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Expression of Protease-Activated Receptor 3 in the Trigeminal and Dorsal Root Ganglion of adult rats using immunohistochemistry

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Abstract

Protease-activated receptors (PARs) have been implicated in a variety of human physiological and pathophysiological processes, including; nociception, neuroinflammation, neuroprotective and neurodegenerative pathways (Peters and Henry, 2009; Adams *et al*. 2011). PAR3 was discovered as a second thrombin receptor in 1997, and since then few papers have been published which assess its expression in the nervous system (Ishihara *et al*. 1997). This paper investigates the expression of PAR3 in sensory neurons of the trigeminal ganglion (TG) and dorsal root ganglion (DRG) of naïve adult rats. Immunohistochemistry showed PAR3 to be strongly expressed in non-neuronal cells of both the TG and DRG. Further analysis showed these cells to be non-myelinating satellite cells and myelinating Schwann cells, both showing strong co-expression of PAR3 and S-100. The expression of PAR3 in sensory neurons proved too difficult to accurately determine. This study concludes that further research, using multiple methods of analysis, is required to accurately describe the distribution and quantification of PAR3 positive sensory neurons within the TG and DRG of naïve adult rats. This study does however show strong PAR3 expression in non-neuronal satellite cells and Schwann cells of both the TG and DRG of naïve adult rats.

Introduction

Protease-activated receptors (PARs) are a family of four seven-transmembrane G protein-coupled receptors (GPCR), activated by protease cleavage (Ramachandran *et al*. 2012). PAR1, PAR3 and PAR4 are preferentially activated by thrombin, whereas PAR2 is primarily activated by trypsin (García *et al*. 2010). Cleavage of the extracellular N-terminal domain results in the exposure of a tethered peptide ligand, which binds and activates the receptor (Vellani *et al*. 2010). This novel mechanism of activation is in contrast with other GPCRs; which, unlike PARs, can be activated by soluble ligand binding *in vivo* (Adams, 2011). PARs have been implicated in a variety of human physiological as well as pathophysiological processes (Peters and Henry, 2009), with *in vitro* and *in vivo* experimental evidence linking PARs to processes involved in the nervous system; including nociception, neuroinflammation, neuroprotective and neurodegenerative pathways (Adams *et al*. 2011). Even though strong experimental evidence link PARs with functional processes in the nervous system, their precise expression in sensory neurons is still relatively unknown. This gap in knowledge is particularly evident when reviewing literature relating to the expression of PAR3 in the sensory neurones of the TG, with relatively few studies confirming its expression in the DRG.

In 1998, Gill and colleagues first reported a functional response to thrombin in sensory neurons of the dorsal root ganglion (DRG) in rat models, where thrombin was shown to reverse stellation in neuroblastoma cell lines and rat astrocytes, respectively, and induce neurite retraction. However it wasn't until 2005 when Zhu and colleagues showed mRNA for all four PAR subtypes are expressed in sensory neurons of the DRG, using rat models. In particular, PAR3 mRNA was shown to be expressed in 40.9± 1.3% of the total neuron population of the DRG, based on scatter plot analysis of hybridisation signals (Zhu *et al*. 2005). Zhu *et al.* (2005), using size-frequency analysis demonstrated PAR3 mRNA was expressed across all neuron sizes in the DRG with no preferential expression. This was the first study, in adult rat DRG, to report the precise expression of PAR-3 mRNA by *in situ* hybridisation. Their work found high co-localisation between CGRP and PAR3 mRNA, suggesting a role for PAR3 in nociceptive mechanisms in the periphery. Many studies in platelets have shown PAR3 acts as a cofactor for PAR4 following activation by thrombin (Nakanishi-Matsui *et al*. 2000). Interestingly however, Zhu and colleagues (2005) found no PAR4 expressing neurons in the DRG, with PAR4 expression shown to be in non-neuronal cells such as satellite cells. The author suggests that the activation of PAR3 in the nervous system may be different from that in platelets, with PAR3 acting independently of PAR4.

More recent work by Vellani and colleagues (2010) used experimental techniques, such as *in situ* hybridisation and immunohistochemistry, to quantify PAR expression in sensory neurones of the DRG of adult mice. Although this study was conducted using a different species, Vellani *et al.* (2010) found few qualitative or quantitative differences between PAR expression in rat and mice DRG. PAR3 was shown to be strongly expressed in 42% of neurones, using immunohistochemistry, predominantly in small neurons (Vellani *et al*. 2010). However a significant reduction in the proportions of neurones expressing PAR3 was found when the authors used functional expression analysis, compared with the histological data. Experimentally induced desensitisation of PAR1 and PAR4 largely ablated neuronal response to thrombin suggesting PAR3 is either not functional in sensory neurones or that it can

only act in combination with other PARs, a hypothesis which has been noted in other studies (Nakanishi-Matsui *et al*. 2000; McLaughlin *et al*. 2007; Vellani *et* al. 2010). The authors suggest this was the first study to assess the localisation of functional thrombin activated PARs in sensory neurons of the DRG.

