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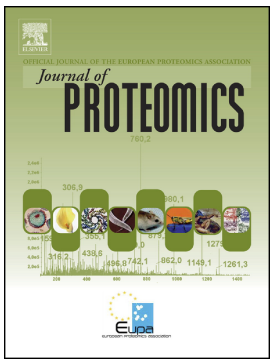

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Characterization of rat primary trigeminal satellite glial cells and associated extracellular vesicles under normal and inflammatory conditions

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Abstract

Satellite glial cells (SGCs) in sensory ganglia contribute to the pathogenesis of chronic pain, potentially through mediating extracellular or paracrine signaling. Recently, extracellular vesicles (EVs) in the form of exosomes have been found to play an important role in cell-cell communication. However, their release from SGCs and extent in modulating pain remain unknown. An *in vitro* cell platform using fresh primary SGCs was used to characterize the shed vesicles by size and proteomic profiling following activation of SGCs by lipopolysaccharide (LPS), simulating neurogenic inflammation *in vivo*. Results demonstrated that SGCs shed vesicles in the size range of exosomes (>150 nm) but with altered protein expression upon LPS-activation. Proteomic profiling of SGCs-shed EVs showed that a number of proteins were differentially regulated upon LPS stimulation such as junction plakoglobin and myosin 9 that are proposed as novel biomarkers of SGCs activation under inflammatory conditions. Findings from this study highlight the utility of using fresh primary SGC cultures as a model to further investigate EVs under normal and inflammatory conditions.

Keywords: satellite glial cells, trigeminal ganglion, inflammation, lipopolysaccharide, extracellular vesicles, exosomes, proteome.

INTRODUCTION

Satellite glial cells (SGCs) are supportive non-neuronal cells that wrap around each neuron in sensory ganglia. SGC-neuron units allow a close bidirectional interaction that facilitates the maintenance of neuronal homeostasis [1]. SGCs are proposed to play a key role in nociception based on their ability to influence the neuronal excitability [2] that occurs under neuropathic and inflammatory pain conditions. This phenomenon is partly mediated by extensive proliferation [1], and cross talk of SGCs after nerve injury or inflammation, which are collectively thought to influence the excitability of adjacent neurons and subsequently modulation of nociception pathways [3]. Expressional changes of nociceptive related proteins in the SGCs and release of pro-inflammatory cytokines have been suggested to affect the firing rate of the neurons and further modulation of nociception [4].

Within the neuronal milieu, extracellular vesicles (EVs) released from cells are known to contribute to intercellular communication via protein moieties [5]. The composition of these proteins undergoes changes when pathological conditions occur and this characteristic makes them potentially suitable for clinical applications in terms of e.g., biomarker development for prognosis or monitoring of treatment responses. Vesicles derived from the late endosome, called exosomes are in the size range of 30 to 150 nm as commonly determined by electron microscopy and measured by Nanoparticle Tracking Analysis (NTA) [5]. Exosomes are believed to contain selected macromolecules involved in cell-cell communication [6]. Currently, the secretion of EVs and alteration of target cells has been extensively studied in immune, cancer, and stem cells [5,7]. However, very little is known about this particular aspect in relation to nociception and pain processing and has remained an open field for investigation in particular with regard to a potential role of SGCs.

In order to have a more in depth understanding of cell-cell communication *in vitro*, primary cell cultures of SCGs can be used [8]. This provides a working model that is not attainable *in vivo*, which would require sampling of EVs (exosomes) directly from sensory ganglia e.g., trigeminal ganglia (TG). In addition, using a cell platform, it is possible to study SGCs under normal and activated conditions for example upon inflammation. The TG has been considered a key component in craniofacial pain nociception and since this structure is located in the peripheral nervous system, it makes it highly attractive for peripheral modulation of pain pathways in associated conditions such as headaches, temporomandibular joint disorder, and trigeminal neuralgia.

Even though studies have proven that primary cultures of SGCs are capable of proliferating and keeping their phenotype *in vitro* for several weeks, there appears to be some diversity in morphology and protein marker expression of these cells over time [8]. One study has identified primary SGCs cultures consisting of 65% small cell bodies with elongated shape, strongly positive for the activation marker glial fibrillary acidic protein (GFAP) and the key phenotypic marker glutamine synthetase (GS), and 35% with an astrocyte like morphology that expressed GFAP and GS to a lower degree [9]. In another study, using mixed neuron-SGCs

primary cultures, the GS expression level declined after two days and the SGCs changed morphology after migration away from the neuron becoming spindle shaped, aligned and forming clusters [10]. Together, these studies indicate that some degree of heterogeneity and plasticity of primary SGCs occurs when culturing, which might be a challenge when translation of in vitro findings is required to describe *in vivo* processes. However, primary SGCs still offer an acceptable way towards identification of unique and deregulated EV proteins that may lead to novel biomarkers of cellular changes under inflammatory pain conditions. Furthermore, the pattern of change may give further insights in to their biological and pathophysiological impact on target cells that may advance our understanding of the molecular mechanisms underlying nociception and pain, in general. Hence, the purpose of this study was to characterize the proteomic profile of EVs (including exosomes) released from fresh primary SGCs under normal condition and after stimulation by lipopolysaccharide (LPS) to resemble inflammatory conditions.

