UNIVERSITY OF NOTTINGHAM

The Effects of Cisplatin on Developing Nociceptive Pathways.

Joseph O. Lloyd, BSc

Thesis submitted to the University of Nottingham
for the degree of Master by Research

December 2016

Abstract

Survivability of paediatric cancers has tripled since the 1970s, however treatment comes with severe side effects. Cisplatin is the main treatment for solid state tumours, the most common form of paediatric cancers. Cisplatin's main dose-limiting factor is Chemotherapy Induced Peripheral Neuropathy (CIPN), a chronic pain disorder that can persist for decades beyond the end of treatment, severely affecting quality of life. This can be especially damaging in children, where early life insults can fundamentally alter the development of spinal nociceptive pathways. This study aimed to determine the extent of glial and neuronal changes in developing spinal nociceptive pathways following chemotherapeutic early life insult.

Sprague Dawley rat pups were given 1mg/kg cisplatin (cis) (0.5mg/ml) or vehicle (veh) via intraperitoneal (IP) injections for 5 days from postnatal day 7 to 11. At a juvenile (P13) and adult (P45) time-point, animals were given IP injection of pentobarbital and fixed via perfusion of 4% paraformaldehyde. Blood, DRGs and spinal cords were removed and stored in 30% sucrose in PBS plus 0.05% sodium azide. Immunohistochemistry performed on 40µm sections of the spinal cord lumbar enlargement. Antibodies for neuronal and glial targets involved in nociceptive pathways used were: neurones (NeuN), Microglia (IBA1), astrocytes (GFAP), Aβ fibres (NF200), non-peptidergic neurones (IB4), peptidergic neurones (CGRP), nerve growth factor receptors (TrkA) and interneurones (vGLUT2). The dorsal horn (DH) was imaged on confocal microscope and regions of interest (ROI) in laminae I-V analysed using ImageJ and Graphpad Prism.

A statistically significant ($p=0.0039$) increase in IL6 was shown in the blood plasma of the cisplatin animals (131.9±31.91 pg/ml) compared to the vehicle animals (38.00±6.748 pg/ml). There were no significant differences between cisplatin and vehicle animals in dorsal horn neurones or astrocytes at either time point. Microglia cell counts in the dorsal horn were significantly decreased at the juvenile time point in the Cisplatin treatment group. Cisplatin induced significant increases in dorsal horn expression of Aβ-fibre and non-peptidergic C-fibre terminations at both time-points, in the Cisplatin treatment group. TrkA expression in dorsal horn peptidetgic C-fibre terminations was increased at

both time-points, however, CGRP expression was only initially increased at the juvenile time-point but showed no difference to the veh at the adult time-point. Interneuron expression was increased at both time-points.

This study has shown that paediatric Cisplatin treatment causes significant chronic changes in PNS and CNS nociceptive histology, potentially underlying the chronic pain behaviours that Cisplatin has previously been shown to produce. Further research is required to fully establish the link between changes in cellular histology and animal behaviour and to understand the full extent of Cisplatin's effect on the CNS.

Acknowledgements

Many thanks to Dr Gareth Hathway and Dr Richard Hulse. As well as to all the members of the Developmental Nociception Laboratory for their help and friendship.

Contents

Abstract 1
Acknowledgements 2
Introduction 5
Cancer statistics 5
Cisplatin 5
Cisplatin action 6
Cisplatin side effects 9
Cognitive Impairment 9
Chemotherapy induced peripheral neuropathy 10
CIPN Mechanisms 11
CIPN treatment 12
Long term quality of life 12
Cisplatin induced allodynia 14
Nociception 14
Peripheral nociception 16
Spinal nociception 17
Ascending tracts 19
Descending control 20
Nociceptive development 21
Sensitisation 23
Peripheral sensitisation 23
Central sensitisation 25
Early life insults 28
Hypothesis 30
Methods 31
Animal model 31
Cisplatin induced neuropathy 31
Tissue preparations 32
Immunohistochemistry 32
Spinal cords 32
DRG's 34
Microscopy 35
IL6 ELISA kit 35

