SOMATOSENSORY & MOTOR RESEARCH
https://doi.org/10.1080/08990220.2021.1941843

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Intracellular potassium depletion enhances apoptosis induced by staurosporine in cultured trigeminal satellite glial cells

Hedie A. Bustamante[a], Marion F. Ehrich[b] and Bradley G. Klein[b]

[a]Faculty of Veterinary Sciences, Veterinary Clinical Sciences Institute, Universidad Austral de Chile, Valdivia, Chile; [b]Department of Biomedical Sciences and Pathobiology, Virginia-Maryland College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA

ABSTRACT

Purpose: Satellite glial cells (SGC) surrounding neurons in sensory ganglia can buffer extracellular potassium, regulating the excitability of injured neurons and possibly influencing a shift from acute to neuropathic pain. SGC apoptosis may be a key component in this process. This work evaluated induction or enhancement of apoptosis in cultured trigeminal SGC following changes in intracellular potassium [K]ic.

Materials and methods: We developed SGC primary cultures from rat trigeminal ganglia (TG). Purity of our cultures was confirmed using immunofluorescence and western blot analysis for the presence of the specific marker of SGC, glutamine synthetase (GS). SGC [K]ic was depleted using hypo-osmotic shock and 4 mM bumetanide plus 10 mM ouabain. [K]ic was measured using the K^+ fluorescent indicator potassium benzofuran isophthalate (PBFI-AM).

Results: SGC tested positive for GS and hypo-osmotic shock induced a significant decrease in [K]ic at every evaluated time. Cells were then incubated for 5 h with either 2 mM staurosporine (STS) or 20 ng/ml of TNF-α and evaluated for early apoptosis and late apoptosis/necrosis by flow cytometry using annexin V and propidium iodide. A significant increase in early apoptosis, from 16 to 38%, was detected in SGC with depleted [K]ic after incubation with STS. In contrast, TNF-α did not increase early apoptosis in normal or [K]ic depleted SGC.

Conclusion: Hypo-osmotic shock induced a decrease in intracellular potassium in cultured trigeminal SGC and this enhanced apoptosis induced by STS that is associated with the mitochondrial pathway. These results suggest that K^+ dysregulation may underlie apoptosis in trigeminal SGC.

ARTICLE HISTORY
Received 4 June 2021
Accepted 8 June 2021

KEYWORDS
Satellite glial cells; apoptosis; staurosporine; neuropathic pain

1. Introduction

Satellite glial cells (SGC) are laminar, multipotent, neural crest-derived glial cells present in the peripheral nervous system (Jessen and Mirsky 2005). SGC surround and form a special sheath around neuronal cell bodies in every sensory ganglion, using gap junctions to connect with one another (Hanani 2005; Ohara et al. 2009). SGC can become activated after diabetes (Rahman et al. 2016), chemotherapy (Warwick and Hanani 2013) and inflammation (Blum et al. 2017). Moreover, SGC play a critical role in maintaining ionic homeostasis by buffering potassium changes in the extracellular space after neuronal depolarization (Hanani, 2010). To properly accomplish this function, SGC in the trigeminal ganglion express potassium selective channels (Cherkas et al. 2004). Inwardly rectifying potassium channels (Kir) in SGC are predominantly responsible for a high potassium permeability, maintenance of the SGC resting membrane potential close to the equilibrium potential of potassium, and indirectly keeping the neuronal resting potential close to this value (Olsen and Sontheimer 2008). After peripheral nerve injury (PNI) or induced peripheral inflammation, reduced immunopositive expression of SGC-associated Kir4.1 inwardly rectifying potassium channels has been reported in the trigeminal ganglion (Vit et al. 2008; Takeda et al. 2011). In addition, suppression of Kir4.1 potassium currents in trigeminal SGCs has been observed after induced inflammation of the rat whisker pad (Takeda et al. 2011). Such changes may lead to a decrease in the K^+ buffering capacity of SGC, decreased intracellular K^+ concentration of SGC, and changes in neuronal extracellular ion concentrations. The latter could affect the excitability of nociceptive primary afferents (Bay and Butt 2012) and contribute to glial cell involvement in the production of neuropathic pain in people and laboratory animals (Vit et al. 2008; Warwick and Hanani 2013; Hanani et al. 2014; Blum et al. 2017).

Changes in the flow of ions in and out of the cell and its intracellular organelles has been linked to the generation of many disease states by leading to pathological apoptosis (Burg et al. 2006). Particularly, changes in intracellular K^+ concentration have been associated with apoptosis

CONTACT Bradley G. Klein bklein@vt.edu Department of Biomedical Sciences and Pathobiology, Virginia-Maryland College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA

regulation (Yu 2003; Remillard and Yuan 2004). Different reports have described the role of K^+ in pro-apoptotic cellular events by both the intrinsic and the extrinsic pathways (Bortner and Cidlowski 1998; Montague et al. 1999; Model 2014). Under normal physiological conditions intracellular K^+ acts as an endogenous modulator of the apoptosis checkpoints, particularly as a potential repressor of executioner caspases and nucleases involved in apoptosis (Bortner et al. 1997; Hughes et al. 1997, Hughes and Cidlowski 1999; Hoffmann, 2011).

Indirect evidence suggests intracellular K^+ concentration may change in SGC after peripheral nerve insult-induced reductions in their expression of K^+ channels or K^+ channel-mediated currents (Vit et al. 2008; Takeda et al. 2011). Therefore, this study evaluated the effect of potassium depletion on induction of apoptosis in trigeminal SGC and relative impact upon the mitochondrial (intrinsic) and death receptor (extrinsic) apoptotic pathways. We hypothesize that a decrease in the intracellular potassium concentration increases apoptosis in trigeminal SGC. Such a mechanism modulating trigeminal SGC survival after peripheral nerve insult could affect K^+ buffering capacity in the trigeminal ganglion, in turn influencing nociceptor excitability.