MATERIALS AND METHODS

Preparation of primary cultures

All protocols were approved in accordance to the ethical guidelines delineated by the Danish Animal Experiments Inspectorate for use of laboratory animals in medical research. Primary SGCs were derived from three male Sprague-Dawley rats (2 months old) provided by the Animal Research Facility, Pathological Institute, Aalborg University Hospital North, Denmark. The rats were deeply anesthetized with a mixture of hypnorm, midazolam and isotonic saline (25%, 25%, 50% v/v. 0.2-0.3 mL/100 g) before cervical dislocation. Both TGs were removed and subsequently placed on ice 1x Phosphate-buffered saline without CaCl2+ and MgCl2+ (DPBS (10x), Gibco, Life Technologys, CA, USA) with 1% Penicillin/Streptomycin (P/S; 15140-122 Gibco, Life Technologies, CA, USA). The remaining fibrous capsule was trimmed away to avoid contamination with fibroblasts before the TGs were finely chopped and digested with 5 mg/mL collagenase (Collagenase; C9891 Sigma-Aldrich, MO, USA) for 15 min at 37°C. The digested ganglia were mixed and centrifuged at 1,300 rpm for 5 min at room temperature (RT) followed by 5 min incubation of the pellet at 37°C with 1 mL 0.125% trypsin (15090-046 Gibco life technologies, Invitrogen, CA, USA). The trypsinization was terminated by adding 5 mL Dulbecco´s Modified Eagle´s Medium (DMEM F-12 1:1 mixture with Hepes, L-Gln, Lonza, Verviers, Belgium) supplemented with 10 % Fetal Bovine Serum (FBS, Gibco, Life Technologies, CA, USA) and 1% P/S, hence named DMEM/F12. A final centrifugation (1,300 rpm, 5 min, 21°C) before new DMEM/F12 was added, and the cell suspension was homogenized by manual pipetting. The cell suspension was divided into two T25 flasks (CELLSTAR® standard TC-treated, Greiner Bio-One GmbH, Frickenhausen, Germany) with DMEM/F12 and incubated in a humidified 5% CO2 incubator for 3 h at 37°C to let the SGCs adhere to the bottom of the flasks. Neurons were carefully removed as they still floated in the media when replaced with new 37°C warm DMEM/F12. Subsequently, media was changed after 24 h and then every second day until day 4 where exosome-depleted DMEM/F12, referred to

as Exo-DMEM/F12 (DMEM/F12 ultra centrifuged at 100,000 x g for 18 h at 4°C, RP70T, Beckman Coulter, Brea, CA, US) was replaced and collected with media change every day.

The cells were kept in culture until confluent or up to 21 days. After, cells were 1) plated with a density of 30,000 cells/well in 8 well chamber-slides (Lab-Tek® System 177445, Thermo Fischer, NY, USA) for three days before immunofluorescens staining, or 2) passaged to T75 flasks with subsequent collection of conditioned media (CM) for NTA or freezing.

Characterization of cultured SGCs by morphology and immunocytochemitry

The morphology of the SGCs culture was observed by phase microscopy (Zeiss Primovert, Germany). For immunocytochemistry, the cells were washed in 1x PBS then fixed in 10% formalin (4% phosphate buffered Histology grade, AppliChem GmbH) for 20 min at RT. An additional wash followed by blocking and permabilization in 5% bovine serum albumin (BSA, Europa Bioproduct Ltd, Cambridge, UK) with 0.2% Triton X-100 for 1 h at RT. After washing in PBS, a cocktail of primary antibodies was added consisting of rabbit anti-rat GS (IgG, Sigma-Aldrich, MO, USA) diluted 1:4,000 in 1% BSA and monoclonal mouse anti-GFAP (Clone G-A-5, Sigma-Aldrich, MO, USA) diluted 1:400 in 1% BSA and these were incubated overnight at 4°C. Cells were washed in PBS and a cocktail of secondary antibodies were added for 1½ h at RT, consisting of donkey polyclonal antibody (Dnk pAb) to mouse IgG, Alexa Fluor 488 (ab150105, Abcam, Cambridge, UK) and Dnk pAb to rabbit IgG, Alexa Flour 555 (ab150106, Abcam, Cambridge, UK) diluted 1:500 in 1% BSA. Excess secondary antibody was washed away in PBS before incubation with the nuclear stain Hoechst 33342 solution diluted 1:5000 in 1% BSA, 20 min at RT. Finally, cells were washed in PBS, milli-Q water and before fluorescent mounting medium was added to the coverslips (S3023 Dako, DK).

Images were obtained by using a Nikon AZ100 microscope (Tokyo, Japan) with a fluorescent illuminator (L200/D, Prior Scientific, Rockland, MA, USA) and a Nikon DS-Vi1 0.6x digital camera. Images were viewed and modified with NIS elements microscope image software.

Activation of cultured SGCs by LPS

Fresh cultures were long-term activated with LPS as a model of inflammation in parallel with untreated SGCs controls. The LPS-induction was initiated at low confluence (~40%) to collect sufficient volume of cell-conditioned media (CM) before reaching confluence. The CM was collected every day and replaced with new Exo-DMEM/F12 with or without LPS until 30-40 mL CM was accumulated or the cells were confluent. An LPS dosage of 50 ng/mL (Escherichia coli 055:B5, Sigma-Aldrich, MO, USA) was selected after titration of LPS (0, 1, 10, 20, 30, 50, 100, 250 ng/mL) and looking at the expression of the glial activation marker GFAP. Furthermore, low levels of LPS (<50 ng/mL) similarly stimulated the cells. After the experiment, a fraction of the cells was subcultured in T75 flasks and treated as usual for 24 h before collection of CM for further protein determination or NTA analysis.