The expression of PAR2 in trigeminal neurons (Dinh *et al*. 2005) has been assessed however, to the best of my knowledge there are no studies assessing the expression of PAR3 in sensory neurones of the TG.

The aim of this study is to describe the distribution and quantification of PAR3 in sensory neurons of the TG and DRG of naïve adult rats (as a model for human expression) using immunohistochemistry. Expression of PAR3 was also investigated in non-neuronal cells of the TG and DRG, such as satellite and Schwann cells.

Method and Materials

Animal/Tissue Preparation

Experiments were carried out on two adult naïve male Wistar rats, with a body weight range between 220-250g (Charles River, Margate, UK). All procedures were conducted in accordance with the UK 1986 Animals (Scientific Procedures) Act. The animals were on a 12:12 hour light/dark cycle, housed in a temperature controlled room. Food and water was available *ad libitum*. Following Schedule 1 killing, the animals were transcardially perfused with 0.01 M phosphate-buffered saline (PBS, pH 7.4), followed by 4% paraformaldehyde in 0.2 M phosphate buffer (pH 7.4).

Trigeminal and Dorsal Root Ganglion (lumbar 4 and 5) were rapidly dissected, postfixed for 2 hours and subsequently stored overnight in 20% sucrose (cryoprotection). The tissue sections were blocked in OCT mounting media (Tissue-Tek), snap-frozen in liquid nitrogen and stored at -80°C until required. Tissue sections, 8µm, were cut on a Leica cryostat (CM1100), serially thaw-mounted onto SuperFrost Plus slides (VWR) and stored in 30% sucrose at -20°C until use.

It should be noted that the tissue sections used in this study were provided, pre-mounted, following this procedure.

Immunohistochemistry Protocol

All stages explained were conducted at room temperature, unless otherwise stated. After each stage which required the removal of liquids the slides were briefly air dried.

The slides were placed in a Coplin jar and washed in phosphate buffered saline (PBS 0.01 M, pH 7.4) on an orbital shaker (160 MOT/1 minute), for 3X10 minute washes. PAP Pen (Sigma) was applied around tissue sections. To decrease the occurrence of non-specific binding the tissue was incubated, in a humidity chamber, with 10% donkey serum (PBS, 0.2% Triton X100, 0.1% sodium azide, 500µl/slide) for 1 hour. The slides were then washed; three 10 min PBS washes as before. The tissue was then incubated with primary antibodies (Goat anti-Rat PAR-3 [M20], Santa Cruz Biotechnology, CA, USA at 1:100; S-100, gift from Peninsular Medical

School at 1:100), diluted in 5% donkey serum (PBS, 0.2 Triton X100, 0.1% sodium azide) and left overnight. PAR-3 (M20) was previously characterised by Wang *et al*. (2004). S-100 was used to identify specific cell types expressing PAR3. The slides were washed (3X10 minutes) in PBS as before and incubated with secondary antibodies (Donkey anti-Goat AlexaFluor-555 [red] at 10µl/ml; Donkey anti-Rabbit AlexaFluor-488 [green] at 1:100) diluted in 5% donkey serum (PBS, 0.2 Triton X100, 0.1% sodium azide) for 3 hours, protected from light. The slides were washed 3X10 minutes in PBS as before, incubated with DAPI (100ng/ml in PBS) for 15 minutes and washed again (1X10 minute PBS wash as before). 2 drops of FluorSave were applied to cover slips and placed over the tissue sections. After being left to dry overnight, protected from light, the slides were sealed with clear nail varnish and stored in a refrigerator at 4°C in the dark.

The images were acquired using a Nikon Eclipse 80i epifluorescence microscope equipped with a Nikon DS-Qi1Mc camera using NIS-Elements Software (BR 3.2, Nikon). Images were taken at X10, X20 and X40 magnification.

Results

PAR3 Expression in TG and DRG neurons

PAR3 immunoreactivity (IR) was detected in both TG (Fig. 1) and DRG (Fig. 2) tissue sections from naïve adult rats. Its precise expression and localisation in neurons however could not be determined due to its strong expression in satellite cells surrounding the neuron cell bodies of both the TG and DRG.

