Gene expression profile of sodium channel subunits in the anterior cingulate cortex during experimental paclitaxel-induced neuropathic pain in mice

Willias Masocha

Paclitaxel, a chemotherapeutic agent, causes neuropathic pain whose supraspinal pathophysiology is not fully understood. Dysregulation of sodium channel expression, studied mainly in the periphery and spinal cord level, contributes to the pathogenesis of neuropathic pain. We examined gene expression of sodium channel subunits by real time PCR in the anterior cingulate cortex (ACC) at day 7 post first administration of paclitaxel, when mice had developed paclitaxel-induced thermal hyperalgesia. The ACC was chosen because increased activity in the ACC has been observed during neuropathic pain. In the ACC of control animals the Ct values for Na\textsubscript{v}1.4, Na\textsubscript{v}1.5, Na\textsubscript{v}1.7, Na\textsubscript{v}1.8 and Na\textsubscript{v}1.9 were above 30 and/or not detectable in some samples. Thus, comparison in mRNA expression between control and paclitaxel treated animals was done for Na\textsubscript{v}1.1, Na\textsubscript{v}1.2, Na\textsubscript{v}1.3, Na\textsubscript{v}1.6, Na\textsubscript{x} as well as Na\textsubscript{v}\beta1-Na\textsubscript{v}\beta4. Paclitaxel treatment significantly increased the mRNA expression of Na\textsubscript{v}1.1, Na\textsubscript{v}1.2, Na\textsubscript{v}1.6 and Na\textsubscript{x}, but not Na\textsubscript{v}1.3, sodium channel alpha subunits compared to vehicle-treated controls. Amongst the sodium channel beta subunits treatment with paclitaxel significantly increased the expression of Na\textsubscript{v}\beta1 and Na\textsubscript{v}\beta3, but not Na\textsubscript{v}\beta2 and Na\textsubscript{v}\beta4, compared to vehicle-treated controls. These findings suggest that during PINP there is differential upregulation of sodium channels in the ACC, which might contribute to the increased neuronal activity observed in the area during neuropathic pain.
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Abstract

Paclitaxel, a chemotherapeutic agent, causes neuropathic pain whose supraspinal pathophysiology is not fully understood. Dysregulation of sodium channel expression, studied mainly in the periphery and spinal cord level, contributes to the pathogenesis of neuropathic pain. We examined gene expression of sodium channel subunits by real time PCR in the anterior cingulate cortex (ACC) at day 7 post first administration of paclitaxel, when mice had developed paclitaxel-induced thermal hyperalgesia. The ACC was chosen because increased activity in the ACC has been observed during neuropathic pain. In the ACC of control animals the Ct values for Na\textsubscript{v}1.4, Na\textsubscript{v}1.5, Na\textsubscript{v}1.7, Na\textsubscript{v}1.8 and Na\textsubscript{v}1.9 were above 30 and/or not detectable in some samples. Thus, comparison in mRNA expression between control and paclitaxel treated animals was done for Na\textsubscript{v}1.1, Na\textsubscript{v}1.2, Na\textsubscript{v}1.3, Na\textsubscript{v}1.6, Na\textsubscript{v} as well as Na\textsubscript{v}\beta1-Na\textsubscript{v}\beta4. Paclitaxel treatment significantly increased the mRNA expression of Na\textsubscript{v}1.1, Na\textsubscript{v}1.2, Na\textsubscript{v}1.6 and Na\textsubscript{v}, but not Na\textsubscript{v}1.3, sodium channel alpha subunits compared to vehicle-treated controls. Amongst the sodium channel beta subunits treatment with paclitaxel significantly increased the expression of Na\textsubscript{v}\beta1 and Na\textsubscript{v}\beta3, but not Na\textsubscript{v}\beta2 and Na\textsubscript{v}\beta4, compared to vehicle-treated controls. These findings suggest that during PINP there is differential upregulation of sodium channels in the ACC, which might contribute to the increased neuronal activity observed in the area during neuropathic pain.