Extracellular vesicle isolation and exosome enrichment

The CM was centrifuged at 3,000 x g for 30 min at 4°C to remove cells and debris, then stored at -80°C to avoid deterioration of exosomes until all CM was collected. The collected supernatant was then thawed in a water bath at 37°C, votexed for 90 sec. to retain all exosomes [11] and centrifuged at 9,050 x g for 1 h, 4°C before filtering through 0.22 μm filters to remove larger vesicles and cell debris. The EVs including the exosomal fraction were pelleted by ultracentrifugation at 100,000 x g (Type RP70T rotor, 37.000 rpm), 4°C for 2½ h. Samples for total protein and particle size distribution were dissolved in 200 μL PBS and stored at -80°C. For proteomic analysis an additional washing step in PBS was performed to remove protein contamination from FBS and ultracentrifuged at 100,000 x g, 4°C for 2 h. The pelleted EVs were diluted in 50 mM ammonium bicarbonate and stored at -80°C until analysis.

Characterization of isolated vesicles

Total protein by BCA Protein Assay

The exosomal protein concentration was determined using Pierce® bicinchoninic acid assay kit (BCA Assay, Thermo Scientific, Rockford, IL, USA). The samples were compared in duplicates against standards of BSA (0, 5, 25, 50, 125, 250 μg/mL), diluted 1 in 8 and measured colorimetrically in a micro plate reader (absorbance 570 nm, Sunrise™, TECAN, Männedorf, CH) with Magellan™ (TECAN) data analysis software. Sample protein concentration was extrapolated from a linear fit of the standard curve, with R2 >0.99.

Particle size and concentration using Nanoparticle Tracking Analysis

The concentration and particle size distribution of isolated vesicles were determined by NTA, consisting of a microscope mounted with a Marlin F-033B scan camera (ATV, Stadtroda, Germany) and a Nanoparticle visualization system HaloTM LM12 (NanoSight Ltd, Salisbury, UK) configured with a Monochromatic laser beam at 638 nm. Particle detection was fixed to a detection threshold of 5 and Blur 7x7. The samples were diluted in DPBS without Ca2+ and Mg2+ to at least 100 particles per frame. For each sample replicate videos of 60 seconds duration were captured of the scattered particles movement and ambient temperature was measured. Graphs of particle size distribution versus intensity were created using NTA 2.1 Analytical Software 2010.

Sample purity by particle to protein ratio

The purity of the isolated particles was evaluated by the particles per μg protein (P/μg) ratio, where high purity >$3x10^{10}$ P/μg, low purity was $2x10^{9}$ to $2x10^{10}$ P/μg and impure was < $1.5x10^{9}$ P/μg [12].

Sample preparation for mass spectrometry

For mass spectrometry analysis, 50 µL of ultracentrifuged sample was homogenized with cooling using steel beads in cold lysis buffer of 5% sodium deoxycholate (SDC) and 50 mM triethylammonium bicarbonate (TEAB), followed by centrifugation at 14,000 x g for 2 min. The denaturated proteins were digested using a modified Filter-Aided Sample Preparation (FASP). Initially, proteins were in solution reduced and alkylated by adding tris(2-carboxyethyl)phosphine and chloroacetamide to a final concentration of 10 mM and 50 mM respectively with incubation for 30 min at 37°C. The sample was transferred to a 30-kDa molecular weight cutoff spin-filter (Millipore, Billerica, MA) and centrifuged at 14,000 x g for 15 min, which was used between all steps to facilitate buffer exchange. Lysates were digested to peptides overnight at 37°C in 0.5% SDC in 50 mM TEAB with 1 µg trypsin (sequencing grade-modified trypsin; Promega, Madison, WI). Peptides were eluted from the spinfilter and purified by phase separation extraction of residual SDC twice by adding ethyl acetate to the sample (3:1) with 1% trifluoroacetic acid (TFA), then vortexed and centrifuged at 14,000-x g for 1 min. The aqueous phase with the peptides was collected and dried completely in a vacuum centrifuge and stored at -20°C until analysis.

Mass spectrometry

The samples were resuspended in 40 µL resuspension buffer (2% acetonitril in 0.1% TFA, 0.1% formic acid) and analyzed by ESI nanoflowLC-MS using a RSLCnano LC system (Thermo Scientific) coupled to QExactive Plus Quadrupole-Orbitrap Mass Spectrometer and a Nanospray Flex ion source (Thermo Scientific). The samples were loaded onto an Acclaim PepMap100 C18 Nano-Trap Column, 5 mm particle and 300 Å pore size (Thermo Scientific) and separated using a 50 cm Acclaim PepMap100 C18 column packed with 1.9 mm particle and 100 Å pore size (Thermo Scientific). The peptides were eluted in 98% solvent A (0.1% TFA, 0.1% formic acid in Ultrapure water) with 2% solvent B (0.1% TFA, 0.1% formic acid in acetonitrile) by a gradient to 30% of solvent B over 35 min at a constant flow rate of 300 nL/min. The mass spectrometer was operated in a TopN data dependent positive mode. A full MS scan of ions with mass/charge (m/z) range of 400 to 1200 were acquired at a resolution of 70,000 followed by MS/MS scans with a dynamic higher-energy collisional dissociation (resolution 17,500; AGC target 4E4; NCE 30 eV).

Data analysis

Raw data from BSA and NTA measurements were further processed in Excel to create standard curves, calculate particle concentration and particle to protein ratio.