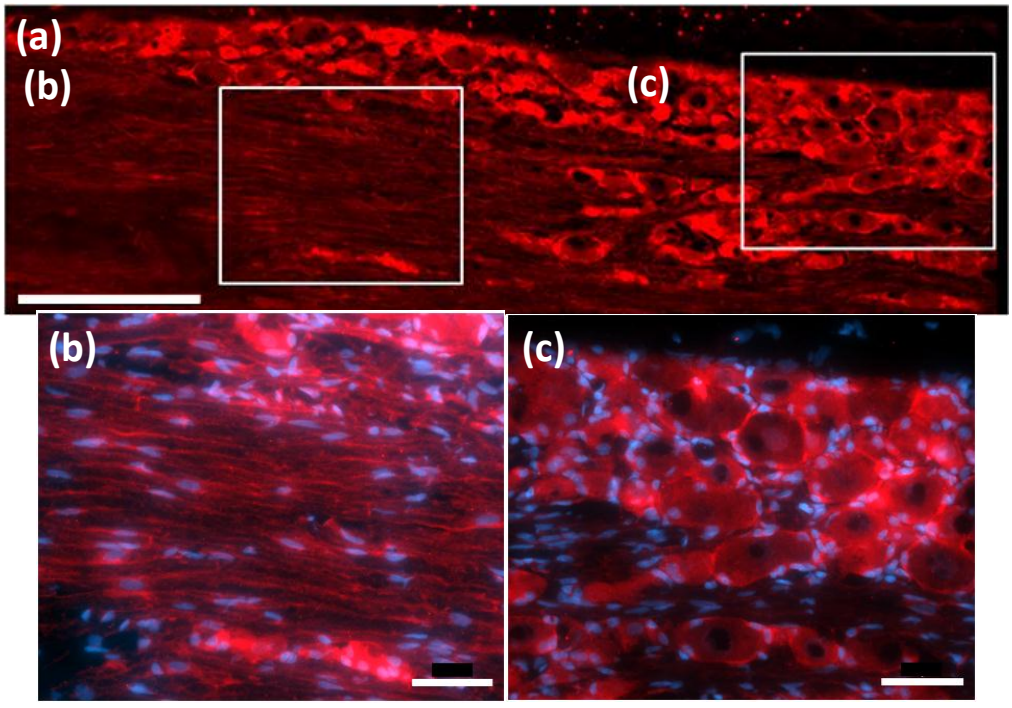

Figure 1: Localisation of PAR3 in the TG of naïve adult rats. PAR3 receptors appear red (a, b and c) and cell nuclei appear blue (b and c). (a) Photomicrograph of section labelled for PAR3 receptor (X10 magnification). (b) Photomicrograph of axons double-labelled for PAR3 and DAPI (X40 magnification). (c) Photomicrograph of sensory neurons double-labelled for PAR3 and DAPI (X40 magnification). Scale bar (a) = 200µm, (b and c) = 50µm.

