**ORIGINAL ARTICLE**

**Vascular changes associated with spinal root avulsion injury**

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**Abstract**

This paper has investigated the hypothesis that spinal root avulsion (SRA) injury produces alterations in blood flow that contribute to avulsion injury induced pain-like behaviour in rodents. Photoplethysmography (PPG) is an established way of assessing blood flow in the central nervous system (CNS) and laser Doppler flowmetry (LDF) is the most widely used technique for measuring tissue perfusion. Using an established model of SRA injury that produces mechanical hypersensitivity, the PPG and LDF signals were recorded in animals 2 weeks post-injury and compared to naive recordings. PPG and LDF measurements were assessed on the ipsilateral and contralateral sides of the spinal cord rostral and caudal to the avulsion injury and at the level of the injury. Two weeks after injury, a time when vascular blood vessel endothelial markers are known to be decreased, no significant changes were seen in the spinal cord blood flow (SCBF) above, at, or below the injury site or when comparing the ipsilateral vs. contralateral side. Assessment of oxygenation levels again revealed no significant differences between naive and spinal root injured animals along the rostrocaudal axis (i.e., above, at, and below the site of injury or its equivalent on the contralateral side). From these experiments it is concluded that SRA does not significantly alter blood flow or tissue oxygen levels and so ischemia may play a less prominent role in avulsion injury induced pain.

**Keywords**

Avulsion, doppler flowmetry, ischemia, pain, photoplethysmography, spinal cord

**Introduction**

Trauma to the spinal roots of the adult brachial or lumbosacral plexi result in limb paralysis, muscular atrophy, sensory impairment, autonomic dysfunction, and chronic pain (Havton and Carlstedt 2009). They most often occur as a result of road traffic accidents or violent acts and create lifelong disability for those suffering the injury. It affects over 1000 patients in the UK annually. Historically, these injuries have been associated with poor clinical outcome and patients most often complain about the severe, intractable pain (Berman et al. 1998). Pharmacological management of the pain is often unsatisfactory and is based on empirical measures (Carvalho et al. 1997; Gordon and Love 2004) rather than a mechanism-based approach.

The mechanisms of neuropathic pain associated with avulsion injury are incompletely understood. Several mechanisms may contribute to the pains. Spontaneous pain may arise due to deafferentation of spinal cord neurons since recordings of neurons within deafferented human and rodent spinal cord models of avulsion injury reveal spontaneous, ongoing activity (Dalal et al. 1999; Guenot et al. 2003) perhaps caused by disinhibition due to neuronal cell death (Chew et al. 2011, 2013). However, at least a third of patients report allodynia and hyperalgesia with avulsion injury and this can be ameliorated by anaesthetic blockade of non-avulsed roots (Htut et al. 2006; Bertelli and Ghizoni 2008) suggesting that non-injured afferents contribute to the pain.

Avulsion injuries also damage the spinal cord resulting in glial changes (Bigbee et al. 2007; Chew et al. 2011, 2013) and it is now well recognised that glia are important contributors to the generation and maintenance of neuropathic pain conditions (Inoue and Tsuda 2009; Chew et al. 2013; Iwasaki et al. 2013). Another possibility is that the pain is driven by lack of adequate vascular supply and that this leads to ischemic cell death. Dorsal root or spinal root injury produces a loss of vascular integrity as assessed by staining of the rat endothelium in both the injured and adjacent spinal segments (Chew et al. 2011, 2013). Neuronal cell counts indicate that approximately 10% dorsal horn cells and 25–40% of motoneurons are lost in avulsed cord (Bergerot et al. 2004; Chew et al. 2011, 2013). However, it is unclear whether the change in vascular endothelial lining staining is
causal to the cell death or merely represents down regulation of the epitope. Therefore, other methods are required to confirm the implication of this observation.

One method of studying changes in blood flow after injury is photoplethysmography (PPG) in which a small fibre optic probe measures the changes in arterial pulsations in the spinal cord (Phillips et al. 2009). It has been used to detect changes in blood flow following spinal cord injury (Phillips et al. 2009) and further advances in probe development now allow estimation of oxygen saturation and blood volume in the tissue (Phillips et al. 2013). Hence, it presents an ideal method to study such changes after avulsion injury. This study has tested the hypothesis that spinal root avulsion (SRA) decreases blood flow in the spinal cord and that this contributes to spinal root evoked pain.