For protein identification and label-free quantification the original files of acquired MS spectra were matched against the theoretical spectra of all reviewed Rattus norvegicus (Rat) reference proteome (Uniprot, November 2015, containing 29,886 protein groups). The database search and matching were performed in the search engine MaxQuant ver. 1.5.3.28 with defaults settings and filtering criteria of 1% false positive rate (FPR) based on target-decoy database search [13]. The identified proteins were entered in Perseus software

ver. 1.5.5.6 for data annotation and statistical analysis. The data were additionally filtered by removal of potential contaminant proteins. All proteins with at least one unique peptide were reported and an estimate of the protein level was based on the intensity-based absolute quantification (iBAQ). The proteins identified were further searched in the Exocarta database (http://www.exocarta.org/) to verify their exosomal origin and searched in PANTHER classification system (http://www.pantherdb.org/) to investigate their gene ontology properties. Protein-protein interaction network was obtained by using STRING software, v. 10 (http://www.string-db.org).

Statistical analysis

The significance of the variation between the proteome from two conditions (LPS-activated vs. normal) was analyzed by combined two-sided *t*-test and fold change. The default settings in the Perseus software were used to identify proteins with high significance and high effect (False Discovery Rate = 0.05 and s0: 0.1). The cutoff curve in the volcano plots was used to identify the proteins-of-interest.

RESULTS

Morphological characterization of SGCs

Morphology of SGCs in fresh primary culture

Using phase contrast microcopy, the morphology of the fresh primary cultures was observed for 21 days or until doubling. During the first 3-4 days in culture, the majority of cells were distributed over the bottom of the flasks as single cells or small clusters. Some cells within the clusters had small cell bodies will long processes, but the majority of cells were flat, in appearance with dispersed cytoplasm from the cell body containing many protruding small processes (Fig. 1, a). After the initial culturing phase, the cells became more spindle-shaped with small cell bodies, 2-3 projections, connecting to form larger networks (Fig. 1, b, c) and this was maintained during the culture period.

In general, growth rate was slow and there was large variation between the culture flasks. There was no visible difference in morphology or growth rate of cells stimulated with LPS when compared to unstimulated populations.

One of the LPS-treated primary cultures was subcultured for an additional week with continued LPS stimulation for NTA analysis of isolated EVs including exosomes.

Immunocytochemical characterization of SGCs

Phenotypic markers of SGCs: expression in subcultured, fresh primary SGCs

Immunocytochemistry was carried out on the SGCs for the expression of GS and GFAP in the cytoplasm. According to published literature, SGCs are the only cells in the trigeminal ganglion that can be stained with

GS, which makes this a suitable phenotypic marker [1]. The level of GFAP, on the other hand, was low or almost undetectable under normal conditions [14].

The primary cultures of fresh SGCs were clearly GS positive with a low expression of GFAP (Fig. 2). With subculturing/passaging the primary cultures were still GS positive and marginally GFAP positive.

The fraction of cells positive for GS and GFAP was not systematically counted. However, counting the GS and GFAP positive cells in the images, the majority of cells were expressing these markers.

Activation marker of SGCs: effect of LPS on GFAP expression

In contrast to GS, the level of GFAP is normally upregulated and it is sensitive to neuronal injury or inflammation; therefore, serving as a suitable marker for SGCs activation [14].

Initially, different concentrations of LPS were assessed by staining for the expression of GFAP to select an effective long-term activation of SGCs. Primary SGCs expressed GFAP at a low level under normal conditions, while the expression was significantly upregulated throughout the whole cytoplasm after stimulation with 1 to 250 ng/mL LPS. It was noted that some of the cells treated with 1 ng/mL LPS were GFAP negative. However, this was difficult to confirm quantitatively as the cytoplasm of several SGCs was overlapping in the field view of the microscope. Based on this observation, the primary cultures were long-term activated with 50 ng/mL LPS (Fig. 2).

Interestingly, the treatment of freshly isolated primary SGCs with LPS led to GFAP expression that radiated from the cell body throughout the longer processes (pattern of GFAP distribution). However, there was no significant difference in the amount of GFAP expression between LPS activated and normal treatment in the subculture of fresh primary cells.

Characterization of isolated vesicles

The EVs were isolated from SGCs conditioned media by several steps of differential centrifugation, filtration and ultrafiltration, before assessing the quality of the isolated fraction.

Sample purity as a particle to protein ratio

The ratio of particles to proteins was calculated based on protein determination and measurement of particle concentration by NTA. The vesicles from fresh primary SGCs had a low purity based on particle to protein ratio ($1.7x10^9$ particle/µg protein). This was measured before the final washing step for mass spectrometry.

BCA analysis was conducted on samples prior to NTA and after. Total protein in µg was determined from a standard curve with r^2 value of 0.9981 using 25 µl of sample. The values obtained were: 8.5, 8.6. 7.6, 6.9, 7.3, 7.7 µg total protein.

Characterization of vesicles by size distribution

The particle size distribution was measured by NTA on a subset of cultured fresh primary SGCs to characterize isolated extracellular vesicles. Two thirds of the particles were in the exosomal size range as shown by the gray area (Fig. 3). The mode size was in the range of 118 – 129.5 nm with variable concentration across samples.

It is important to note that the exsosomal fractions were from primary rat cells with very low yield, which permitted size distribution and quantification with the nano-particle tracking analysis (NTA), BCA determination for total protein, and dot-blot of the tetraspanins CD9, CD63, and CD81. However, the analysis with tetraspanins was not optimal with rat protein extracts. This was the first time it has been investigated in rat SGCs and the standard methodology for exosome characterization with transmission electron microscopy (TEM) was not conducted since the prerequisite to have a good protein detection of tetraspanins on blotting was not met.

