, respectively. Analysis of calcium imaging footage showed no difference in overall frequency of calcium-dependent signalling per microscopic field, between SCSC within MSC CM compared to controls, which were 4 ± 1.1 peaks per 204s and 4 ± 0.6 peaks per 204s, respectively ($p = 0.738$) (Fig. 3C and D). There was a significant decrease in the number of active cells in SCSC within MSC CM compared to controls, i.e., 3 ± 2 active cells (per field of view) and 10 ± 2 (per microscopic field), respectively ($p \leq 0.01$) (Fig. 3E). In those active cells, there was a significant increase in calcium oscillations in MSC CM compared to controls, which were 1.6 ± 1 peaks per 204s and 0.4 ± 0.1 peaks per 204s, respectively ($p \leq 0.01$) (Fig. 3F).

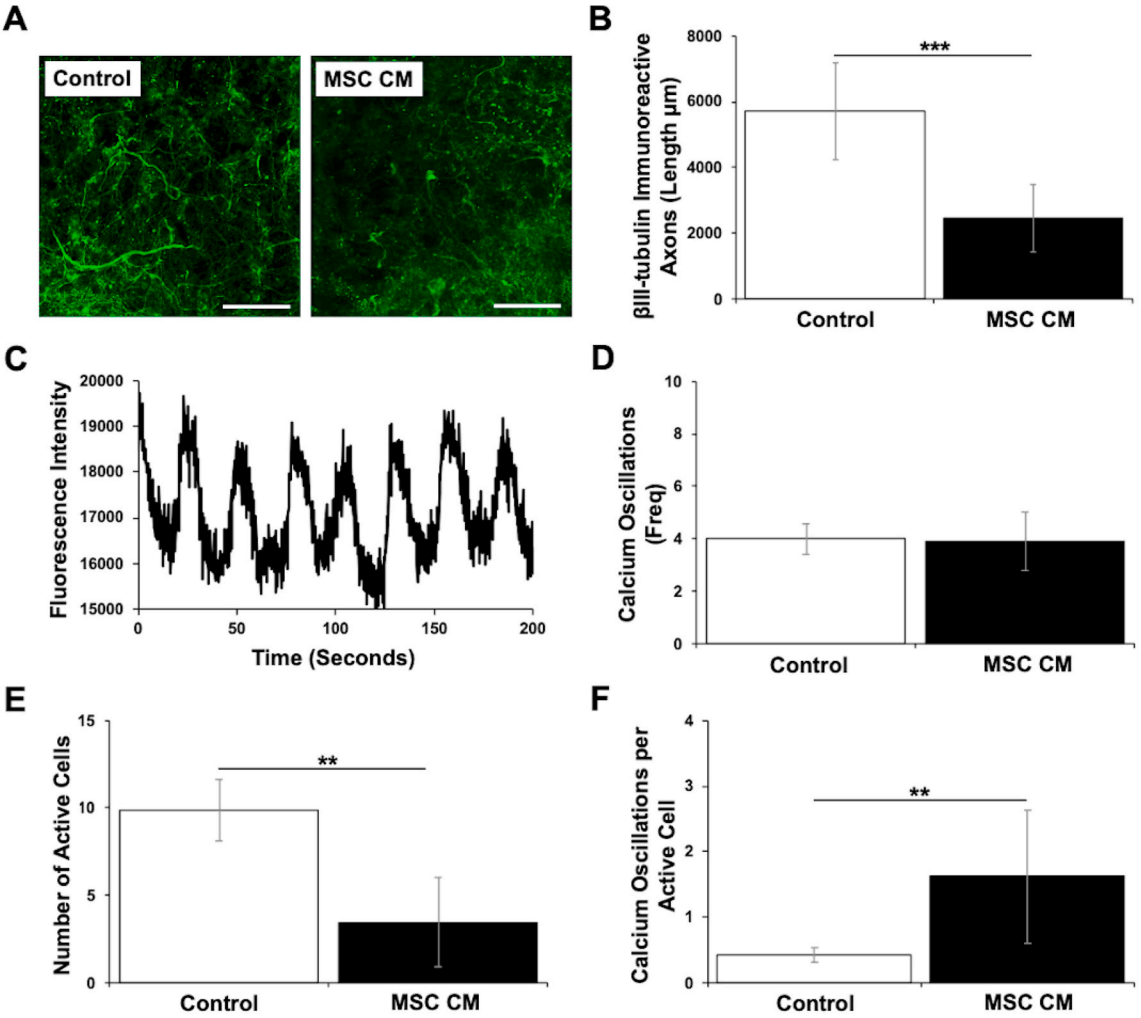

Fig. 3. MSC CM reduced neurite extension in cultured neurons and the number of active cells but increased the frequency of Ca^{2+} transients in active cells. (A) Representative fluorescent images of neurite outgrowth visualised by βIII-tubulin antibody, of SCSC cultured in control medium (left) or MSC CM (right). A greater number of longer neuronal cell processes were observed under control conditions. (B) Data analysis revealed a significant decrease in neuronal cell process length between slices in control medium and MSC CM. Data are means ± s.d. (*t*-Test; n = 3 donors) ***$p \leq 0.001$. Scale bar = 100 μm. (C) Graphical representation of fluorescence changes denoting calcium oscillations over time (204 s). (D) Recording analysis revealed no difference in the overall frequency of calcium transients in active cells from each field of view between SCSC within MSC CM or control medium. (E) A significantly reduced number of active cells (per field of view) was observed in SCSC within MSC CM but (F) these active cells displayed a significantly higher frequency of calcium transients (per 204 s) when cultivated in MSC CM. Data are means ± s.d. (Mann-Whitney U, *t*-Test; n = 3 donors) **$p \leq 0.01$.