2. Materials and methods

2.1. Animals and trigeminal satellite glial cell primary cultures

The Institutional Animal Care and Use Committee of Virginia Polytechnic Institute and State University approved the present study. Satellite glial cells (SGC) were cultured from trigeminal ganglia (TG) of 200–250 g male Sprague Dawley rats (Harlan Laboratories, Dublin, VA). Both TG were aseptically removed after euthanasia by cervical decapitation and collected in a Petri dish containing 4°C sterile phosphate buffered saline (PBS) without Ca^{++} and Mg^{++} (Invitrogen, Carlsbad, CA). After careful removal of any remaining connective tissue, individual TG were minced and digested for 60 min at 37°C in 5 ml of dissociation buffer containing 0.2 U/ml of collagenase (Liberase TM®, Roche, Indianapolis, IN) and 0.05% (*w/v*) DNAse I (Sigma, St. Louis, MO, USA). After completion of digestion, the tissue was mechanically dissociated using a polished tip Pasteur pipette and filtered through a 70 μm cell strainer (BD Biosciences, Franklin Lakes, NJ, USA). The filtrate was collected in a medium containing 5 ml of 89% Ham's F12 medium (CellGrow®, Mediatech, Inc, Manassas, VA, USA), 10% heat-inactivated foetal bovine serum (FBS) (Atlanta Biologicals Inc. Lawrenceville, GA, USA) and 1% antibacterial/antifungal antibiotics (CellGrow®, Mediatech, Inc, Manassas, VA, USA). SGC were isolated according to Capuano et al. (2007) and Capuano et al. (2009). Briefly, SGC were allowed to pre-plate for 3–4 h in a 25 cm^2 flask at 37 °C in a humidified atmosphere with 5% CO_2. SGC attached firmly to the bottom of the uncoated flask, leaving neurons and other remaining cells to float freely in the medium. At the end of this step, the medium was removed and SGC remained attached to the flask to which 5 ml of fresh media were added. SGC were incubated and media changed every other day until full confluence (10–14 days).

2.2. Confirmation of SGC primary culture by immunofluorescence

Immunofluorescence staining of primary cell culture SGC was performed to qualitatively verify the presence of glutamine synthetase (GS), which is a specific marker characteristic of SGC (Hanani 2005). SGC were obtained from each of 5 different 25 cm^2 culture flasks. At full confluence, SGC were washed with PBS and then detached from the flasks by adding 1.5 ml of 0.125% trypsin followed by incubation for 5 min at 37°C. Cells were then re-suspended in media and plated at a density of 10,000 cells/well in six-well plates to which German coverslips coated with poly-D-lysine and laminin (BD Biocoat TM, BD Biosciences, Franklin Lakes, NJ) were added. After 4 days of culture coverslips were removed, washed three times with PBS and fixed for 3 min with pre-warmed 4% paraformaldehyde. Cells were then washed three times with PBS and permeabilized using a fresh 0.3% Triton X-100 solution in PBS for 5 min. After three successive PBS washes, cells were incubated in a 5% bovine serum albumin (BSA)/PBS blocking solution for 1 h at room temperature. Primary antibody for mouse monoclonal anti-glutamine synthetase (GS) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) was diluted 1:500 (*v/v*) in 1% BSA/PBS. Coverslips were incubated overnight at 4°C. A fluorescein isothiocyanate (FITC) coupled goat anti mouse IgG (Invitrogen, Carlsbad, CA, USA) was used as secondary antibody. Secondary antibody was diluted 1:200 (*v/v*) in 1% BSA/PBS and incubated for 2 h at room temperature. Coverslips were washed in PBS and mounted using a 4′,6 diamidino-2-phenylindole (DAPI) mounting medium (Vectashield TM, Vector Lab, Burlingame, CA, USA). Primary antibody deletion served as a negative control.

2.3. Confirmation of SGC primary culture by western blot

Ganglion cells from 5 additional flasks were dissociated as previously described, pre-plated and grown until full confluence. These were used for western blot of SGC and neuronal markers to further evaluate the composition of SGC cultures. The presence or absence of neurons in our primary cultures was evaluated using the specific neuronal marker neuron-specific nuclear protein (NeuN) (Mullen et al. 1992). Briefly, SGC from 5 different flasks were detached using 0.125% trypsin after washing with ice-cold PBS, then lysed for 10 min using 200 μl of an ice-cold lysis buffer solution that contained 150 mM NaCl, 10 mM trizma base, 0.5% *v/v* triton X-100 and 0.5% *w/v* deoxycholate. Positive control for western blot analysis included tissue extracts from rat cortical hemispheres and trigeminal ganglia. Cell lysates and homogenized positive control tissues were centrifuged for 8 min at 14,000*g*. The supernatant was collected and frozen at −70°C until further analysis. Before western blot experiments were carried out, total protein concentration was analyzed using the bicinchoninic acid (BCA) protein assay reagent method

(Thermo Scientific, Rockford, IL, USA). 10 mg of total protein was separated by electrophoresis in 10% SDS-PAGE gels and then transferred to a 0.45 μm nitrocellulose membrane by electroblotting. After blocking with Odyssey blocking buffer (LI-COR Biotechnology, Lincoln, NE, USA) for 1 h at room temperature, respective membranes were incubated at 4 °C overnight with 1:1000 (*w/v*) anti-glutamine synthetase (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) or 1:1000 (*w/v*) anti-NeuN mouse monoclonal antibodies (Millipore, Billerica, MA, USA). Membranes were washed twice (15 min each) using a 0.1% Tween-20/PBS (pH 7.4) solution and then incubated for 1 h with the fluorescent secondary antibody (IRDye 800CW donkey anti-mouse IgG (H + L); LI-COR Biotechnology, Lincoln, NE, USA) at 1:5000 w/v dilution. After washing, membranes were developed using a digital imaging device (LI-COR Biotechnologies Lincoln, NE, USA). For each antibody, gels for analysis were prepared in duplicate for each primary culture.

2.4. Potassium depletion and intracellular potassium measurement

SGC from 24 different primary cultures, originating from 12 different animals, were used. Cells were detached from flasks using 0.125% trypsin, washed with PBS, resuspended in 1 ml of media, and counted using an automatic cell counter (Cellometer AutoX4 Cell Counter, Nexcelom Bioscience LCC, Lawrence, MA, USA). Potassium depletion was accomplished by hypo-osmotic shock (Elperin et al. 2011). Briefly, 100,000 SGC originating from each primary culture were incubated in iso-osmotic PBS or in hypo-osmotic PBS (136 mOsm) alone, or in each of these solutions along with a combination of the K^+ influx inhibitors ouabain (10 μM) plus bumetanide (10 μM). Incubations in hypo-osmotic solutions were performed for 2, 5 or 10 min. Hypo-osmotic shock induces an increase in cell volume, which is followed by a regulatory volume decrease (RVD) as a form of compensation after removal of the hypo-osmotic PBS.