Proteomic profile of SGCs-shed vesicles

A total of 43 proteins were identified; of which, 30 proteins were excluded as potential contaminants such as trypsin and proteins of human or bovine origin. We selected a cut-off to only include significantly differentially regulated proteins. Therefore, we have presented 13 proteins. The selected proteins are based on ranking of abundance. Identified exosomal proteins of fresh primary SGCs are listed in Table 1 in ranking of abundance.

The majority of the isolated proteins have been previously identified in exosomes according to the online ExoCarta database. These are highlighted in bold font (Table 1). Five proteins: E3 ubiquitin-protein ligase RNF181, protein Rlbp1, elongation factor 1-alpha, beta-enolase and protein Dsp had not previously been found in exosomes.

Half of the proteins identified were of cytoskeletal origin (actin, myosin-9, vimentin, tubulin, junction plakoglobin, protein desmoplakin) with structural molecular activity when analyzed by PANTHER gene ontology database. Actin, the most abundant protein and known to be linked to inflammation, was the center of ten predicted protein-protein interactions visualized by STRING database, v. 10 (Fig. 4).

In fresh primary SGCs exosomes, upon LPS-stimulation, four proteins: histone H2B, ubiquitin-60S ribosomal, myosin-9, elongation factor 1-alpha, were exclusively expressed compared to normal treatment. The cellular myosin had the strongest identification in terms of high number of unique peptides. In human, this protein plays an important role in determining cell morphology (focal contact formation and lamellipodial retraction), cytokinesis and specialized functions as secretion/transport of proteins [15]. Junction plakoglobin and Dsp proteins belong to the desmoplakin family and these were found significantly upregulated in LPS-stimulated, primary SGCs by a factor of 2.6 and 3, respectively, when compared to the

normally treated SGCs. In humans, these proteins play a role in cell-cell adhesion since desmoplakin organizes the cluster of desmosomal cadherin-plakoglobin complex where intermediate filaments are coupled to the plasma membrane [16]. Using UniProt database, fibronectins like junction plakoglobin are involved in cell adhesion, migration, cell shape and the binding of various compounds [17].

DISCUSSION

In this in vitro study, freshly isolated primary SGCs were used as a model for characterization of morphology and phenotypic marker expression after stimulation with the inflammatory substance, LPS. The experimental conditions chosen were based on background theory of neurogenic inflammation. Here, it was proposed that protein maker expression and release of molecules by SGCs, would be measurable in the extracellular milieu.

The shed EVs from trigeminal SGCs, predominantly exosomes, based on size distribution, were characterized after stimulation with LPS. These EVs were thought to play a key role in cell-cell communication by means of their molecular cargo, which is suggested to change upon disease and affect nearby target cells [18]. Here, we identified one exosomal protein (Myosin 9) that could be a potential novel marker of SGC activation in the fresh primary cells.

Morphology and phenotype of fresh primary SGCs

In accordance with previous studies, the primary cultured SGCs appeared with small cell bodies and 2-3 long processes [8,10]. The cells were not multilayered after 21 days in cultures as observed by Poulsen [8]. This was due to the fact that the SGCs from the two trigeminal ganglia were divided into two T25 flasks to avoid overgrowth and they were treated with serum that was exosome depleted - a factor known to slow down growth [19]. Following the initial phase of culturing, the cells continued to have a visible cell body but it was larger in form. Long processes were no longer present and the cells gathered as clumps/islands from which they proliferated. This morphology was similar to another in vitro study, which subcultured primary SGCs cells twice with further expansion before experiments were carried out. Roughly, 35% of the cells were described as having an astrocyte-like morphology [9]. These results indicate that SGCs are plastic with respect to morphological changes during cultivation. One interesting feature was that as time progressed, in culture or by subculturing, they looked more like astrocytes.

It has been suggested that SGCs do not proliferate under normal in situ conditions because of a form of a contact inhibition, diffusible factors and by inhibitory signals from parental neurons. However, upon injury the inhibitory signals are overcome by other signals and SGCs migrate away from their associated neurons and change morphology [20]. A similar disturbance in cell-cell contact and loss of inhibitory signals might explain the proliferation and change in SGCs morphology during in vitro culture time. Furthermore, astrocytes exhibit a different morphology under control conditions (polygonal cells) compared to stimulation

with TGFα (long thin processes as SGCs) or with neuregulin-1 (increased number of short, thick processes) [21]. Thus, it is speculated if the autocrine secretion of factors maintains the characteristic SGCs morphology in primary culture, which is gradually lost due to lack of neuronal feed-back upon passaging. The lack of autocrine signals might reduce the cells into a more "basic", astrocyte-like morphology that is more disorganized compared to cells of primary and first passage cultures that tend to cluster together in islands.

SGCs selectively express GS, which makes this a useful marker for SGCs identification [1]. In this study, the SGCs preserved their phenotypic GS expression regardless of the culture status they were in. This is in line with other studies using primary and subcultured SGCs [8,9]. As explained in the previous section, the SGCs would return to a more basic morphology but keep their phenotype when the influence of neurons or SGC-SGC signaling is missing.

Another glial marker, GFAP, is displayed in low levels under resting conditions [1]; but increased upon injury or inflammation in animal models of pain [14]. In this study, treatment with different LPS concentrations showed a markedly higher GFAP expression even by a very low concentration of LPS (1 ng/mL).