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Introduction

Voltage-gated sodium channels are responsible for action potential initiation and propagation in neurons and other excitable cells. Sodium channels are composed of a pore-forming α subunit associated with one or more auxiliary β subunits that modulate channel gating, expression and localisation (Catterall et al. 2005; Isom 2001). There are ten sodium channel α subunits Na\textsubscript{v}1.1-3 Na\textsubscript{v}1.9 and Na\textsubscript{x} encoded by genes SCN1A-SCN11A, and four β subunits Na\textsubscript{v}β1-Na\textsubscript{v}β4, encoded by genes SCN1B-SCN4B (Brackenbury & Isom 2008; Cummins et al. 2007; Yu & Catterall 2003) expressed at different levels in a wide variety of tissues.

Sodium channels play an important role in the propagation of nociceptive signals, and altered pain sensitivity and perception in various conditions including neuropathic pain (Bagal et al. 2015; Cummins et al. 2007). Dysregulated expression of sodium channels in both the periphery and the central nervous system (CNS), as well as frequent and ectopic firing in neurons have been associated with the pathogenesis of neuropathic pain (Craner et al. 2002; Lindia et al. 2005; Pertin et al. 2005; Rogers et al. 2006).

In the periphery the expression all sodium channel α subunits was downregulated, except for Na\textsubscript{v}1.2, in the dorsal root ganglia (DRG) of rats with spared nerve injury (SNI) (Laedermann et al. 2014). Another study observed downregulation of Na\textsubscript{v}1.8 and Na\textsubscript{v}1.9 in the DRG of a chronic constriction injury (CCI) model of neuropathic pain ((Dib-Hajj et al. 1999). However, other studies have observed upregulation of sodium channel subunits such as Nav1.3, Nav1.6, Nav1.9, Na\textsubscript{v}β2 and Na\textsubscript{v}β3 in the DRG of animal models of neuropathic pain (Craner et al. 2002; Lindia et al. 2005; Pertin et al. 2005; Shah et al. 2001; Shah et al. 2000).
In the spinal cord Na\textsubscript{v}1.3 was also found to be upregulated in the dorsal horn neurons of CCI and spinal cord injury (SCI) models of neuropathic pain (Hains et al. 2003; Hains et al. 2004).

Sciatic nerve injury (axotomy) resulted in upregulation of Nav1.7 in the spinal cord, which had strong correlation with the level of pain behaviour (Persson et al. 2009). In a model of painful diabetic neuropathy there was upregulation of Na\textsubscript{v}1.3 expression in spinal cord (Shah et al. 2001). Na\textsubscript{v}1.1 expression increased whereas Na\textsubscript{v}1.2 decreased in the spinal cord of neuropathic rats (Blackburn-Munro & Fleetwood-Walker 1999).

In the brain dysregulation of sodium channel expression has been observed in different areas during neuropathic pain. In the prefrontal cortex Na\textsubscript{v}1.1 expression was upregulated in mice with SNI (Alvarado et al. 2013). The expression of Na\textsubscript{v}1.3 was upregulated in the ventral posterolateral (VPL) nucleus of the thalamus of rats with CCI or spinal cord contusion injury (Hains et al. 2005; Zhao et al. 2006).

Recently, we observed increased excitability of the anterior cingulate cortex (ACC) to electrophysiological stimulation in a rat model of paclitaxel-induced neuropathic pain (PINP) (H Nashawi, IO Edafiogho, SB Kombian, W Masocha, unpublished data). Paclitaxel is a chemotherapeutic drug whose therapeutic use is sometimes limited by the development of dose-dependent painful neuropathy (Scripture et al. 2006; Wolf et al. 2008). The ACC is an area in the brain involved in pain perception and modulation, and has increased activity during neuropathic pain (Hsieh et al. 1995; Vogt 2005; Xie et al. 2009; Zhuo 2008). We have observed changes in the expression of gamma-aminobutyric acid (GABA)-ergic and glutamatergic molecules in the ACC of a mouse model of PINP (Masocha 2015a; Masocha 2015b). However, the expression of sodium channels in the ACC during PINP has not been studied as yet. Studying the expression of sodium channels in the ACC during PINP is important as they might contribute to the increased
neuronal excitability we observed in the ACC during PINP. Thus, the gene expression of sodium channel subunits in the ACC was evaluated in mice at a time point when the mice had paclitaxel-induced thermal hyperalgesia and gene expression changes of other molecules in the ACC (Masocha 2015a; Masocha 2015b; Nieto et al. 2008; Parvathy & Masocha 2013).