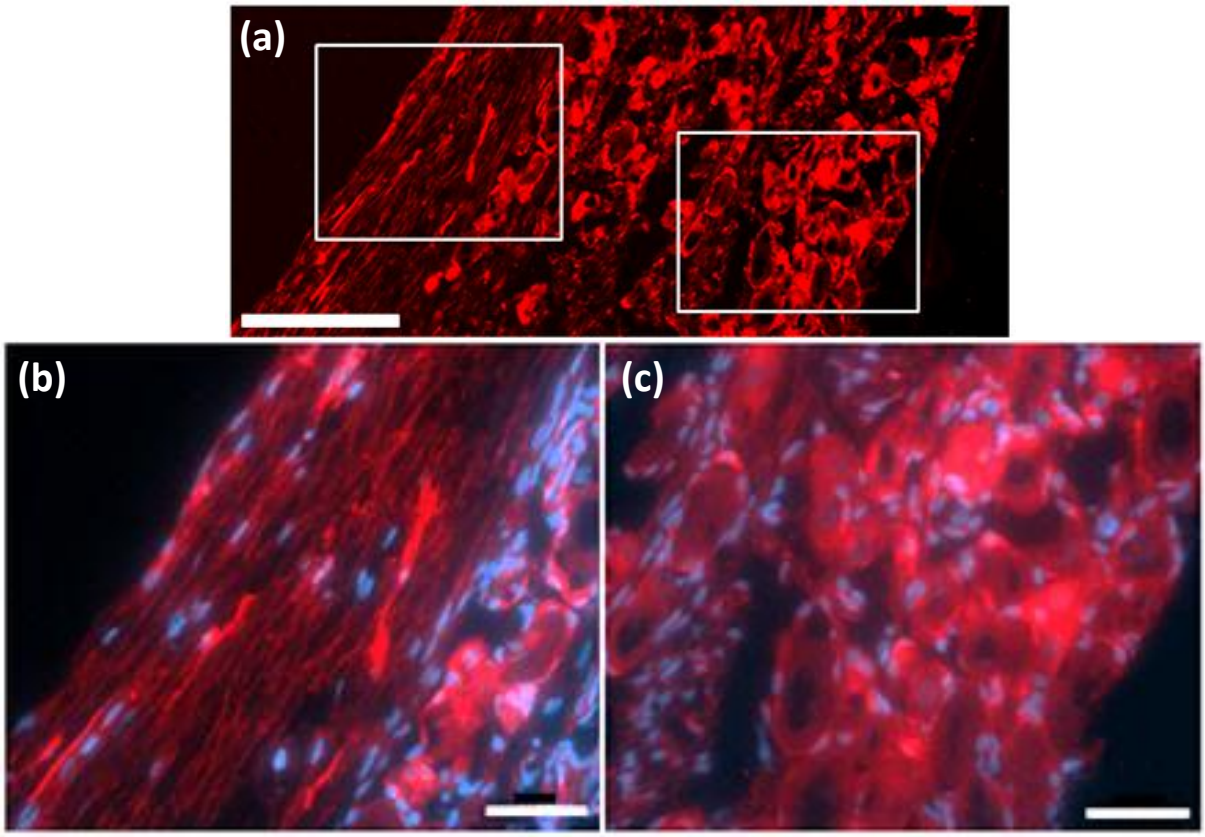

Figure 2: Localisation of PAR3 in L4/5 DRG of naïve adult rats. PAR3 receptors appear red (a, b and c) and cell nuclei appear blue (b and c). (a) Photomicrograph of section labelled for PAR3 receptor (X10 magnification). (b) Photomicrograph of axons double-labelled for PAR3 and DAPI (X40 magnification). (c) Photomicrograph of sensory neurons double-labelled for PAR3 and DAPI (X40 magnification). Scale bar (a) = 200µm, (b and c) = 50µm.

PAR3 Expression in Non-Neuronal cells of the TG and DRG: Satellite Cells

PAR3 immunoreactivity was found in satellite cells of both the TG (Fig. 1c) and DRG (Fig. 2c) of naïve adult rats. Primary staining appeared to be in non-neuronal cells; shown in 'ring like' structures surrounding sensory neuron cell bodies and associated with satellite cell nuclei in both the TG and DRG. A primary antibody, S-100, was used to identify satellite cells within the TG and DRG. The S-100 phenotypic marker appears green (Fig. 3a and d), the PAR3 receptor in red (Fig. 3b and e) and overlapping expression in yellow (Fig. 3c and f). Areas of strong PAR3 and S-100 co-expression in the satellite cells of both the DRG and TG can be clearly seen in the photomicrographs taken (Fig 5a and c). PAR3-immunoreactivity appears to be found in both the cytoplasm and the plasma membrane. PAR3 was found to be expressed in all satellite cells observed, surrounding neurons in both the TG and DRG.