Characterization of isolated vesicles by particle to protein ratio and vesicle size distribution

Ultracentrifugation is a widely-used method to isolate exosomes and preferable compared to some commercial available kits [22]. This was confirmed by previous work in our laboratory (unpublished). However, it is known that ultracentrifugation might result in co-sedimentation of non-vesicular proteins among others [5]. This was assessed by the particle to protein ratio [12]. The selected isolation method and ultracentrifugation parameters resulted in acceptable purity post isolation but some smaller contaminants were apparent. This was equivalent to results obtained by Webber [12] when analyzing similar sample types of conditioned media. Thus, protein aggregates and non-vesicular proteins would probably pellet together with the extracellular vesicles. Therefore, to reduce the impurities, the pellet was washed once more in PBS with additional ultracentrifugation before mass spectrometry. This stringent washing resulted in loss of sample, which had an effect on our pool of identified proteins.

To our knowledge, no literature is available on documenting the size or yield of exosomal particles from isolated SGCs in culture. Here, we found approximately 2/3 of the particles were in the exosomal size of 30 – 150 nm measured by NTA with low and variable concentration across samples.

Therefore, this study is the first to present that it is possible to isolate extracellular vesicles from primary cultures of trigeminal SGCs and that the isolated vesicles contain the majority of particles in the exosomal size range. Analysis of proteomic composition of the enriched fraction can reveal the extent of non-vesicular proteins.

Global proteomic profile of exosomes

Here, we report the first global screening of exosomal proteins isolated from rat trigeminal primary SGCs conditioned media. As stated above, few proteins were identified after initial filtration of the data, but they had several protein-protein interactions indicating a non-random population, suitable to conclude on by statistical tests.

Most proteins identified had previously been isolated from exosomes, indicating a relatively pure vesicle fraction after the additional washing step. Cytoskeletal proteins were likely to have been involved in the morphological changes observed when grown under different culturing conditions. Five new exosomal proteins were identified through mass spectrometry in SGCs. All but one had a unique peptide indicating low confidence of protein identification. Only the protein Dsp had 2 unique peptides and moreover, this was significantly upregulated and four exosomal proteins were uniquely expressed in fresh primary SGCs upon LPS-activation. Out of the four, myosin-9 could serve as a novel marker of SGCs activation. The proteins Dsp and junction plakoglobin were significantly increased in exosomes; they play a role in cell-cell adhesion and possibly contribute in some way to increasing SGC-SGC cross-talk upon injury or inflammation which is thought to influence the excitability of nearby neurons [1,3].

In summary, it is likely that the exosomal proteins are involved in mediating some of the changes in cell morphology that was observed culturing the primary cells under the different experimental conditions. The protein composition was different when comparing significantly altered proteins under normal conditions and LPS treatment. Together, this supports the notion that fresh primary SGCs would be a good choice for performing experimental *in vitro* inflammatory studies.

Methodological considerations

The observed molecular alteration upon LPS stimulation in vitro might be more pronounced than in vivo since in vitro primary SGCs lack neuronal regulation of the inflammatory process. A more recent study suggests that LPS activation of the toll-like receptor 4 is inhibited in vivo, since their expression on SGCs is downregulated by neuronal contact [23]. On the other hand, the lack of neuronal suppression in the in-vitro primary SGCs culture might model uncontrolled inflammatory response by SGCs upon neuronal injury.

In light of the small pool of proteins, there might be additional proteins expressed at low levels that were not identified, since only a fraction of the original sample was analyzed by mass spectrometry. Dilution of starting material is a standard procedure in sample preparation for mass spectrometry and in this study, the initial amount of primary SGCs was low. Thus, analysis of undiluted samples might have been more applicable for a larger scale proteomic study.

CONCLUSION

This study demonstrated that primary SGCs do express the phenotypic marker GS. This finding supports the future use of fresh primary SGCs for experimental inflammatory studies. Interestingly, we found the

concentration of 1 ng/mL LPS was sufficient enough to activate SGCs in vitro without any form of toxicity. SGCs shed a population of extracellular vesicles in the exosomal size range and the identified proteins have a potential effect on SGCs morphology upon activation by LPS. For the first time, myosin-9 was identified as a possible novel marker of LPS-activated SGCs in fresh primary SGCs that can be applicable for inflammatory pain conditions.

ACKNOWLEDGEMENTS

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The authors declare that they have no conflict of interest.

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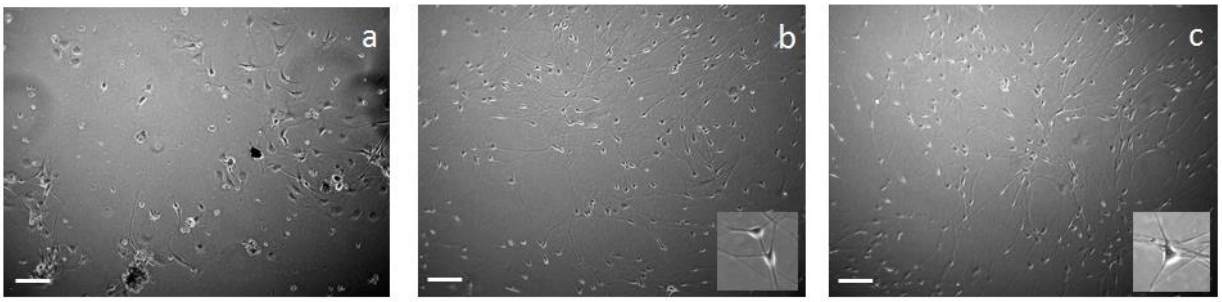
Fig. 1. The morphology of rat trigeminal SGCs, following cultivation of freshly isolated, SGCs. Images were obtained from fresh primary culture of SGCs from day 3 (a), day 10 (b), and day 21 (c) (10x objective; insert: 20x objective). Notice the formation of multiple processes from a single SGC from an initial spherical appearance to long processes after additional growth. Images taken using phase contrast microscope, Zeiss Primovert, Germany. Scale bar: 50 µm