To assess the amount of potassium depletion induced by hypo-osmotic shock, 10,000 SGC from each treatment condition mentioned above were plated in triplicate in a 96-well plate and wells were loaded with 10 μM of the cell permeant acetoxymethyl esterified derivative of potassium benzofuran isophthalate (PBFI-AM) (Molecular Probes, Carlsbad, CA). PBFI-AM is hydrolyzed intracellularly to a non-permeable fluorescent indicator for K^+. PBFI-AM was diluted in DMSO containing 25% Pluronic-127 (Molecular Probes, Carlsbad, CA, USA). After dye loading, cells were incubated for 60 min at 37°C and PBFI-AM excess was washed away using PBS. SGC were plated and signal intensity was detected using a fluorescence plate reader (SpectraMax M5 TM, Molecular Devices, Sunnyvale, CA, USA). Cells were excited at 340 and 380 nm and emission was recorded at 500 nm. Potassium-associated fluorescence was determined according to the following formula: Fluorescence = (E 340 – E 340 auto)/(E 380 – E 380 auto), where E = emission after 340 and 380 nm excitation, and E 340 auto and E 380 auto are respective autofluorescence values (Grishin et al. 2005). The average of the triplicates for each treatment, from a given primary culture, was considered a single data point.

2.5. Stimulation, and flow cytometric analysis, of SGC apoptosis

A separate complement of cells from each of the 24 primary cultures used above was employed in our analysis of apoptosis. About 100,000 potassium depleted (2 min hypo-osmotic exposure) cells and non-depleted (iso-osmotic) cells were plated in triplicate in a 24 well cell culture plate (CostarTM, Corning Life Sciences, Lowell, MA, USA). No K^+ influx blockers were used in this analysis, and the mean of each triplicate was considered a single experimental unit. Iso-osmotic and hypo-osmotic triplicates were incubated for 5 h with PBS vehicle, or with either 2 mM staurosporine (STS) in PBS or 20 ng/ml TNF-α in PBS to, respectively, differentiate apoptosis induced by the intrinsic and extrinsic pathways. After this incubation period cells were washed with cold PBS to stop the reaction and resuspended in 100 ml of annexin V binding buffer to which 5 ml of Pacific Blue coupled annexin V (Molecular Probes, Carlsbad, CA, USA) and 1 ml of propidium iodide (PI) (Molecular Probes, Carlsbad, CA, USA) were added. Cells were incubated for 15 minutes at room temperature and then apoptosis was analyzed by flow cytometry (FACSAria, BD Biosciences, Franklin Lakes, NJ, USA). The above procedure was repeated four times on separate days. Annexin V and PI were used for detection of early apoptotic (EA) cells and late apoptotic/necrotic (LA/N) cells, respectively.

2.6. Statistical analysis

All analyses were performed using SAS version 9.2 (SAS Inc., Cary, NC, USA). To verify that data followed a normal distribution, probability plots for each variable's data were generated. Data were analyzed using a mixed-model analysis of variance (ANOVA). For the potassium depletion data the linear model included osmolarity (hypo-osmotic and iso-osmotic), potassium blockers and time (0, 2, 5 and 10 min), and the interaction between them, as fixed effects. For flow cytometry data the linear model included apoptosis induction (control, STS and TNF-α), osmolarity (hypo-osmotic and iso-osmotic), and the interaction between them, as fixed effects. Interactions were investigated using the slice option of the GLIMMIX procedure of SAS. For each model, residual plots were inspected to verify that the errors followed a normal distribution with constant variance. Effects of treatment and conditions were assessed using Tukey's procedure for multiple comparison. Overall alpha was set to $p < 0.05$.

3. Results

3.1. Characterization of SGC primary culture

SGC primary cultures grew to full confluence at day 14 and appeared morphologically homogeneous (Figure 1(A)). Figure 1(B) demonstrates a representative immunofluorescence