Statistical Analysis 36
Results 39
Juvenile Blood Plasma IL6 Concentration 39
Juvenile DRG Immunohistochemisty 39
Juvenile Spinal Cord Immunohistochemistry 41
Adult Spinal Cord Immunohistochemistry 45
Discussion 50
Cisplatin induced peripheral sensory alterations 50
Cisplatin induced central sensory alterations 54
Cisplatin induced systemic inflammation 57
Human Comparison 57
Limitations and Improvements 59
Future Directions 62
Impact statement 63
Conclusion 63
References 64
Appendix 1 73
Abbreviations 73

Introduction

Cancer statistics

The latest statistics from Cancer Research UK estimate that 2.5 million people in the UK alone are living with cancer, 400,000 more than 5 years previously. It is estimated that by 2030 this will continue to rise, reaching 4 million people. The greatest increase in cancer rates has been in the 0-24-year-old age group where the incidence of cancer has increased by 43% since the 1970s. However, improvements in detection and treatment mean that the prognosis for those diagnosed is far better than it once was. In the early 1970s the mean survival time following diagnosis was 1 year; as of 2011 the mean is now 10 years (Cancer Research UK 2016).

Regarding paediatric cancers specifically, the survival statistics are especially promising. Paediatric patient 10 year or more survival rates have more than doubled since the 1970s from 36% to 76%, and survival for at least 1 year has reached 91% of patients (Cancer Research UK 2016). The most commonly diagnosed type of cancer in patients 0-14 years old are solid mass tumours which make up 52% of cases. This type of cancer forms a discreet solid mass, as opposed to leukaemia (which makes up 31% of diagnosed cancers in 0-14 year olds) or lymphomas (a further 10% of paediatric diagnoses) which diffusely infiltrate tissue without forming a single mass. There are many varieties of solid state tumour, in 0-14 year olds the most common include: Central Nervous System (CNS) tumours (26% of all paediatric cancer diagnoses), neuroblastomas (6%) and soft tissue tumours (7%) (Cancer Research UK 2014).

Cisplatin

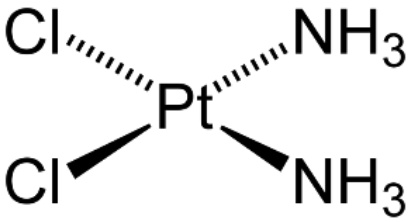


Figure 1: Cisplatin structure (Morgan 2010)

Cisplatin (Cis–diamminedichloroidoplatinum(II) (CDDP)) has been used as chemotherapeutic treatment for solid state tumours since 1972. It is a member of the group of alkylating agent chemotherapeutics. It is especially used in paediatric cases where radiotherapy can cause severe side effects on developing systems. It was the first platinum based chemotherapeutic agent developed, the others being

Oxaliplatinum and Carboplatinum. It consists of a central platinum atom surrounded by 2 chlorine atoms and 2 ammonia molecules (Figure 1) (Siddik 2003).

Cisplatin action

Cisplatin is an orange-yellow powder dissolved in saline solution and delivered via intravenous infusion. The dose of cisplatin depends on the type of cancer being treated and thus varies a lot between patients, however examples of recommended doses in paediatric and adult patients are; 60-100 mg/m^2 every 3-4 weeks for neuroblastoma, 60 mg/m^2 once a day for 2 consecutive days every 3-4 weeks for brain tumours and 75-100 mg/m^2 once every 4 weeks for ovarian cancer (Medscape 2016) (Drugs.com 2016).

In large part cisplatin is actively transported into the cell via several membrane transporters including efflux ATPases and solute carrier importers (Arnesano, Losacco and Natile 2013). High affinity copper uptake protein 1 (CTR1) found in yeast and mammals has been particularly associated with Cisplatin influx into cells. Genetic knock-out of CTR1 results in resistance to Cisplatin (Lin, et al. 2002) and cells that overexpress CTR1 show increased sensitivity to Cisplatin.

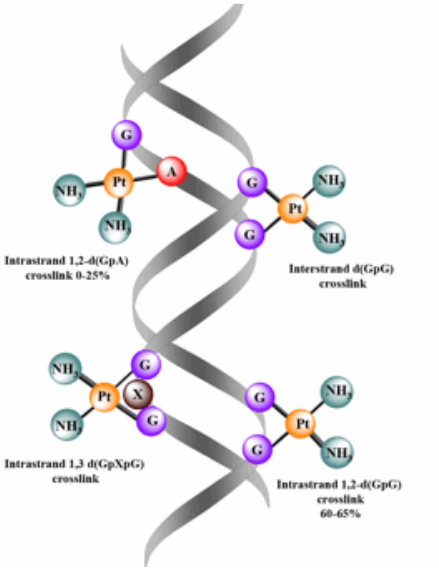


Figure 2: Cisplatin DNA adducts

However, exposure to Cisplatin causes a rapid decrease in CTR1, suggestive of protein degradation (Cancer Drug Resistance 2007), resulting in diminished uptake to the cell which contributes to Cisplatin resistance (Larson, et al. 2009).