Fig. 2. Expression of GS and GFAP in rat trigeminal SGCs in fresh primary culture. The expression of GS (green) and GFAP (red) in fresh primary cells under normal and LPS-activated conditions. Trigeminal rat SGCs activated with different LPS concentrations (1, 50, 250 ng/mL; only 50 ng/mL is shown). The up-regulation of GFAP (red) was used to assess if SGCs were activated upon LPS-treatment. Images were captured with an immunfluorescent microscope (Nikon AZ100 microscope, Tokey, JP; L200/D fluorescent illuminator, Prior Scientific, Rockland, MA, USA and DS-Vi1 Nikon digital camera, Tokey, JP) at a fixed auto exposure in order to compare fluorescence intensity (30x objective). Scale bar: 50 µm.

Fig. 3. SGCs particle concentration versus size distribution by NTA. Particle size distribution was used to characterize isolated particles from control (A) and LPS treated (B) cultures (only one example has been depicted). Exosomal size range of 30 – 150 nm is shown. Solid line depicts average of two measurements. Distribution of particle size in percentage (%) showed that 67% were in the exosomal range of <150 nm.

Fig. 4. Protein-protein interaction network by STRING v. 10. Extra cellular vesicles (EVs) with particles in the expected exosomal range isolated from fresh rat primary SGCs conditioned media. The nodes are proteins and the line/color represents the predicted mode of action between protein pair: blue line (binding), purple line (post-translational interaction) and black line (reaction). Abbreviations and names of proteins are displayed in alphabetical order to the right.



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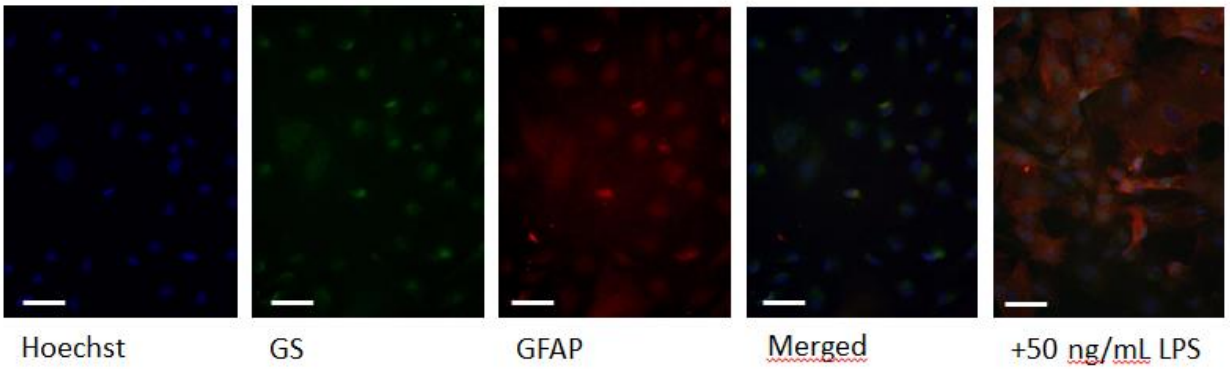

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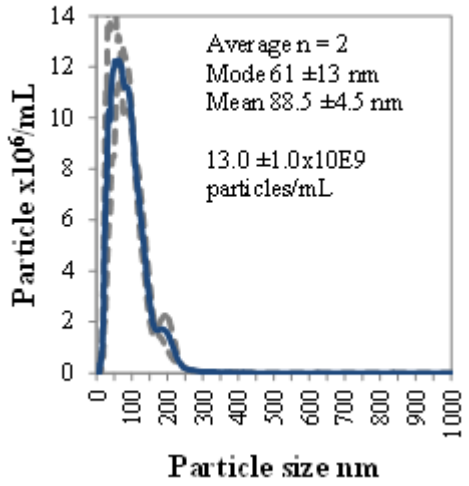
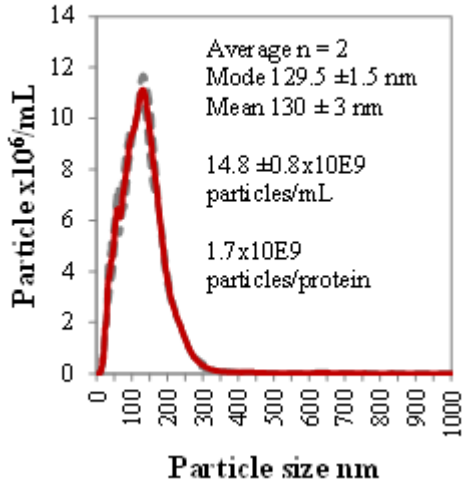
A. Control