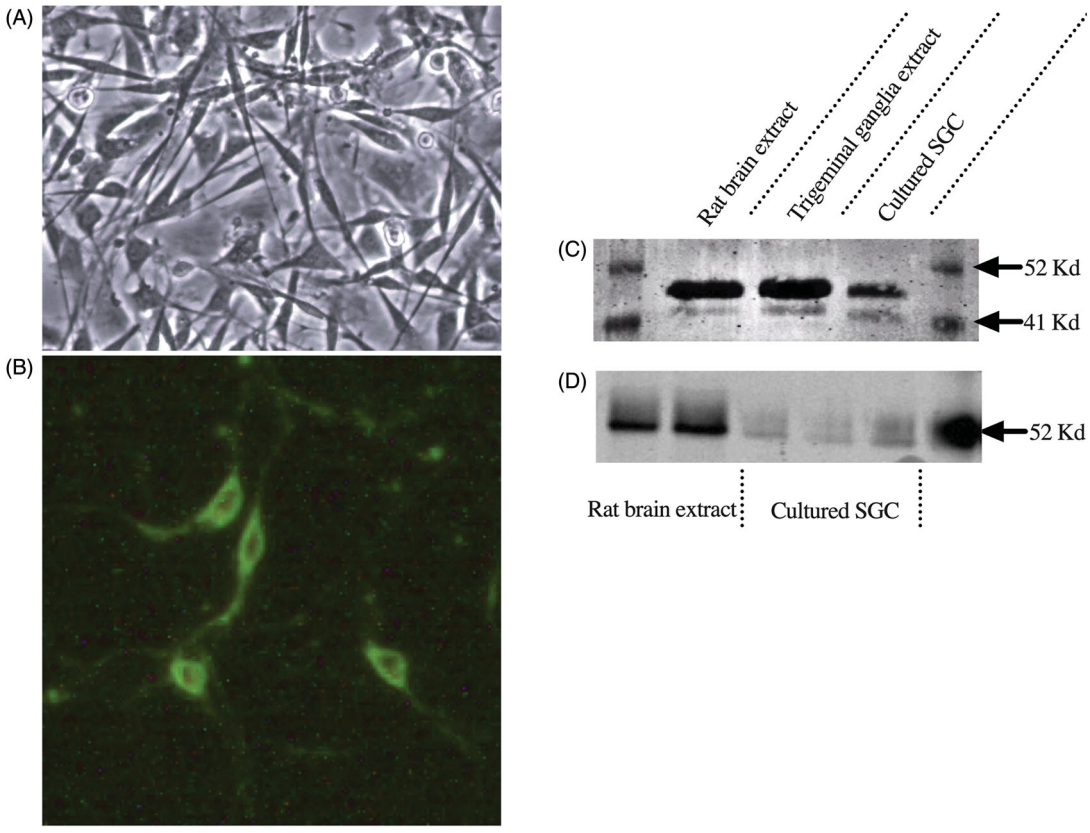

Figure 1. Characterization of SGC primary cultures. (A) Phase contrast photomicrograph of a representative 14-day SGC culture grown in 25 cm^2 flasks (10×). (B) Immunofluorescence image showing glutamine synthetase positive SGC grown on coverslips. (C) Immunoblot analysis, for GS, of SGC primary cell culture compared with positive controls of rat brain extract and rat trigeminal ganglion extract. A similar band pattern for glutamine synthetase across the three sample populations can be observed at approximately 49 kd. (D) Immunoblot analysis, for NeuN, of SGC primary cell culture compared with positive controls of rat brain extract. Band pattern of approximately 50 kd showing that SGC primary cell cultures did not express notable levels of neuronal-specific marker NeuN when compared to rat brain extract.

image for the SGC molecular marker GS, confirming that SGC were present in these trigeminal ganglion cultures. There was no convincing evidence of unstained cells on coverslips after the fixation and staining procedure. In addition, no recognizable fluorescent signal was evident on negative control coverslips. A similar pattern of results was obtained from all 5 trigeminal ganglia primary cultures.

Western blot analysis of protein extracts from SGC primary cell cultures showed an easily detectable band for GS, similar in position to that of GS-containing positive controls from rat brain and trigeminal ganglia extracts (Figure 1(C)). GS positive control bands were located in a position of approximately 49 Kd. In contrast, there was little indication of positive bands for NeuN in SGC protein obtained from primary cultures when compared to easily recognizable banding at the position of NeuN-containing positive control extracts from brain (Figure 1(D)). NeuN positive control bands were located in an expected position of approximately 48 Kd. Therefore, western blots suggested the presence of a ganglionic SGC molecular marker in the cultured cells, while showing little evidence for a molecular marker of neurons.

3.2. Intracellular potassium depletion

Hypo-osmotic shock by itself significantly reduced [K]ic of SGC by 58% compared to the iso-osmotic control as early as 2 min after the start of incubation (Figure 2) with a maximum reduction of 64% ($p < 0.05$) observed at 10 min. However, no significant differences in [K]ic among different hypo-osmotic incubation times was observed. Addition of the K^+ influx blockers ouabain plus bumetanide to all experimental conditions significantly reduced [K]ic of SGC relative to the absence of blockers for the iso-osmotic condition ($p < 0.05$). However, this addition failed to significantly improve the magnitude of [K]ic reduction in SGC induced by incubation in hypo-osmotic solutions at any time point (Figure 2).

3.3. Apoptosis induction and potassium depletion

We stimulated apoptosis in cultured SGC through the intrinsic pathway using staurosporine and through the extrinsic pathway using TNF-α and examined the effect of [K]ic reduction by hypo-osmotic shock upon the progression of cell death. Figure 3(A) shows the percentage of SGC in early apoptosis because of these conditions. Percentages of early apoptotic cells did not differ between K^+-depleted and non-depleted SGC in PBS control nor in TNF-α stimulated SGC. In contrast, after stimulation with staurosporine (STS), there was a significant ($p < 0.05$) increase in the percentage of cells in early apoptosis to 38% in [K]ic-depleted cultures compared to 16% in non-depleted SGC. This percentage for [K]ic depleted SGC in early apoptosis in the staurosporine

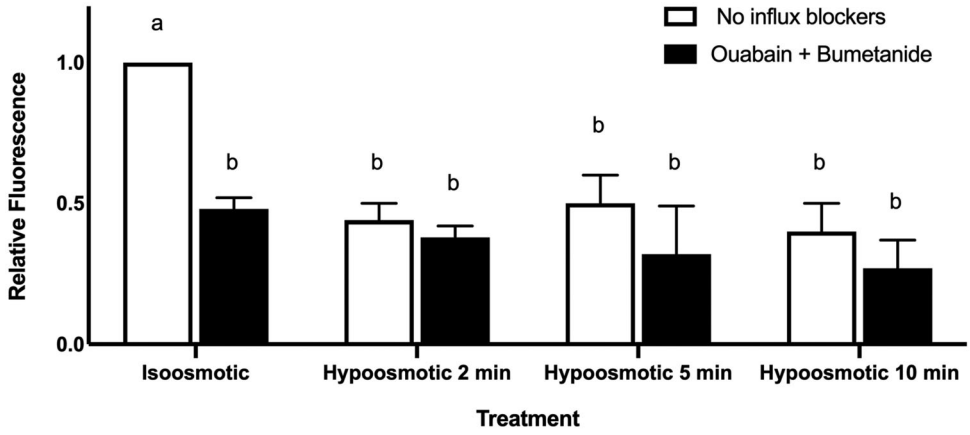

Figure 2. Relative fluorescence intensity indicating [K]ic (mean ± SEM) under iso-osmotic and hypo-osmotic conditions, with or without potassium influx blockers ouabain + bumetanide at different times ($N = 4$). Different letters indicate significant differences among treatment groups ($p < 0.05$).