Materials and Methods

Animals

Female BALB/c mice (8 to 12 weeks old; 20 – 30 g; n = 23) supplied by the Animal Resources Centre (ARC) at the Health Sciences Center (HSC), Kuwait University were used. The animals were kept in temperature controlled (24 ± 1°C) rooms with food and water given ad libitum. Animals were handled in compliance with the Kuwait University, HSC, ARC guidelines and in compliance with Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. All animal experiments were approved by the Ethical Committee for the use of Laboratory Animals in Teaching and in Research, HSC, Kuwait University.

Paclitaxel administration

Paclitaxel (Cat. No. 1097, Tocris, Bristol, UK) was dissolved in a solution made up of 50% Cremophor EL and 50% absolute ethanol to a concentration of 6 mg/ml and then diluted in normal saline (NaCl 0.9%), to a final concentration of 0.2 mg/ml just before administration. Mice were treated intraperitoneally (i.p.) for 5 consecutive days with paclitaxel 2 mg/kg, the cumulative dose was 10 mg/kg, or its vehicle. This treatment regimen produces painful
neuropathy and thermal hyperalgesia in mice on day 7 post first administration (Nieto et al. 2008; Parvathy & Masocha 2013).

Tissue preparation and Real time RT-PCR

Mice were anesthetized with isoflurane, sacrificed by decapitation on day 7 post first administration of paclitaxel. The ACC was dissected and prepared for RNA extraction as described previously (Masocha 2015b).

Gene transcripts of the 10 sodium channel alpha subunits (Naᵥ1.1, Naᵥ1.2, Naᵥ1.3, Naᵥ1.4, Naᵥ1.5, Naᵥ1.6, Naᵥ1.7, Naᵥ1.8, Naᵥ1.9 and Naᵥx) and 4 sodium channel beta subunits (Naᵥβ1, Naᵥβ2, Naᵥβ3 and Naᵥβ4) were quantified in the ACC of vehicle-treated or paclitaxel-treated by real time PCR. Total RNA was extracted from the fresh frozen ACC using the RNeasy Kit (Qiagen GmbH), reverse-transcribed, and the mRNA levels were quantified on an ABI Prism® 7500 sequence detection system (Applied Biosystems) as previously described (Masocha 2009; Masocha 2015a). The primer sequences which were used, listed in Table 1, were ordered from Invitrogen (Life Technologies) and/or synthesized at the Research Core Facility (RCF), HSC, Kuwait University. Threshold cycle (Ct) values for all cDNA samples were obtained and the amount of mRNA of individual animal sample (n = 7 to 12 per group) was normalized to cyclophilin (housekeeping gene) (ΔCt). The relative amount of target gene transcripts was calculated using the $2^{-\Delta\Delta C_t}$ method as described previously (Livak & Schmittgen 2001). These values were then used to calculate the mean and standard error of the relative expression of the target gene mRNA in the ACC of paclitaxel- and vehicle-treated mice.

Statistical analyses
Statistical analyses were performed using unpaired two-tailed Student’s t-test using Graph Pad Prism software (version 5.0). The differences were considered significant at p < 0.05. The results in the text and figures are expressed as the means ± S.E.M.
Results

The mRNA expression of sodium channel subunits were analysed in the ACC at day 7, a time when the mice treated with paclitaxel had developed thermal hyperalgesia (~36% and 31%, reduction in reaction latency compared to the baseline latency and vehicle-treated mice, respectively) as we described previously (Masocha 2014; Parvathy & Masocha 2013).