In the form in which it is administered cisplatin in unable to act as a cytotoxin. Cisplatin is activated once inside the cell; water molecules replace the chloride atoms to create electrophilic sites. DNA adducts are formed between consecutive purine bases (guanine and adenine), as the electrophilic sites on the cisplatin molecule interact with the nucleophilic purine nitrogen on the imidazole ring of cisplatin. This forms an intrastrand cross-link that creates a kink in the DNA chain (Figure 2).

Guanine-guanine intrastrand cross-links account for 90% of the DNA adducts, with the remaining 10% being adenine-guanine cross-links (Dasari and Tchounwou 2014). Cisplatin causes damage to both the neuronal and the mitochondrial DNA (Han and Smith 2013).

This intrastrand cross-link disrupts transcription of the DNA by blocking both RNA-polymerase to decrease protein synthesis and DNA-polymerase to stop DNA replication during cell mitosis. In addition, damage recognition proteins, in particular high-mobility group (HMG) 1 proteins and mismatch repair (MMR) complex, bind to the DNA distortion (Siddik 2003). These damage recognition proteins directly promote cytotoxicity by blocking DNA repair. Overexpression of the HMG1 protein has been shown to cause sensitisation to Cisplatin in breast cancer cells (He, Liang and Lippard 2000). In addition to blocking DNA repair, damage, recognition proteins also initiate a series of cellular cascades that promote cellular apoptosis. The activation of tumour suppressor p52 protein and mitogen-activated protein kinase (MAPK), both of which are activated directly by damage recognition proteins and indirectly via ataxia-telagiectasia-mutated related (ATR) protein. p53 activates the apoptotic regulator Bax in the mitochondrial cytosol, which promotes pro-apoptotic factors such as cytochrome C. Interestingly, p53 also promotes Gadd45, a protein that promotes DNA repair; however, the repair mechanisms appear to become overwhelmed as DNA damage increases. MAPK regulates cell proliferation, differentiation and apoptosis, so can directly promote apoptosis, but also further activates the p53 apoptotic cascade (Siddik 2003).

Unfortunately, apoptotic resistance can be formed to counter the DNA damage creating a major limitation to Cisplatin treatment. There are several molecular mechanisms that contribute to Cisplatin resistance including: reduced drug uptake (as described above), increased Cisplatin inactivation by thiol containing molecules and increased DNA adduct repair via overexpression of HER-2/neu and antiapoptotic bcl-2, activation of P13-K/Akt pathway and inactivation of P53 pathway (Siddik 2003). As a consequence of the formation of resistance the survivability of tumour cells increases limiting Cisplatin's effectiveness. The initial ovarian cancer response rate to Cisplatin up to

70%. Unfortunately, this isn't sustained, the development of resistance causes the response rate drops to just 15-20%, 5 years later (Siddik 2003).

Current research suggests that Cisplatin has difficulty crossing the blood-brain barrier (BBB) and entering the central nervous system (CNS). A study by Jacobs et al. (2010) used micro-dialysis to measure the Cisplatin concentrations in various tissues of rhesus monkeys. In the CNS they found low concentrations of Cisplatin, 1.1-2.8 µM per hour in the brain and 4.0-4.8 µM per hour in the CNS following a systemic dose of 10 mg/kg of Cisplatin. This compares to peripheral concentrations of 89-92 µM per hour in the plasma, 89-112 µM per hour in peripheral veins and 72-103 µM per hour in the temporalis muscle. These finding were observed to be dose independent (Jacobs, et al. 2010). This suggests that the BBB severely limits the entry of Cisplatin to the CNS. Nakagawa et al (1996) also looked at Cisplatin levels in the cerebrospinal fluid (CSF) in patients with malignant gliomas using Ommaya reservoirs in the anterior horn of the lateral ventricle of the brain or the postoperative cavity following the removal of the tumour. They found levels that were not as high as those considered to be cytotoxic to either cells in the CSF or neuronal cells. However, far higher Cisplatin levels were found in post-operative patients where the BBB had been disrupted (Nakagawa, et al. 1996).