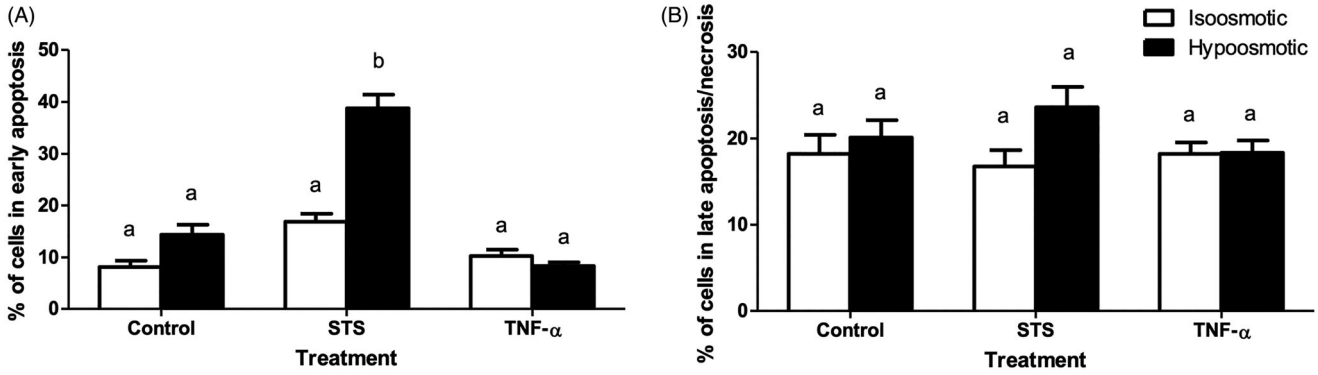

Figure 3. Percentage of cultured SGC in early apoptosis as demonstrated by annexin V binding (A) and late apoptosis/necrosis determined by PI binding (B) under iso-osmotic and hypo-osmotic conditions after treatment with apoptosis inducers ($N = 4$, SGC primary cultures originating from different animals for each treatment). Different letters indicate significant difference among treatment groups ($p < 0.05$). STS: staurosporine; TNF-α: tumour necrosis factor alpha.

condition was also significantly greater ($p < 0.05$) than that for the PBS vehicle and TNF-α exposed, [K]ic depleted cells. The percentage of SGC in late apoptosis or necrosis after STS and TNF-α exposure is presented in Figure 3(B). No significant differences between treatments, or between depletion conditions within treatments, were observed.

4. Discussion

The difficulty of studying SGC derives from their small size and complications associated with their isolation (Hanani 2005; Vit et al. 2006; Capuano et al. 2009; Poulsen et al. 2014). In the present study, we developed a primary cell culture of trigeminal SGC that was then used to examine the effects of a decrease in their intracellular potassium concentration on apoptosis induced through the intrinsic and extrinsic pathways. The issue is important considering prior evidence for changes in potassium channel expression in SGC following trigeminal nerve constriction (Vit et al. 2008).

Qualitative observation of our cultures demonstrated a high degree of confluence and morphological homogeneity among cells that was verified using glutamine synthetase (GS), a frequently used specific marker of uninjured ganglionic SGC (Weick et al. 2003; Procacci et al. 2008; Belzer et al. 2010; Laursen et al. 2014; Poulsen et al. 2014). As seen by others (Belzer et al. 2010; Poulsen et al. 2014), the SGC character of our primary cell cultures was supported by Western blot detection of intense banding corresponding with positive controls at the molecular weight of GS and comparatively negligible signal for the neuronal marker NeuN. This suggests that we were able to successfully produce viable SGC cultures.

We successfully reduced intracellular potassium in cultured trigeminal SGC using hypo-osmotic shock. Hypo-osmotic shock results in cell swelling, but cells then return towards their iso-osmotic volume through a variety of actions collectively known as regulatory volume decrease (RVD) (Pasantes-Morales 1996; Simard and Nedergaard 2004; Hoffmann et al. 2009). Astrocytes are particularly sensitive to hypo-osmotic changes, exhibiting a rapid RVD response that is critical for the fine control of volume in the brain (Benfenati et al. 2011). RVD has been extensively studied in primary cultures of astrocytes and neurons (Pasantes-Morales et al. 1994), but not in SGC. In astrocytes, as well as neurons, a prominent efflux of potassium ions is a feature of the RVD that follows hypo-osmotic swelling (Simard and Nedergaard 2004; Pasantes-Morales et al. 2006; Hoffmann et al. 2009). We exploited this feature of the RVD response, considering previously reported similarities in physiology and pharmacology between astrocytes and satellite glial cells (Hanani 2005; Ohara et al. 2009), to produce a decrease in SGC [K]ic for purposes of addressing its modulation of SGC apoptosis.

Previous studies of the behaviour of potassium ions in astrocytes under hypo-osmotic conditions have relied on measurements of radioactive rubidium release (a congener surrogate for potassium release), or on whole cell patch clamp currents (Sanchez-Olea et al. 1993; Quesada et al. 1999; Ordaz et al. 2004). Reductions in osmolarity, like those used in our experiment, have been shown to produce a 60% increase in cultured cerebellar astrocyte volume within 1 min, followed as little as 1–2 min later by an RVD that progressed to within 20% of control volume by 15 min. This was mirrored by an increase in rubidium release that peaked within 2–3 min and then gradually decreased almost to control values over the next 10 min, resulting in a loss of about 30–35% of internal rubidium (Sanchez-Olea et al. 1993). The large rapid decrease (58%) in [K]ic that we observed in our cultured SGC by 2 min after exposure to hypo-osmotic conditions is similar to the above noted latency of the rapid initial increase in rubidium release in astrocytes under similar conditions. The lack of significant change in [K]ic that we observed for our cultured SGC between 2 and 10 min after hypo-osmotic exposure is not surprising given the above noted gradual nature of the return towards baseline of rubidium release in astrocytes during a comparable time period after hypo-osmotic exposure. Although the PBFI-AM method has been used to determine changes in [K]ic in astrocytes, both *in vitro* and *in vivo*, these studies were not concerned with changes following hypo-osmotic shock (Muyderman et al. 2001; Kozoriz et al. 2010; Dufour et al. 2011). Our data utilizing the PBFI-AM technique in cultured trigeminal SGC, following hypo-osmotic shock, indicated a large rapid decrease in [K]ic concordant with the potassium efflux reported to accompany the robust RVD seen in cerebellar astrocytes and several other cell types following hypo-osmotic challenge (Pasantes-Morales 1996; Mongin and Orlov 2001; Lauf et al. 2008).

Additionally, we evaluated the use of potassium transport blockers in conjunction with hypo-osmotic shock intending to prevent the re-entry of potassium effluent, thereby maximizing the amount of potassium depletion. Bumetanide is a well know inhibitor of the ubiquitous $Na^+/K^+/Cl^-$ influx symporter that normally plays an important role in glial cell potassium buffering (Hoffmann et al. 2009). Similarly, ouabain is a well-known inhibitor of the ubiquitous Na^+-K^+-ATPase exchange pump that is an indirect driver of so many critical cell functions. The activity of both pumps can be affected by changes in cell volume (Mongin and Orlov 2001). As we reported above, using the blockers failed to further reduce the [K]ic that we observed in our cultured SGC following hypo-osmotic shock. It is unlikely that this resulted from an ineffective concentration of these agents given that they were able to induce a significant reduction in [K]ic in our SGC under iso-osmotic conditions. Such effects of these agents have been reported in other cells under iso-osmotic conditions, although often with a longer time course. Our findings suggest a K^+ efflux response to hypo-osmotic swelling in trigeminal SGC that was similar in time course and magnitude to other glial cells, including astrocytes and microglia, which are known to show a brisk RVD response (Chvátal et al. 2007; Ducharme et al. 2007). Although we used the specific K^+ influx blockers ouabain and bumetanide to avoid K^+ influx as a compensatory mechanism that counteracts K^+ loss during RVD, they did not appear to affect the magnitude of [K]ic reduction induced by hypo-osmotic shock. However, the reduction of [K]ic when these blockers were employed under iso-osmotic conditions suggested that these blockers were active in our preparation.