Expression of sodium channel alpha subunits transcripts in the ACC at 7 days after paclitaxel administration

In control animals the Ct values for Na\textsubscript{v}1.4, Na\textsubscript{v}1.5, Na\textsubscript{v}1.7, Na\textsubscript{v}1.8 and Na\textsubscript{v}1.9 were above 30 and not detectable in some samples, whereas the Ct values for Na\textsubscript{v}1.1, Na\textsubscript{v}1.2, Na\textsubscript{v}1.3, Na\textsubscript{v}1.6 and Na\textsubscript{x} were below 30. Thus, comparison in mRNA expression between control and paclitaxel treated animals was done for Na\textsubscript{v}1.1, Na\textsubscript{v}1.2, Na\textsubscript{v}1.3, Na\textsubscript{v}1.6 and Na\textsubscript{x}.

Amongst the 5 sodium channel alpha subunits (Na\textsubscript{v}1.1, Na\textsubscript{v}1.2, Na\textsubscript{v}1.3, Na\textsubscript{v}1.6 and Na\textsubscript{x}) treatment with paclitaxel did not significantly alter the mRNA expression of the Na\textsubscript{v}1.3 (p = 0.1228), but significantly increased the expression of Na\textsubscript{v}1.1 (p<0.0001), Na\textsubscript{v}1.2 (p = 0.0077), Na\textsubscript{v}1.6 (p = 0.0079), compared to vehicle-treated controls (Figure 1). Na\textsubscript{x} was significantly upregulated (p = 0.0174) in the ACC by treatment with paclitaxel compared to treatment with vehicle (Figure 2). The most upregulated sodium channel alpha subunits were Na\textsubscript{v}1.2 and Na\textsubscript{x}, which were increased by more than sixfold.

Expression of sodium channel beta subunits transcripts in the ACC at 7 days after paclitaxel administration

Amongst the 4 sodium channel beta subunits analysed treatment with paclitaxel significantly increased the expression of Na\textsubscript{v}β1 (p = 0.0166) and Na\textsubscript{v}β3 (p = 0.0145), but not Na\textsubscript{v}β2 (p = 0.2411) and Na\textsubscript{v}β4 (p = 0.0742), compared to vehicle-treated controls (Figure 3). The most
upregulated sodium channel beta subunit was Na,β3, which was increased by more than fourfold.

Discussion

This study presents the first comprehensive analysis of the expression of transcripts of sodium channel subunits in the ACC during neuropathic pain, specifically paclitaxel-induced neuropathic pain (PINP). The ACC is associated with pain perception and modulation (Vogt 2005; Xie et al. 2009; Zhuo 2008).

No reports about the expression of sodium channels in the ACC specifically were found. However, Na,1.1, Na,1.2, Na,1.3, Na,1.6 and also Na, have been reported to be expressed predominantly (but not exclusively) in the brain with differential expression in different brain areas such as hippocampus, thalamus, cerebellum etc. (Beckh et al. 1989; Catterall 2000; Gautron et al. 1992; Levy-Mozziconacci et al. 1998; Schaller & Caldwell 2003; Westenbroek et al. 1989; Whitaker et al. 2000; Whitaker et al. 2001). In the current study using real time PCR all the 10 α subunits and 4 β subunits were detected in the ACC with different degrees of expression. Na,1.1, Na,1.2, Na,1.3, Na,1.6 and Na, as well as Na,β1 – Na,β4 were highly expressed in the ACC. On the other hand, although Na,1.4, Na,1.5, Na,1.7, Na,1.8 and Na,1.9 were detected in the ACC they were lowly expressed and/or were not detectable in some samples. Thus, the findings of this study are in agreement with studies described above.