Dietrich et al. showed that the extent of Cisplatin's toxicity was tissue dependant by exposing a range of *in vitro* rat and human cell cultures (including cancer cells, neuronal precursor cells and neuronal cell lines) to 1µM of Cisplatin for 20 hours, a dose was well within the range of concentrations found in the CSF during treatment. They showed that oligodendrocytes and their precursors, oligodendrocyte-type-2 astrocytes (O-2A) as well as neurone restricted precursor (NRP) cells and glial restricted precursor (GRP) cells, had a 60-90% reduction in viability, as opposed to less than 40% drop in viability seen in most of the cancer cell lines tested. Even when the Cisplatin dose was dropped to 0.1µM there was a 40% reduction in viability of O-2As, Oligodendrocytes and NRP cells. When the dose of Cisplatin was increased to cause a 40-80% reduction in cancer cells lines

there was a 70-100% reduction in viability for O-2A, GRP, NRP and neuroepithelial stem cells (NSCs) (Dietrich, Han, et al., 2006).

These findings were then confirmed using an *in vivo* mouse study. Following 5mg/kg IP Cisplatin treatment for 3 days' significant increases in apoptotic cells (shown using a terminal deoxynecleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) assay) were shown in the dentate gyrus (DG) and corpus callosum (CC) up to 42 days following treatment and in the sub-ventricular zone (SVZ) up to 10 days after treatment. Immunohistochemistry was used to confirm that the same precursor cells Cisplatin had previously been confirmed to have the greatest toxic action on were lost in the affected areas. (Dietrich, Han, et al., 2006)

Cisplatin side effects

Cisplatin shares the side effects common to many chemotherapeutic agents including nausea and vomiting. In children, particularly below 5 years of age, there is a high risk of ototoxicity, affecting the cochlea and auditory nerves, causing hearing loss which is bilateral and permanent (Rybak, et al. 2010). Patients complained of tinnitus 2-36% after being treated with cisplatin (Reddel, et al. 1982). Cisplatin is excreted following accumulation in the kidneys, Cisplatin may cause significant renal toxicity (Dasari and Tchounwou 2014). In the proximal tubular epithelial cells, the concentration of Cisplatin can be as high as 5 times the concentration found in the blood serum (Kuhlmann, Burkhardt and Kohler 1997).

Cognitive Impairment

Although more usually associated with breast cancer treatments such as 5-fluorouracil and cyclophosphamide, emerging evidence suggests that platinum based chemotherapeutics also cause cognitive impairments or 'chemobrain'. This condition is characterised by decreased memory, attention and processing speed and like chemotherapy induced peripheral neuropathy (CIPN) can persist long after the end of treatment (Zhou, Kevelaars and Heijnen 2016).

Chemobrain has been investigated in neuroimaging studies which have shown structural decreases in grey and white matter tracts and decreases in the activation of the frontal regions (Simo, et al. 2013). *In-vitro* studies have shown related losses of NSCs and differentiated hippocampal neurones (Andres, Gong, et al., 2014).

Andres *et al.* (2014) investigated the effect of sub-neurotoxic levels of Cisplatin (0.1µM) on cultured hippocampal neurones. They found that even in doses that didn't effect NSC viability or promote apoptosis, after 30 minutes of exposure there was a loss of dendritic spines and synapses. This also proved to be true in further *in vivo* experiments. Neuronal connectivity and function is linked to dendritic density; therefore, dendritic damage could account for some of the Cisplatin induced cognitive impairment (Andres, Gong, et al., 2014).