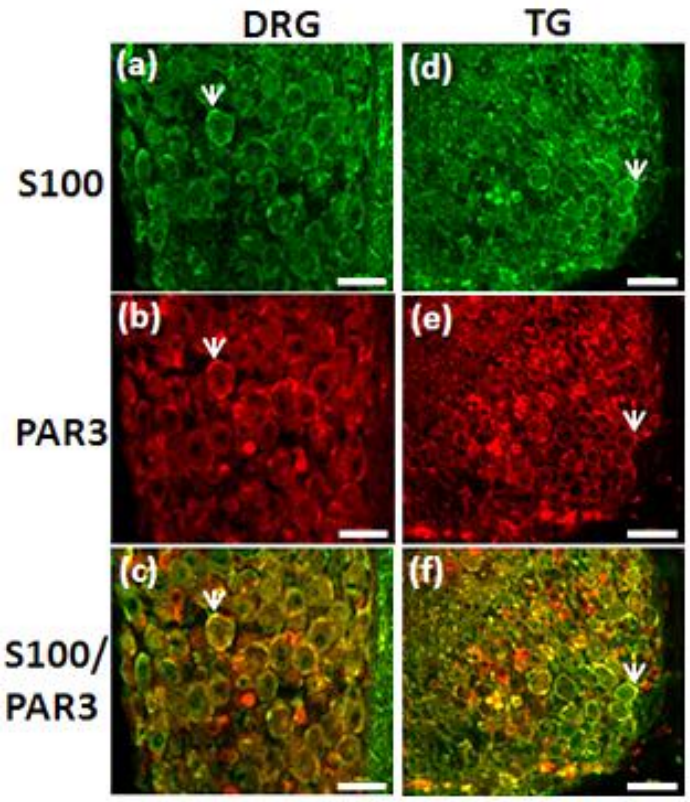

Figure 3: Co-localisation of S-100 and PAR3 in L4/5 DRG (a, b and c) and TG (d, e and f) sensory neurones. Satellite cells appear green, PAR3 receptors appear red and overlapping of these two markers appears yellow. (a and d) Photomicrographs of section labelled for S-100. (b and e) Photomicrographs of section labelled for PAR3 receptor. (c and f) Photomicrographs of section double-labelled for S-100 and PAR3 receptor. Arrow shows example of co-expression. Images taken at X20 magnification. Scale bar = 100µm.

PAR3 Expression in Non-Neuronal cells of the TG and DRG: Schwann Cells

PAR3 immunoreactivity was found in the axonal regions of both the TG and DRG (Fig. 4). Co-staining with S-100 (Fig. 4a and d), a phenotypic marker of Schwann cells, and PAR3 (Fig. 4b and e) showed strong evidence of overlapping expression (Fig. 4c and f). This co-expression can be seen clearly in the photomicrographs of both the TG and DRG (Fig. 5b and d). Axonal expression of PAR3 is specifically localised to Schwann cells. PAR3-immunoreactivity appears to be in both the plasma membrane and cytoplasm of axonal Schwann cells of the TG and DRG.

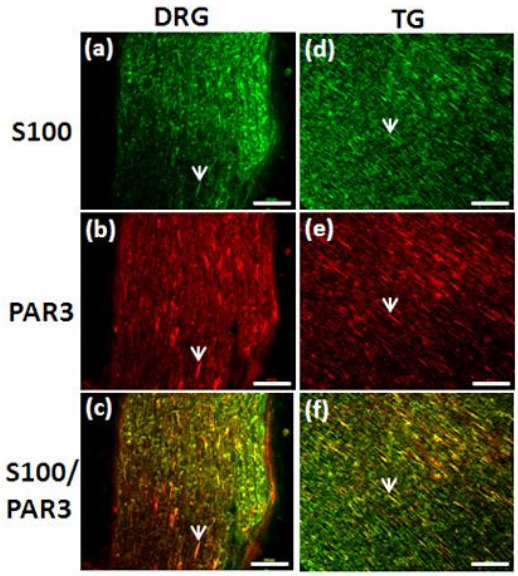

Figure 4: Co-localisation of S-100 and PAR3 in L4/5 DRG (a, b and c) and TG (d, e and f) axons. Satellite and Schwann cells appear green, PAR3 receptors appear red and overlapping of these two markers appears yellow. (a and d) Photomicrographs of section labelled for S-100. (b and e) Photomicrographs of section labelled for PAR3 receptor. (c and f) Photomicrographs of section double-labelled for S-100 and PAR3 receptor. Arrow shows example of co-expression. Images taken at X20 magnification. Scale bar = 100µm.