Na,1.1, Na,1.2, Na,1.6 and Na, as well as Na,B1 and Na,B3 were upregulated in the ACC of mice with paclitaxel-induced thermal hyperalgesia. Upregulation of sodium channel expression has been observed in other areas of the brain during neuropathic pain. In the prefrontal cortex Na,1.1 expression was upregulated in mice with SNI (Alvarado et al. 2013). The expression of
Na\textsubscript{v}1.3 was upregulated in the ventral posterolateral (VPL) nucleus of the thalamus of rats with CCI (Zhao et al. 2006). Na\textsubscript{v}1.3 expression was also upregulated in the VPL of rats with spinal cord contusion injury (Hains et al. 2005). The findings of the current study suggest that upregulation of sodium channel subunits might contribute to hyperexcitability in the ACC. Hyperexcitability has been associated with dysregulation in sodium channels (Devor 2006). A link between upregulation of Na\textsubscript{v}1.3 and hyperexcitability of neurons in the spinal cord was found in neuropathic pain after spinal cord injury (Hains et al. 2003). Recently, we observed increased excitability of the anterior cingulate cortex (ACC) to electrophysiological stimulation in a rat model PINP (H Nashawi, IO Edafiogho, SB Kombian, W Masocha, unpublished data), which could be in part be due upregulation of sodium channels amongst other mechanisms such as decreased GABA availability at the synapse because of increased GABA transporter 1 (GAT-1) expression (Masocha 2015b). Changes in the expression of other molecules such as those of the GABAergic, glutamatergic, muscarinic dopaminergic systems have been observed in the ACC during experimental neuropathic pain (Masocha 2015a; Masocha 2015b; Ortega-Legaspi et al. 2011; Ortega-Legaspi et al. 2010).

Conclusions

In conclusion, the findings of this study show that during experimental paclitaxel-induced neuropathic pain there is increased expression of various sodium channel subunit transcripts in the ACC, which could contribute to the increased excitability and activity observed in this brain region during neuropathic pain.
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228 Expression of the mRNA for the beta 2 subunit of the voltage-dependent sodium channel in rat


Effects of paclitaxel on sodium channel alpha subunits transcript levels in the anterior cingulate cortex (ACC)

Relative mRNA expression of sodium channel alpha subunits Na\textsubscript{v}1.1, Na\textsubscript{v}1.2, Na\textsubscript{v}1.3 and Na\textsubscript{v}1.6 in the ACC of BALB/c mice on day 7 after first administration of the drug or its vehicle. Each point represents the mean ± S.E.M of the values obtained from 9-11 vehicle-treated control mice and 12 paclitaxel-treated mice ** \( p < 0.01 \) compared to vehicle-treated control mice.
Control

Paclitaxel

Relative expression of mRNA

Na\textsubscript{v}1.1

Na\textsubscript{v}1.2

Na\textsubscript{v}1.3

Na\textsubscript{v}1.6

**
Effects of paclitaxel on the sodium channel alpha subunit Na\(_x\) transcript levels in the anterior cingulate cortex (ACC)

Relative mRNA expression of Na\(_x\) in the ACC of BALB/c mice on day 7 after first administration of the drug or its vehicle. Each point represents the mean ± S.E.M of the values obtained from 11 vehicle-treated control mice and 12 paclitaxel-treated mice. * p < 0.05 compared to vehicle-treated control mice.
Relative expression of mRNA

Control

Paclitaxel
**Figure 3** (on next page)

Effects of paclitaxel on sodium channel beta subunits transcript levels in the anterior cingulate cortex (ACC)

Relative mRNA expression of sodium channel beta subunits Na\(_\beta\)1 to 4 in the ACC of BALB/c mice on day 7 after first administration of the drug or its vehicle. Each point represents the mean ± S.E.M of the values obtained from 8-11 vehicle-treated control mice and 8-12 paclitaxel-treated mice. * p < 0.05 compared to vehicle-treated control mice.
Relative expression of mRNA

- **Naᵥβ1**
- **Naᵥβ2**
- **Naᵥβ3**
- **Naᵥβ4**

- **Control**
- **Paclitaxel**

* indicates a statistically significant difference.
Table 1 (on next page)

PCR primer sequences of cyclophilin, and sodium channel subunits
**Table 1. PCR primer sequences of cyclophilin, and sodium channel subunits**

<table>
<thead>
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<th>Gene</th>
<th>Polarity</th>
<th>Sense</th>
<th>Antisense</th>
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<tr>
<td></td>
<td></td>
<td>Sequence 5’ to 3’</td>
<td>Sequence 5’ to 3’</td>
</tr>
<tr>
<td>Cyclophilin</td>
<td>GCTTTTCGCGCTTGCT</td>
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