4. Discussion

The slice culturing technique used for this study is a well characterised interface static method [44] whereby slices receive oxygen from the top and nutrients across the porous membrane at the bottom, allowing for interaction of the slices with soluble molecules contained in culture medium. Likewise, standardised methods of analysis were used to assess the effects of the MSC CM compared with control medium.

Previous studies [45] demonstrated that after an initial phase (four days) of cell death in SCSC, PI-stained dead cell numbers drop to remain relatively stable thereafter. In keeping with these observations, we allowed SCSC to recover for at least four days before treatment with MSC CM. The latter was associated with enhanced cell survival compared to control medium, indicating possible neuroprotective effects, although the difference was not statistically significant. Still, this observation (together with the results of calcium imaging experiments – see below) demonstrated that our SCSC were perfectly viable after several days *in vitro*. Increased GFAP immunopositivity and astrocytic process length was observed in these conditions, suggesting that MSC CM increased glial cell survival and reactivity.

Astrocytic growth has been repeatedly reported in CNS organotypic cultures [45]. However, unchanged NG2 levels in the increased presence of astrocytic growth seen in MSC CM was an unexpected finding. A major component of SCI pathology is the occurrence of astrogliosis, glial proliferation, atrophy, increased GFAP expression, and CSPG secretion (an inhibitor of neuronal growth and wound repair) [46,47]. In this study, we observed no increase in NG2 levels, as a marker of CSPGs, which may be due to loss of CSPG into surrounding medium, cell mediated degradation of CSPG molecules [48,49] or because NG2 has been reported to increase at later time points after SCI [50].

Observation of a significant decrease in βIII-tubulin immunoreactive neurites in response to MSC CM treatment was initially surprising, due to previous studies proving the neurogenic properties of MSC CM [35,51,52]. *In vivo* axotomy of primary afferent fibres, which is necessary to prepare SCSC, was long ago reported to cause death of spinal cord neurons receiving monosynaptic input from these fibres [53]. Therefore, it seems reasonable to hypothesise that the spinal neurons reached a steady state of lower survival during equilibration in plain medium and before cultivation in MSC CM. If so, the presence of MSC CM would have been unable to protect them from death.

Another possible explanation for βIII-tubulin immunoreactivity reduction is that the SCSC microenvironment may not be permissive for neuronal maturation, or that MSC CM could potentially secrete bioactive molecules in a paracrine fashion, potentially causing differentiation of resident neural precursors towards a glial lineage. Supporting evidence includes previous experiments where, after transplantation of neural stem cells into organotypic slices, survival but no increased neuronal maturation was observed [7]. Furthermore, increased GFAP immunopositivity coupled with no change in βIII-tubulin immunopositivity were similarly found elsewhere, demonstrating transplanted neural stem cells had a greater affinity for endogenous glial cells in SCSC, and differentiated towards a glial lineage [54].

To our knowledge, nobody has investigated effects of MSC CM on cell activity in SCSC. Calcium imaging showed that although overall cell activity was unchanged (measured as calcium transients), the signalling frequency of individual active cells increased in response to MSC CM. The type of calcium responses and temporal pattern [43] are strongly suggestive that spontaneous calcium oscillations in SCSC reflect increased neuronal network activity. However, neuronal cell process outgrowth was decreased in response to MSC CM and, although initially unexpected, a previous *in vitro* study on *Xenopus* embryonic spinal cord found similar results - neurons that exhibited slower rates of axonal outgrowth signalled more frequently [55].

A key question that is raised in this study is by which mechanism of action the MSC CM is causing the observed effects onto SCSC. Previous studies which have revealed potential mechanisms can help to provide some speculation as to possible factors at play in this study. Various experiments have shown that MSC transplantation causes an increase in neurotrophic factors such as NGF and BDNF [19,20], and causes shifts in the immune response through increases of the interleukins (IL) 4 and 13 [12,15,21–24]. Of note is that throughout many of these studies MSC survival was low, indicating that the observed changes were due to their secretome, rather than the cells themselves. *In vitro* studies on MSC CM have used both ELISA and mass spectrometry, revealing that the MSC secretome contains a myriad of factors that promote both neurogenesis and immune modulation [56–58]. This knowledge, combined with the data from this study, suggests that the here observed changes are a response to neurotrophic factors (that have yet to be identified) present within the MSC CM.

In conclusion, further studies are required to fully understand why the effects of MSC CM differ between *in vitro* cell cultures and SCSC, why they contrast with MSC transplants into SCI sites, and to ascertain the specific mechanism(s) of action(s). These differences highlight the importance of model systems to examine effects of cell transplants and secretion on the CNS. SCSC were cultured without corresponding circulatory models, which could alter how resident cells respond to the MSC, i.e., no immune cell influx nor a nutrient provision/waste removal system. Hence, the results from this study suggest that effects of MSC and derived factors may depend on more systemic factors than those seen within the CNS alone.

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CRediT authorship contribution statement

Chelsea R. Wood: Conceptualization, Investigation, Formal analysis, Visualization, Writing – original draft, preparation, Writing – review & editing. **Esri H. Juárez:** Investigation, Formal analysis. **Francesco Ferrini:** Conceptualization, Investigation, Formal analysis, Resources. **Peter Myint:** Conceptualization, Resources, Funding acquisition. **John Innes:** Conceptualization, Resources, Funding acquisition. **Laura Lossi:** PI staining. **Adalberto Merighi:** Conceptualization, Supervision, Visualization, Funding acquisition, Writing – review & editing. **William E.B. Johnson:** Conceptualization, Supervision, Visualization, Funding acquisition, Writing – review & editing, All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study, the collection, analyses, or interpretation of data, writing of the manuscript, or decision to publish results.

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