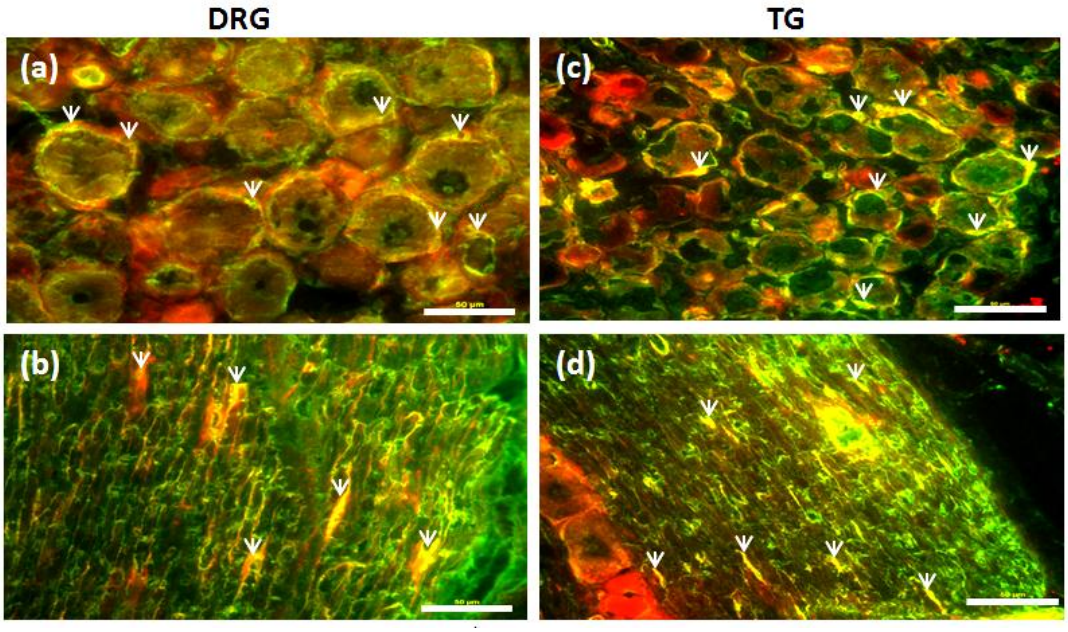

Figure 5: Photomicrographs showing co-localisation of S-100 and PAR3 in L4/5 DRG (a and b) and TG (c and d) of sensory neurons (a and c) and axons (b and d). PAR3 receptors appear red, satellite and Schwann cells appear green, overlap between these two markers appears yellow. Arrows show examples of co-expression. Images taken at X40 magnification. Scale bar = 50µm.

Discussion

This study used immunohistochemistry to describe the distribution and quantification of PAR3 in the TG and DRG of naïve adult rats. This study found clear evidence for the expression of PAR3 in satellite and Schwann glial cell types of both the TG and DRG. However it was not possible to accurately determine PAR3 expression in sensory neurons, of these regions.

PAR3 Expression in Glial Cells

A preliminary experiment, using immunohistochemistry, showed PAR3 expression to be strong in the areas directly surrounding neuron cell bodies, shown to be non-myelinating satellite cells, and in specific regions within the axons, shown to be myelinating Schwann cells (Figs 1 and 2). Both satellite cells and Schwann cells express S100β (Woodhoo *et al*. 2004) and therefore this phenotypic stain was used to distinguish both cell types *in vitro* immunohistochemical analysis. Co-staining of PAR3 receptors and S-100 resulted in strong overlapping-expression in both satellite cells and Schwann cells of the TG and DRG of naïve adult rats (Figs 3, 4 and 5).

Satellite Cells

PAR3 was found to have strong co-expression with S-100 in satellite cells of both the TG and DRG in naïve adult rats (Figs 3 and 5). A previous study by Zhu *et al*. (2005) found PAR4 mRNA expression was seen in non-neuronal cells, such as satellite cells, however made no comment regarding PAR3. PAR2 and PAR4 expression have been described in satellite cells of both the skeletal muscle and DRG of mature rats, respectively (Zhu *et al*. 2005; Duchesne *et al*. 2011). Literature covering PAR3 expression in satellite cells of the TG and DRG, to the best of my knowledge, is not available. This would therefore be the first study to describe the expression of PAR3 in satellite cells of the TG and DRG in naïve adult rats.

Schwann Cells

PAR3 was found to have strong co-expression with S-100 in Schwann cells of both the TG and DRG in naïve adult rats. To the best of my knowledge there are no studies which have assessed PAR3 expression in Schwann cells. This study would therefore be the first to describe the expression of PAR3 in Schwann cells of both the TG and DRG of naïve adult rats.

PAR3 Expression in Sensory Neurons

Determining the expression of PAR3 in sensory neurons of the TG and DRG, in this study, was not possible. PAR3 was found to be strongly expressed in the satellite cells surrounding the sensory neuron cell bodies. As this study used an epifluorescence microscope, only a single focal plane though the tissue sample was able to be viewed. Due to the close distribution of satellite cells around the neuron cell bodies, neurons which appeared positive may have actually been viewed through an overlying positive satellite cell. It was therefore not possible to describe with certainty, using this technique, whether a neuron was PAR3 positive.