B. LPS



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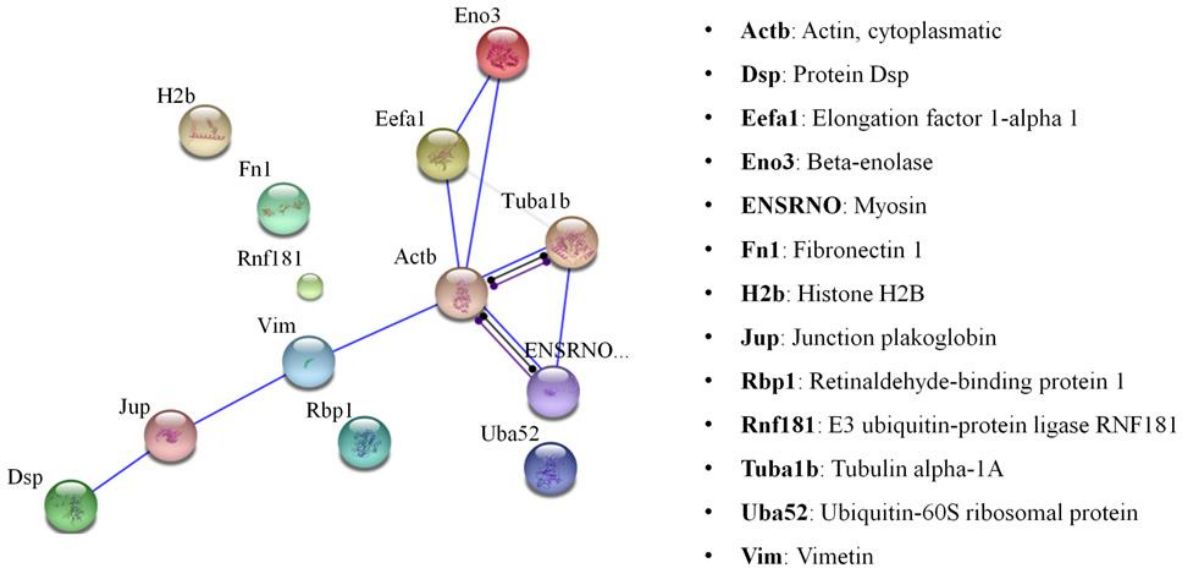

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Table 1. List of exosomal proteins identified from fresh primary cultures of rat trigeminal SGCs conditioned media by rank of abundance

Rank [1)]	Uniprot Acc. [2)]	Protein Name [3)]	Protein Function according to UniProtKB	Peptides (Unique), Seq. Cov. [4)]
1	P63259	**Actin, cytoplasmic 2**	Involved in cell motility	5 (5), 25.6%
2	Q6AXU4	E3 ubiquitin-protein ligase RNF181	Accept and transfere ubiquitin to target substrates	1 (1), 4.8%
3	P04937	**Fibronectin**	Binds cell surfaces and various compounds; involved in cell motility, opsonization, wound healing and maintenance of cell shape.	24 (24), 16.6%
4	Q00715	**Histone H2B type 1**	Central role in transcription regulation, DNA repair/replication and chromosomal stability.	1 (1), 12.7%
5	D3Z956	Protein Rlbp1	Transporter activity	1 (1), 7.3%
6	P62986	**Ubiquitin-60S ribosomal protein L40**	Functions depend on linked protein: damage response, degradation and cell-cycle regulation	1 (1), 10.7%
7	Q62812	**Myosin-9**	During cell spreading: cytokinesis, cell shape, secretion and capping	10 (8), 6.9%
8	P62630	Elongation factor 1-alpha	Binds the promotor of IFN-gamma and regulate its transcription	1 (1), 5.9%
9	P68370	**Tubulin alpha-1A chain**	Major constituent of microtubules. Binds GTP.	2 (2), 5.4%
10	P31000	**Vimentin**	Intermediate filament attached to the nucleus, ER and mitochondria	2 (2), 4.9%
11	Q6P0K8	**Junction plakoglobin**	Influences the arrangement and function of cytoskeleton and the cells within the tissue	1 (1), 1.5%
12	P15429	Beta-enolase	Functions in striated muscle development and regeneration	1 (1), 4.1%
13	F1LMV6	Protein Dsp	Poly(A) RNA binding; structural molecule activity	2 (2), 1%

[1]Rank of abundance based on iBAQ value = intensity-based absolute quantification.
[2]Uniprot accession number is a unique identifier assigned to each entry
[3]Protein name written with bold font have previously been identified in exosomes according to ExoCarta exosome database.
[4]Peptide = total number of peptide sequences identified in the protein group; Unique = number of peptide that are found only in one protein group; Seq. Cov. % = Percentage of the protein sequence covered by identified peptides.

Graphical abstract
The authors do not consider any graphical abstract.

Highlight for review

- Satellite glial cells (SGCs) in sensory ganglia contribute to the pathogenesis of chronic pain.
- This study characterized extracellular vesicles shed from SGCs in the form of exosomes.
- SGCs shed vesicles in the size range of exosomes (>150 nm) showed an altered protein expression under inflammatory condition.
- Proteomic profile of exosomal SGCs-shed vesicles showed that junction plakoglobin and myosin 9 might be novel biomarkers of SGCs under inflammatory conditions.

Significance

This study demonstrated that

1. Primary SGCs do express the phenotypic marker.
2. One ng/mL LPS is sufficient enough to activate SGCs in vitro without any form of toxicity.
3. SGCs shed a population of extracellular vesicles in the exosomal size range and the identified proteins have a potential effect on SGCs morphology upon activation by LPS.
4. Myosin-9 was identified as a possible novel marker of LPS-activated SGCs in fresh primary SGCs that can be applicable for inflammatory pain conditions.