Chemotherapy induced peripheral neuropathy

One of the principle side effects of Cisplatin, as well as other chemotherapeutic treatments, is CIPN. The neurotoxic and gliotoxic properties of chemotherapeutics cause cell death in peripheral nerve fibres and neuronal bodies, in particular the dorsal root ganglion (DRG) (Argyriou, Bruna, et al., 2012). This leads to CIPN, a chronic pain condition that can persist for years or decades following the conclusion of the treatment. In some cases, CIPN never resolves and patients are left in permanent pain. Initially (in the first month) 68.1% of patients receiving chemotherapy suffer from CIPN, this drops over time to 60% after 3 months and 30% after 6 months (Seretny, et al. 2014). However, beyond 6 months, 30% of patients continue to suffer from CIPN, one study looking at the long-term toxicity of patients treated with Cisplatin found that 28% of patients had symptomatic neuropathy and 38% had asymptomatic neuropathy 15 years following the end of treatment. In 6% of patients the neuropathy was disabling (Strumberg, et al. 2002). Patients experiencing CIPN reported simultaneous pain and loss of sensation in the upper and lower extremities, also numbness, muscle weakness and loss of balance. Emotionally patients reported feeling frustrated and depressed (Tofthagen 2010).

The reported rate of paediatric CIPN is between 3-13% for all cancers (Moore and Groninger 2014), in patients being treated for acute lymphoblastic leukemia the rate is far higher around 29.7% (Ramchandren, et al. 2009). However, while standardised measures are available for the characterisation of CIPN in adults, their use is limited in children. This, along with communication problems associated with young children that have been shown to be a problem between patients, parents and medical professionals, means that CIPN is underdiagnosed for paediatric cancer patients so incidence is likely higher than the figures reported (Gilchrist 2012).

The early symptoms of CIPN in adults include; numbness, tingling, and pins and needles in the fingers and toes. These symptoms progress symmetrically up the arms and legs as CIPN worsens in a glove and stocking pattern. Deep tendon reflexes, such as found in the knee twitch reflex, are lost with prolonged treatment and in severe cases an ataxic gait may develop due to the loss of proprioception (Argyriou, Bruna, et al., 2012).

CIPN Mechanisms

CIPN develops due to the toxic nature of cancer chemotherapeutics and is primarily due to the degeneration and loss of axonal processes from the peripheries. The exact cellular and molecular mechanisms that produce CIPN remain unclear. Due to their differing mechanisms of action, subtle differences exist between the effects of different chemotherapeutic agents, which is further complicated, as they are often given in combination. However, the different chemotherapies do appear to share underlying mechanisms, that contribute towards neuronopathy, axonopathy and myelinopathy (Han and Smith 2013).

The longest nerves are the first to be affected by chemotherapy, possibly due to their higher metabolic requirements. This is why the most peripheral sensory neurones (innervating the fingers and toes) are the first to be affected, progressing up the hands and feet, then the arms and legs, in a glove stocking pattern. Cisplatin causes structural and functional mitochondrial abnormalities, inducing apoptosis via oxidative stress, as described above. The more mitochondrial activity, the

greater the oxidative stress (Han and Smith 2013). The administration of antioxidant α-lipoic acid, to *in vitro* cultured DRG neurones reversed Cisplatin induced mitochondrial damage (Melli, et al. 2008).

Myelinated primary afferent fibres appear to be most affected in CIPN, showing reduced nerve conduction velocities (Han and Smith 2013). In cultured cells, Cisplatin shows increased toxicity to oligodendrocytes, the myelinating cells of the central nervous system (Dietrich, Han, et al., 2006). This doesn't appear to be the case in the peripheries, the myelin sheaths arising from Schwann cell show no structural changes after Cisplatin (or Paclitaxel and Bortezomib) induced CIPN. The alterations in conduction velocity may instead be due to axonal degeneration (Windebank and Grisold 2008).

The extent of peripheral axonal damage is highly dependent on the dose of chemotherapeutic. Higher doses of Paclitaxel and Bortezomib chemotherapeutics, either given at once or as cumulative dose, resulted in a degeneration of both axonal processes and DRG neurones (Han and Smith 2013).

CIPN treatment

Currently, dose-reduction and increased dose intervals are the best way of reducing the incidence of CIPN (Kaley and DeAngelis 2009) (Gutiérrez-Gutiérrez, et al. 2010), but many clinical studies have been conducted which are investigating possible agents for the prevention and treatment of CIPN. A 2014 review by Hershman *et al.* of 48 randomised control trials recommended none of the agents examined for CIPN prevention due to a lack of high-quality consistent evidence. For CIPN treatment, the trials of drugs, such as tricyclic antidepressants and gabapentin, used for the treatment of other neuropathic conditions, were inconclusive in the case of CIPN and required further research. It does however moderately recommend the serotonin-noradrenaline reuptake inhibitor (SNRI) Duloxetine as a first line agent for CIPN treatment (Hershman, et al. 2014).