A previous study conducted by Zhu and colleagues (2005) also found the expression of PAR protein in neurons to be equivocal, finding PAR4 expression in glial cells impossible to distinguish from neuronal staining. Zhu *et al*. (2005) did however find PAR3 mRNA expression in sensory neurons of the DRG of naïve adult rats, with distribution shown to be irrespective of neuron size. These findings are inconsistent with a study conducted by Vellani *et al.* (2010) in which immunohistochemistry found

PAR3 expression was seen predominantly in small neurons of adult mice DRG. Although these studies were carried out on different species, Vellani *et al.* (2010) found few qualitative or quantitative differences between PAR expression in rat and mice DRG. Vellani *et al*. (2010) found that the PAR2 antibody used in their study was non-specific on Western blots, resulting in the removal of PAR2 immunohistochemistry analysis from the paper. The antibody used in this study may also have had questionable specificity, resulting potentially in non-uniform staining; as found on some tissue samples of this study. An antibody specificity test, such as conducting the immunohistochemistry procedure with only the secondary antibody would have helped to determine this. Western blotting could have also been used to ascertain the specificity of the antibodies being used.

The use of multiple analytical techniques, such as *in situ* hybridisation, immunohistochemistry and methods ascertaining functional expression as used by Vellani *et al*. (2010) may have proved advantageous. As in other studies the use of different microscopy techniques, such as confocal microscopy, may have also aided the precise assessment of PAR3 expression in sensory neurons (Vellani *et al*. 2010; Shavit *et al*. 2011; Sokolova *et al*. 2012). This technique would have proved particularly useful when determining neuronal PAR3 expression.

This study may also have benefited from an increased number of sample animals. This would help create a wider, more accurate assessment of PAR3 expressing cells and allowed for statistical analysis. To the best of my knowledge, no studies assessing the expression of PAR3 have been conducted in the TG.

Functional Implications

Glial-Neuron Interaction

Glial cells have been shown to actively interact with neurons, contributing to neuronal function, neuroarchitecture, as well as maintenance of physiological homeostasis; under physiological and pathophysiological conditions (Eulenburg and Gomeza, 2010; Gwak *et al*. 2011). This glial-neuron interaction can directly affect neuron function and has been implicated in the development and maintenance of pathological pain, such as seen following peripheral nerve and spinal cord injury (Nesic *et al*. 2005; Watkins *et al*. 2007; Gwak and Hulsebosch, 2009).

In recent years evidence has accrued which shows the immune system can directly alter neuronal function (Watkins *et al*. 2007). Both satellite cells and Schwann cells of the DRG have been found to be immune-like glial cells, comprising the resident immunocompetent cells of the peripheral nervous system; providing immune surveillance of neurons (Moalem and Tracy, 2006; Myers *et al*. 2006; Watkins *et al*. 2007). Both cell types are capable of producing what are considered classical immune substances, such as proinflammatory cytokines and chemoattractants (Watkins and Maier, 2002) that can interact with both CNS and PNS neurons.

Satellite cells

In recent years many studies have assessed the interactive role between satellite cells and neurons. Satellite cells of the DRG and TG are connected by gap junctions and supply neurons with nutrients as well as buffer the extracellular ion and neurotransmitter levels (Sakuma *et al*. 2001; Ren and Dubner, 2010). Following nerve injury, invasion of the DRG by macrophages and T cells occurs, and macrophages move across the satellite cells forming perineuronal rings around large

and medium sized neurons (Hu and McLachlan, 2002; Hu *et al*. 2007; Ren and Dubner, 2010). Their presence increases satellite cell and neuron interaction by paracrine signalling, known to be a major mechanism which underlies peripheral sensitization in the DRG (Thalakoti *et al.* 2007; Miller *et al*. 2009; Ren and Dubner, 2010). Recent studies suggest, following injury, satellite cells of both the TG and DRG show increased gap junction coupling (Dublin and Hanani, 2007; Durham and Garrett, 2010). More interestingly, in the absence of noxious stimulation, no gap junction coupling between either DRG neurons or neurons and satellite cells can be seen (Ledda *et al*. 2009). Therefore following peripheral noxious stimulation, increased satellite cell to satellite cell and satellite cell to neuronal interaction occurs, enhancing primary afferent input and increased neuronal excitability (Ren and Dubner, 2010). This mechanism of increased communication can also affect neurons and satellite cells in the surrounding area (Thalakoti *et al*. 2007).