We stimulated the intrinsic/mitochondrial (STS) and extrinsic/death receptor (TNF-α) apoptotic pathways to determine if intracellular K^+ reduction can facilitate apoptosis in trigeminal SGC. Apoptosis induction has been strongly associated with changes in ion fluxes, particularly K^+ efflux (Yu et al. 1997; Krick et al. 2002; Platoshyn et al. 2002). The involvement of K^+ currents in apoptosis has been demonstrated in neurons and some glial cells other than SGC (Nadeau et al. 2000; Yu 2003). The involvement of K^+ regulation in SCG apoptosis is an important question considering evidence for changes in K^+ channel expression in SGC associated with models of neuropathic pain (Vit et al. 2008; Ohara et al. 2009). Manipulations designed to enhance outward K^+ currents, such as use of the selective K^+ ionophore vanillomycin, or the K^+ channel opener chromakalin, induced apoptosis among immune, tumour and neuronal cells (Yu et al. 1997). Furthermore, high intracellular K^+ protects from entering apoptosis, inhibiting both the intrinsic and extrinsic pathways (Thompson et al. 2001). However, there is evidence that the direction of the relationship between K^+ currents or [K]ic with apoptosis may vary among cell types and apoptotic paradigms (Bortner et al. 2007) highlighting the importance of investigating the effect of altered [K]ic on apoptosis in SGC induced by different routes. In the present study, we observed that cultured trigeminal SGC with lowered [K]ic presented a higher incidence of early apoptosis compared to non-depleted cells only after stimulation with staurosporine (STS). Potassium efflux may not only be capable of triggering or facilitating apoptosis in some cells but is an important event in the apoptotic volume decrease phase once apoptosis has been initiated. Apoptotic cells have been shown to have reduced [K]ic, ranging from 50 to 35 mmol/L (Barbiero et al. 1995; Hughes and Cidlowski 1999). The enhanced early apoptosis we observed in K^+ depleted trigeminal SGC following STS stimulation, in conjunction with the failure of K^+ depletion to increase apoptosis in non-stimulated cells, suggests that in trigeminal SGC, osmotically induced K^+ depletion may not be sufficient to induce apoptosis, but may interact with the K^+ loss of AVD to enhance early apoptosis.

Current results for STS-induced apoptosis suggest that the role of changing K^+ ion flux may be more important for the mitochondrial pathway than the death receptor pathway in trigeminal SGC. STS is often used as an apoptogenic inducer (Zhang et al. 2004) as it inhibits a broad spectrum of protein kinases affecting vital metabolic mitochondrial pathways (Ruegg and Burgess 1989; Yin et al. 2010). The mitochondrial pathway is one of the major executioner pathways, acting through permeabilization of the outer membrane and release of cytochrome c from mitochondria. In addition to this, mitochondria respond to proapoptotic stimuli by several

reactions, including a disturbance of the voltage homeostasis of the inner membrane (Cheng et al. 2011). Several theories have emerged over the years, including the potential role of K^+ in activation of caspases 1 and 3, DNA fragmentation, and activation of cytochrome c-apoptosome formation (Walev et al. 1995; Hughes et al. 1997; Cain and Freathy 2001). Studies have evaluated the counter regulation of K^+ transport by Bcl-2 and Bid in the mitochondrial membrane (Eliseev et al. 2003). It is also known that K^+ fluxes can affect the mitochondrial membrane potential and volume, but its association to Bcl-2 deregulation and cytochrome c release remains to be further clarified (Yu 2003).

A limitation of this study includes the fact that although our SGC culture procedure was a modification of previously published methods, and there was little evidence for the presence of non-fluorescent cells in our immunostaining, the choice of antibodies used for our western blots does not allow us to completely rule out the presence of fibroblasts or Schwann cells in our cultures. However, the removal of the fibrous outer capsule after ganglion harvesting likely reduced the probability of any fibroblast contamination. Similarly, the RVD procedure used for decreasing [K]ic could have allowed the efflux of other intracellular ions or molecules that might have played a role in apoptosis.

In summary, this study showed that hypo-osmotic shock induced a decrease in intracellular potassium in cultured trigeminal SGC and this decrease enhanced STS-induced early apoptosis associated with the mitochondrial pathway.

Acknowledgements

The authors of this paper would like to acknowledge Stephen Werre for statistical analysis and Thomas Rogers-Cotrone for technical support.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the Virginia-Maryland College of Veterinary Medicine under Internal Research Competition (IRC) [Grant 4-41588].

References

Barbiero G, Duranti F, Bonelli G, Amenta JS, Baccino FM. 1995. Intracellular ionic variations in the apoptotic death of L cells by inhibitors of cell cycle progression. Exp Cell Res. 217(2):410–418.

Bay V, Butt AM. 2012. Relationship between glial potassium regulation and axon excitability: a role for glial Kir4.1 channels. Glia. 60(4): 651–660.

Belzer V, Shraer N, Hanani M. 2010. Phenotypic changes in satellite glial cells in cultured trigeminal ganglia. Neuron Glia Biol. 6(4):237–243.

Benfenati V, Caprini M, Dovizio M, Mylonakou MN, Ferroni S, Ottersen OP, Amiry-Moghaddam M. 2011. An aquaporin-4/transient receptor potential vanilloid 4 (AQP4/TRPV4) complex is essential for cell-volume control in astrocytes. Proc Natl Acad Sci USA. 108(6):2563–2568.

Blum E, Procacci P, Conte V, Sartori P, Hanani M. 2017. Long term effects of lipopolysaccharide on satellite glial cells in mouse dorsal root ganglia. Exp Cell Res. 350(1):236–241.