Material and methods

Surgery

All experimental procedures were carried out in accordance with the UK Scientific Procedures Act (1986). Twenty-five male Wistar rats (150–175 g) were anaesthetised with 4% isoflurane in 1.5 l/min of O2. All animals underwent a week of behavioural training to mechanical stimuli using an automated plantar anaesthesiometer (Ugo Basile, Varese, Italy). Following this period, animals were split into two groups: Group 1 = SRA (N = 16) and Group 2 = naive control rats (N = 9). Under sterile conditions, the L5 spinal nerve was exposed and avulsed using exactly the same methods as described by Chew et al. (2013). These animals recovered uneventfully and then underwent behavioural assessment for the development of mechanical hypersensitivity over the following 14 days using an automated plantar anaesthesiometer (Ugo Basile).

Photoplethysmography and laser flowmetry

On day 14 the terminal experiment was conducted; rats of both groups were deeply re-anaesthetised with isoflurane and a laminectomy of the lumbar spinal cord (T13–L3 vertebrae) performed to expose the lumbar enlargement. The spinal cord was stabilised using vertebral clamps and a fibre optic probe (PPG followed by Doppler) was lowered onto the surface of the spinal cord (dura intact) as previously described (Phillips et al. 2009, 2013) at the level of the avulsion (identified by a missing root). In control animals, the L5 root was identified as the largest root caudal in the lumbar enlargement region. The amplitudes of the PPG and Doppler flow signals were then measured at the lesion site for 3 min. Measurements were subsequently made at a similar position to the root entry zone (REZ) on the contralateral side of the cord as well as rostrally (L4 REZ) and caudally (L5 REZ) to the avulsion lesion site on both the ipsilateral and contralateral sides of the cord. Data was collected on a laptop for subsequent offline analysis (Phillips et al. 2013). Following the last recordings, animals were overdosed with terminal anaesthetic and perfused with 0.1 M phosphate buffer (pH 7.4) followed by 4% paraformaldehyde. Post-mortem dissection confirmed the completeness of the lesion and then the L4–L5 spinal cord region was removed, post-fixed for 2 h, and then stored overnight in 30% phosphate buffered saline (PBS).

Histology and microscopy analysis

Fourteen micron thick transverse sections of the spinal cord were cut on a cryostat and thaw mounted onto Superfrost™ slides. Slides were processed for immunofluorescence staining as described in detail previously (Chew et al. 2011). Briefly, slides were incubated in 10% donkey serum before staining with mouse anti-RECA antibody (1:200; Serotec, Oxford, UK). Following PBS washes donkey anti-mouse fluorescein isothiocyanate (FITC 1:400; Sigma, Poole, UK) for 2 h. Slides were rinsed with PBS and then coverslipped with glycerol–PBS.

The ipsilateral and contralateral dorsal horns of three to four sections of the L4–L5 spinal cord were image captured with a digital camera (Hamamatsu) attached to a Leica DMRD microscope. Quantitative analysis of the percentage density staining in an area of 200 000 pixels2 of the dorsal horn was carried out using Leica Qwin software (Leica, Milton Keynes, UK).

Statistical analysis

Statistical analysis of data was performed by ANOVA followed by post hoc tests where appropriate using GraphPad prism 5. Behavioural statistics were achieved using two-way ANOVA with Repeated Measures, followed by All Pairwise Multiple comparison to reveal the differences at individual time points as indicated. T-tests were used to assess significance associated with differences in RECA staining or alterations in blood flow.

Results

Behaviour

In naive rats there was no significant difference between sides over the course of the 2 weeks of behavioural testing to mechanical thresholds. The mean response thresholds were similar between left and right hind paws (p = 0.4306) and were thus merged for simplicity (Figure 1). L5 SRA injury generated ipsilateral mechanical allodynia in rats as evidenced by the decreased threshold response to mechanical stimulation of the ipsilateral hind paw compared to the contralateral paw (p < 0.0001) or naive controls (p < 0.0001) (Figure 1). Post hoc analyses showed significant differences from days 2 to 10 (p < 0.05). SRA mechanical thresholds remained at a similar level on day 12 even though this was not statistically significant from naive rats (p = 0.066).