TG satellite cells also promote neuron sensitisation though autocrine/paracrine stimulation, creating a positive feedback loop (Capuano *et al*. 2009). A major mediator of TG nociceptive signalling is calcitonin gene-related peptide (CGRP) (Corato *et al*. 2011). CGRP released by neurons induces IL-1β (Interleukin-1 beta) production by satellite cells, which in turn activates the satellite cell COX2 (Cyclooxygenase-2) pathway, increasing PGE_2 (Prostaglandin E2) production (Capuano *et al*. 2009; Ren and Dubner, 2010). NO (Nitric oxide) production by TG neurons also increases PGE_2 production possibly though the activation of COX1 (Cyclooxygenase-1) in satellite cells (Capuano *et al*. 2009; Ren and Dubner, 2010). PGE_2 stimulates CGRP production in TG neurons increasing sensitisation of nociceptors (Miller *et al*. 2009; Ren and Dubner, 2010).

Sensitisation of sensory neurons of the DRG, following injury, can also occur via the increased levels of pro- and anti-inflammatory cytokines and chemokines (Ren and Dubner, 2010). These act upon their specific receptor on DRG neurons, to generate ectopic discharges and enhance primary afferent input to the dorsal horn; by coupling to specific transient receptor potentials and sodium channels (Ren and Dubner, 2010). Reviewed by Miller *et al* (2009).

Schwann cells

Surprisingly, unlike satellite cells, few papers have been published which assess the interaction between Schwann cells and neuron function. Schwann cells have however been implicated in the recruitment of macrophages, resulting in nociceptor sensitisation following injury. TNF-α is released by Schwann cells following nerve injury which is known to induce matrix metalloproteinase-9 (MMP-9) (Shubayev *et al*. 2006; Ren and Dubner, 2010). MMP-9 increases macrophage migration to injury site via mechanisms associated with the breakdown of the blood-brain barrier (Shubayev *et al.* 2006; Ren and Dubner, 2010).

As explained satellite and Schwann cell types have been implicated as contributors to neuropathic pain (Watkins *et al*. 2007). An increased understanding of the glial-neuron interaction may lead to the discovery of novel therapeutic targets for the treatment of neuropathic pain (Ledeboer *et al*. 2006; Myers *et al*. 2006; Ledeboer *et al*. 2007; Bernardo and Minghetti, 2008; Capuano *et al*. 2009; Vergnolle, 2009).

Thrombin and PAR3 in the nervous system

Thrombin is the primary activator of PAR1, PAR3 and PAR4. Thrombin activated PARs have been shown to have multiple roles in the central (CNS) and peripheral nervous system (PNS) these include; nociceptive signalling interaction, neurite out-growth and neuronal death regulation as well as microglia activation (García *et al*. 2010). The majority of the literature, however, specifically covers PAR1 and PAR4, with few studies specifically assessing PAR3. This is likely due to the order of PAR discovery and the general view that PAR3 acts as a cofactor for PAR4, although this has recently been questioned (Zhu *et al*. 2005; Ostrowsaka and Reiser, 2008). There is currently a lack of information on the effect of PAR3 on synaptic function and behaviour (Adams *et al*. 2011; Almonte and Sweatt, 2011). The current understanding of thrombin and PARs in pain mechanisms are reviewed by García and colleagues (2010). Both PAR1 and PAR4, of the thrombin activated PARs, have been implicated in having a potentiating effect on TRPV1 (Transient Receptor Potential Vanilloid, the noxious thermal transducer protein) activity (Vellani *et al*. 2010).

Although PARs have been implicated in many roles within the nervous system, the functional role of PAR3 remains generally unclear (Ostrowska and Reiser, 2008). As shown glial neuron interaction can result in increased nociception following injury. Thrombin's release is known to occur following cell damage, and PARs have been strongly implicated in the response to pain in both the CNS and PNS (García *et al*. 2010). It is therefore possible that although mechanistic links have yet to be made, PAR3 may play a role in these pathways; i.e. PAR3 mediated glial activation with subsequent neuron-glial interaction.

Conclusion

Of the PAR family, PAR3 remains the most elusive, with its functional role relatively unknown (Ostrowsaka and Reiser, 2008).

To the best of my knowledge, there are no studies which specifically assess the potential role of PAR3, or any PARs, in interactions between neurons and glial cells. The unequivocal evidence of interaction between these two cell types, as well as the knowledge PAR3 is activated by thrombin; a serine protease released following injury, suggests that there is the possibility of an interactive role of PAR3 expressing glial cells with neurons of the DRG and TG. Depending on PAR3 expression within neurons, this receptor may also play a major role in other mechanisms within the nervous system; such as nociception, inflammation and neurodegeneration.

In summary this study found PAR3 to be strongly expressed in non-neuronal glial cells (satellite cells and Schwann cells) of the TG and DRG of naïve adult rats. However the expression of PAR3 in sensory neurons of both the TG and DRG proved inconclusive.

Acknowledgements

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