Bortner CD, Cidlowski JA. 1998. A necessary role for cell shrinkage in apoptosis. Biochem Pharmacol. 56(12):1549–1559.

Bortner CD, Hughes FM, Jr, Cidlowski JA. 1997. A primary role for K + and Na + efflux in the activation of apoptosis. J Biol Chem. 272(51):32436–32442.

Bortner CD, Sifre MI, Cidlowski JA. 2007. New approaches for determining apoptotic volume decrease in cells. Methods Enzymol. 428: 161–181.

Burg ED, Remillard CV, Yuan JX. 2006. K + channels in apoptosis. J Membr Biol. 209(1):3–20.

Cain K, Freathy C. 2001. Liver toxicity and apoptosis: role of TGF-beta1, cytochrome c and the apoptosome. Toxicol Lett. 120(1–3):307–315.

Capuano A, De Corato A, Lisi L, Tringali G, Navarra P, Dello Russo C. 2009. Proinflammatory-activated trigeminal satellite cells promote neuronal sensitization: relevance for migraine pathology. Mol Pain. 5: 43.

Capuano A, Curro D, Dello Russo C, Tringali G, Pozzoli G, Di Trapani G, Navarra P. 2007. Nociceptin (1-13)NH2 inhibits stimulated calcitonin-gene-related-peptide release from primary cultures of rat trigeminal ganglia neurones . Cephalalgia. 27(8):868–876.

Cheng Y, Gulbins E, Siemen D. 2011. Activation of the permeability transition pore by Bax via inhibition of the mitochondrial BK channel. Cell Physiol Biochem. 27(3–4):191–200.

Cherkas PS, Huang TY, Pannicke T, Tal M, Reichenbach A, Hanani M. 2004. The effects of axotomy on neurons and satellite glial cells in mouse trigeminal ganglion. Pain. 110(1–2):290–298.

Chvátal A, Anděrová M, Hock M, Prajerová I, Neprašová H, Chvátal V, Kirchhoff F, Syková E. 2007. Three-dimensional confocal morphometry reveals structural changes in astrocyte morphology in situ. J Neurosci Res. 85(2):260–271.

Ducharme G, Newell EW, Pinto C, Schlichter LC. 2007. Small-conductance Cl- channels contribute to volume regulation and phagocytosis in microglia. Eur J Neurosci. 26(8):2119–2130.

Dufour S, Dufour P, Chever O, Vallee R, Amzica F. 2011. In vivo simultaneous intra- and extracellular potassium recordings using a microoptrode. J Neurosci Methods. 194(2):206–217.

Eliseev RA, Salter JD, Gunter KK, Gunter TE. 2003. Bcl-2 and tBid proteins counter-regulate mitochondrial potassium transport. Biochim Biophys Acta. 1604(1):1–5.

Elperin AB, Pore SA, Evans JM, Naditz AL, Light B. 2011. Swelling-induced Ca^{2+} influx and K^+ efflux in American alligator erythrocytes. J Membrane Biol. 240(1):1–12.

Grishin A, Ford H, Wang J, Li H, Salvador-Recatala V, Levitan ES, Zaks-Makhina E. 2005. Attenuation of apoptosis in enterocytes by blockade of potassium channels. Am J Physiol Gastr Liver Physiol. 289(5): G815–G821.

Hanani M, Blum E, Liu S, Peng L, Liang S. 2014. Satellite glial cells in dorsal root ganglia are activated in streptozotocin-treated rodents. J Cell Mol Med. 18(12):2367–2371.

Hanani M. 2005. Satellite glial cells in sensory ganglia: from form to function. Brain Res Brain Res Rev. 48(3):457–476.

Hanani M. 2010. Satellite glial cells: more than just 'rings around the neuron'. Neuron Glia Biol. 6(1):1–2.

Hoffmann EK, Lambert IH, Pedersen SF. 2009. Physiology of cell volume regulation in vertebrates. Physiol Rev. 89(1):193–277.

Hoffmann EK. 2011. Ion channels involved in cell volume regulation: effects on migration, proliferation, and programmed cell death in non-adherent EAT cells and adherent ELA cells . Cell Physiol Biochem. 28(6):1061–1078.

Hughes FM, Jr. Bortner CD, Purdy GD, Cidlowski JA. 1997. Intracellular K^+ suppresses the activation of apoptosis in lymphocytes. J Biol Chem. 272(48):30567–30576.

Hughes FM, Jr., Cidlowski JA. 1999. Potassium is a critical regulator of apoptotic enzymes *in vitro* and *in vivo*. Adv Enzyme Regul. 39: 157–171.

Jessen KR, Mirsky R. 2005. The origin and development of glial cells in peripheral nerves. Nat Rev Neurosci. 6(9):671–682.

Kozoriz MG, Church J, Ozog MA, Naus CC, Krebs C. 2010. Temporary sequestration of potassium by mitochondria in astrocytes. J Biol Chem. 285(41):31107–31119.

Krick S, Platoshyn O, Sweeney M, Mcdaniel SS, Zhang S, Rubin LJ, Yuan JX. 2002. Nitric oxide induces apoptosis by activating K^+ channels in pulmonary vascular smooth muscle cells. Am J Physiol Heart Circ Physiol. 282(1):H184–H193.

Lauf PK, Misri S, Chimote AA, Adragna NC. 2008. Apparent intermediate K conductance channel hyposmotic activation in human lens epithelial cells. Am J Physiol Cell Physiol. 294(3):C820–C832.

Laursen JC, Cairns BE, Dong XD, Kumar U, Somvanshi RK, Arendt-Nielsen L, Gazerani P. 2014. Glutamate dysregulation in the trigeminal ganglion: a novel mechanism for peripheral sensitization of the craniofacial region. Neuroscience. 256:23–35.

Model MA. 2014. Possible causes of apoptotic volume decrease: an attempt at quantitative review. Am J Physiol Cell Physiol. 306(5): C417–C424.

Mongin AA, Orlov SN. 2001. Mechanisms of cell volume regulation and possible nature of the cell volume sensor. Pathophysiology. 8(2): 77–88.

Montague JW, Bortner CD, Hughes FM, Jr, Cidlowski JA. 1999. A necessary role for reduced intracellular potassium during the DNA degradation phase of apoptosis. Steroids. 64(9):563–569.

Mullen RJ, Buck CR, Smith AM. 1992. NeuN, a neuronal specific nuclear protein in vertebrates. Development. 116(1):201–212.