Doppler flow

Doppler flowmetry was used to explore whether the spinal cord blood flow (SCBF) was altered in the lumbar spinal cord 2 weeks post-L5 SRA injury. SCBF was not significantly changed in SRA vs. naive animals (Figure 2). Very stable recordings were made along the exposed area of the spinal cord dorsum suggesting good reproducibility of the method. No differences were observed between flow in the ipsilateral and contralateral lumbar spinal cord after SRA at all three levels investigated. This suggests that the SCBF is at a “normal” level in these animals 2 weeks post-injury.
Oxygenation

Injury also does not appear to affect the oxygenation levels recorded at all three levels (immediately above injury, i.e., L4 cord, immediately below injury, i.e., L6–S1 cord, and at the level of injury, i.e., L5 cord) of the spinal cord (Figure 3). Indeed, the RD/IR ratio (RD and IR are the normalised amplitudes of the red and infrared PPG signals respectively) is very stable throughout all the areas investigated in both naive and injured animals, demonstrating that the method does provide very stable readings along the cord dorsum. The ratio value is purely a measure of the oxygen content of the tissue, that is, how oxygenated the blood is, and has nothing to do with the volume of blood going through an area (SCBF) or the tissue perfusion index (PPG). The results suggest that the spinal cord is not ischemic 2 weeks post-injury.

Photoplethysmography (PPG)

PPG measurements record blood volume changes by measuring changes in light absorption and the amplitude of the signal indicates the availability of blood supply to the tissue. By calculating the perfusion index (PI), conclusions can be drawn about whether or not the cord is hypoperfused or not.

In naive animals, the PI appears reliably stable throughout all the positions where recordings were made (Figure 4). In the L5 SRA animals, the PI remains comparable to that of naive rats below injury; however, on the ipsilateral side above the injury, the PI is significantly increased at the ipsilateral side above the injury. Note that the contralateral above level and ipsilateral side at level values approach significance vs. naive (p value equal to 0.07 and 0.08, respectively), thus suggesting a potentially larger area of the spinal cord is affected by the lesion.
the implication of this observation and PPG and laser Doppler
staining on the epitope. Other methods were required to confirm
the present study. However, it was unclear whether the change in
that staining for the blood vessel marker RECA is decreased
significant differences in perfusion or tissue oxygenation.
A hypothesis that L5 SRA results in ischemia of the spinal cord,
neuropathic pain. The results of this study do not support the
of local spinal cord ischemia that would trigger cell death and
factors (Sko¨ld et al. 2004; Chew et al. 2011, 2013), suggestive
demonstrated that root injury leads to decreased staining of
the affected region.
Spinal root injuries are known to damage the spinal cord
and produce bleeding which can lead to ischemia by
damaging the blood–spinal cord barrier interface (Mautes
et al. 2000; Echeverry et al. 2011). It has previously been
demonstrated that root injury leads to decreased staining of
blood vessel markers and upregulation of hypoxia inducible
factors (Sköld et al. 2004; Chew et al. 2011, 2013), suggestive
of local spinal cord ischemia that would trigger cell death and
neuropathic pain. The results of this study do not support the
hypothesis that L5 SRA results in ischemia of the spinal cord,
unless a single root avulsion injury that SCBF significantly decreased at the site of lesion
within 90 min post-injury. It is therefore unlikely that the
SCBF remained unaffected at any point in our model.
Additionally, expression of the inducible factor HIF1α was
shown as being only transient in intraspinal axotomised
motoneurons with levels declining after a week to become
non-existent after 3 weeks (Sköld et al. 2004). However,
considering that these measurements took place 2 weeks after
injury, the possibility of an “adaptive system” with changes
in the overall structure of the vasculature remains probable.
Increased HIF expression correlates with increased vascular
endothelium growth factor (VEGF) production (Sköld et al.
2004) and this is known to produce angiogenesis allowing
tissue perfusion and blood flow to return to normal. The
consequences of acute avulsion injury on PPG and LDF
would thus deserve further investigation.
Another possibility is that the extent of a single root
avulsion lesion is too limited to allow detection of changes in
a rat model. Patients who suffer from avulsion injuries
generally have multiple root injuries (Berman et al. 1998;
Hut et al. 2006). A single spinal root injury produces very
selective spinal cord damage at the REZs (Carlstedt 1997;
Chew et al. 2013) and the methodology used presently might
be insensitive to such a restricted lesion. This seems unlikely
as PPG has been shown to be sensitive enough to measure
acute transient changes following segmental compression of
the spinal cord (Phillips et al. 2009). PPG is now, along with
LDF, being applied to assess the vascular integrity as well as
correlation studies with neuroprotective effects of acute drug
treatments in spinal cord contusion injuries (Yip et al. 2012,
2013).

Discussion
The present study has investigated the contribution of
potential changes in vascular flow and tissue oxygenation to
the generation of mechanical hypersensitivity associated with
avulsion injuries. The results confirm that SRA leads to
mechanical hypersensitivity 2 weeks post-injury but that this
is not due to changes in tissue perfusion or blood flow through
the affected region.
Spinal root injuries are known to damage the spinal cord
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Declaration of interest
The authors report no conflicts of interest.

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