Muyderman H, Sinclair J, Jardemark K, Hansson E, Nilsson M. 2001. Activation of beta-adrenoceptors opens calcium-activated potassium channels in astroglial cells. Neurochem Int. 38(3):269–276.

Nadeau H, Mckinney S, Anderson DJ, Lester HA. 2000. ROMK1 (Kir1.1) causes apoptosis and chronic silencing of hippocampal neurons. J Neurophysiol. 84(2):1062–1075.

Ohara PT, Vit JP, Bhargava A, Romero M, Sundberg C, Charles AC, Jasmin L. 2009. Gliopathic pain: when satellite glial cells go bad. Neuroscientist. 15(5):450–463.

Olsen ML, Sontheimer H. 2008. Functional implications for Kir4.1 channels in glial biology: from $K+$ buffering to cell differentiation. J Neurochem. 107(3):589–601.

Ordaz B, Vaca L, Franco R, Pasantes-Morales H. 2004. Volume changes and whole cell membrane currents activated during gradual osmolarity decrease in C6 glioma cells: contribution of two types of $K+$ channels. Am J Physiol Cell Physiol. 286(6):C1399–C1409.

Pasantes-Morales H, Lezama RA, Ramos-Mandujano G, Tuz KL. 2006. Mechanisms of cell volume regulation in hypo-osmolality. Am J Med. 119(7 Suppl 1):S4–S11.

Pasantes-Morales H, Murray RA, Lilja L, Moran J. 1994. Regulatory volume decrease in cultured astrocytes. I. Potassium- and chloride-activated permeability. Am J Physiol. 266(1):C165–C171.

Pasantes-Morales H. 1996. Volume regulation in brain cells: cellular and molecular mechanisms. Metab Brain Dis. 11(3):187–204.

Platoshyn O, Zhang S, Mcdaniel SS, Yuan JX. 2002. Cytochrome c activates K^+ channels before inducing apoptosis. Am J Physiol Cell Physiol. 283(4):C1298–1305.

Poulsen JN, Larsen F, Duroux M, Gazerani P. 2014. Primary culture of trigeminal satellite glial cells. A cell-based platform to study morphology and function of peripheral glia. Int J Physiol Pathophysiol Pharmacol. 6(1):1–12.

Procacci P, Magnaghi V, Pannese E. 2008. Perineuronal satellite cells in mouse spinal ganglia express the gap junction protein connexin43 throughout life with decline in old age. Brain Res Bull. 75(5):562–569.

Quesada O, Ordaz B, Morales-Mulia S, Pasantes-Morales H. 1999. Influence of Ca^{2+} on K^+ efflux during regulatory volume decrease in cultured astrocytes. J Neurosci Res. 57(3):350–358.

Rahman HM, Kumar Jha M, Suk K. 2016. Evolving insights into the pathophysiology of diabetic neuropathy: implications of malfunctioning glia and discovery of novel therapeutic targets. CPD. 22(6):738–757.

Remillard CV, Yuan JXJ. 2004. Activation of K^+ channels: an essential pathway in programmed cell death. Am J Physiol Lung Cell Mol Physiol. 286(1):L49–L67.

Rodriguez-Lozano FJ, Sanchez-Perez A, Moya-Vilaescusa MJ, Rodriguez-Lozano A, Saez-Yuguero MR. 2010. Neuropathic orofacial pain after dental implant placement: review of the literature and case report. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 109(49):8–12.

Ruegg UT, Burgess GM. 1989. Staurosporine, K-252 and UCN-01: potent but nonspecific inhibitors of protein kinases. Trends Pharmacol Sci. 10:218–220.

Sanchez-Olea R, Moran J, Martinez A, Pasantes-Morales H. 1993. Volume-activated Rb^+ transport in astrocytes in culture. Am J Physiol. 264(4 Pt 1):C836–C842.

Simard M, Nedergaard M. 2004. The neurobiology of glia in the context of water and ion homeostasis. Neuroscience. 129(4):877–896.

Takeda M, Takahashi M, Nasu M, Matsumoto S. 2011. Peripheral inflammation suppresses inward rectifying potassium currents of satellite glial cells in the trigeminal ganglia. Pain. 152(9):2147–2156.

Thompson GJ, Langlais C, Cain K, Conley EC, Cohen GM. 2001. Elevated extracellular [K+] inhibits death-receptor- and chemical-mediated apoptosis prior to caspase activation and cytochrome c release. Biochem J. 357(Pt 1):137–145.

Vit JP, Jasmin L, Bhargava A, Ohara PT. 2006. Satellite glial cells in the trigeminal ganglion as a determinant of orofacial neuropathic pain. Neuron Glia Biol. 2(4):247–257.

Vit JP, Ohara PT, Bhargava A, Kelley K, Jasmin L. 2008. Silencing the Kir4.1 potassium channel subunit in satellite glial cells of the rat trigeminal ganglion results in pain-like behavior in the absence of nerve injury. J Neurosci. 28(16):4161–4171.

Walev I, Reske K, Palmer M, Valeva A, Bhakdi S. 1995. Potassium-inhibited processing of IL-1 beta in human monocytes. Embo J. 14(8): 1607–1614.

Warwick RA, Hanani M. 2013. The contribution of satellite glial cells to chemotherapy-induced neuropathic pain. Eur J Pain. 17(4):571–580.

Weick M, Cherkas PS, Hartig W, Pannicke T, Uckermann O, Bringmann A, Tal M, Reichenbach A, Hanani M. 2003. P2 receptors in satellite glial cells in trigeminal ganglia of mice. Neuroscience.

Yin J, Howe J, Tan KSW. 2010. Staurosporine-induced programmed cell death in blastocystis occurs independently of caspases and cathepsins and is augmented by calpain inhibition. Microbiology. 156(Pt 5): 1284–2193.

Yu SP, Yeh CH, Sensi SL, Gwag BJ, Canzoniero LM, Farhangrazi ZS, Ying HS, Tian M, Dugan LL, Choi DW. 1997. Mediation of neuronal apoptosis by enhancement of outward potassium current. Science. 278(5335):114–117.

Yu SP. 2003. Regulation and critical role of potassium homeostasis in apoptosis. Prog Neurobiol. 70(4):363–386.

Zhang XD, Gillespie SK, Hersey P. 2004. Staurosporine induces apoptosis of melanoma by both caspase-dependent and -independent apoptotic pathways. Mol Cancer Ther. 3(2):187–197.