Long term quality of life

Chemotherapy induced side effects can be severely detrimental to the long term wellbeing and quality of life of patients that beat cancer. The Childhood Cancer Survivor Survey (CCSS) is cited

when investigating the long term health effects of adult survivors of childhood cancers. It is a retrospective cohort study that tracked the health states of 35,923 survivors of childhood cancers (and over 5,000 of their siblings) that were diagnosed with paediatric cancer between 1970 and1999 (St. Jude Children's Reseach Hospital 2016)

Pain is one of the long term side effects studied by the CCSS. In patients that survived for at least 5 years after diagnosis, 12.3% of patients in the CCSS reported pain, 15.5% reported migraines and 20.5% reported other headaches, for comparison, 6.3%, 15.2% and 15.3% respectively were reported by siblings. 21% thought their cancer or its treatment was the cause of their pain, with 3.4% describing their cancer-related pain as excruciating (Lu, et al. 2011). Some gender differences were also reported with females exhibiting higher risk for developing pain. Females were more likely to report migraines and other headaches, and 1.8x more likely to use analgesics than their male counterparts (Lu, et al. 2011).

Other chronic conditions are also affected by childhood cancer and its treatment. Childhood cancer Survivors were 3.3 times more likely to have a chronic health conditions than their siblings. They were also more likely to have a severe or life-threatening condition (27.5% vs. 5.2%). The conditions with the greatest relative risk compared with siblings include; major joint replacement (16.1% vs. 0.03%), congestive heart failure (1.24% vs. 0.1%) and second malignant neoplasm (2.38% vs. 0.33%). The risk of multiple health conditions was also increased for ≥2 conditions (37.6% vs. 13.1%) and ≥3 conditions (23.8 vs. 5.4%). Female survivors were also 1.5 times more likely to have a severe or life threatening condition than their male counterparts (Oeffinger, et al. 2006).

Many studies find that most survivors report few or no symptoms of psychological distress (Zebrack, et al. 2004). However, a subgroup with risk factors like ‘perception of worsening health over time’ and ‘increased cancer-related pain’ showed an increased likelihood of depression (8.9%), anxiety (4.8%) and somatisation (7.2%) (Brinkman, et al. 2013). Conversely, another study found that survivors of childhood cancer were 1.5 times more likely to have symptoms of depression and

anxiety and 1.7 times more likely to show antisocial behaviours (Schultz, et al. 2007). A study by Recklitis *et al.* evaluated the risk of suicide ideation (SI) in adult survivors of childhood cancer. Survivors had a rate of 7.8% for SI compared to 4.6% of siblings. It suggested that risk factors included a diagnosis of CNS cancer, depression, chronic pain and poor health (Recklitis, et al. 2009).

The fact that chronic pain plays such a major role in so many of the chronic physical and psychological conditions suffered by adult survivors of childhood cancer suggests that further research is required to fully understand the peripheral and central nociceptive mechanisms in play.

Cisplatin induced allodynia

Previous research from the University of Nottingham Developmental Nociception laboratory, investigated Cisplatin induced allodynia. Neonatal Sprague Dawley rats were given intraperitoneal (IP) injections of Cisplatin. Significantly decreased thresholds for mechanical and thermal stimuli where shown in the cisplatin animals compared to vehicles from Postnatal day 22 to the end of testing at postnatal day 42. Suggesting a permanent change in the processing of sensory information induced by early life cisplatin exposure. It also suggests a lag from the end of treatment at postnatal day 11 to the onset of pain at postnatal day 22, this has also been shown in other experiments and may be due to intercellular anterograde transportation from the sensory cell body to the peripheral axons (Han and Smith 2013). Neuroimmune suppression of the inflammatory response may be another explanation for the delay in Cisplatin's effect. (McKelvey, et al. 2015) showed nerve injury to infant rats triggered an increase in the anti-inflammatory cytokines IL4 and IL10, the blocking of which produced pain hypersensitivity. The immune response switches at adolescence (P25-P30) accompanied by the onset of pain behaviour. This switch at adolescence correlates with the delayed onset of pain behaviours in the Cisplatin seen at P22.

Nociception

In order to fully understand how Cisplatin causes these profound changes in nociceptive processing, an in depth explanation of nociceptive systems is required. The cellular and molecular systems that

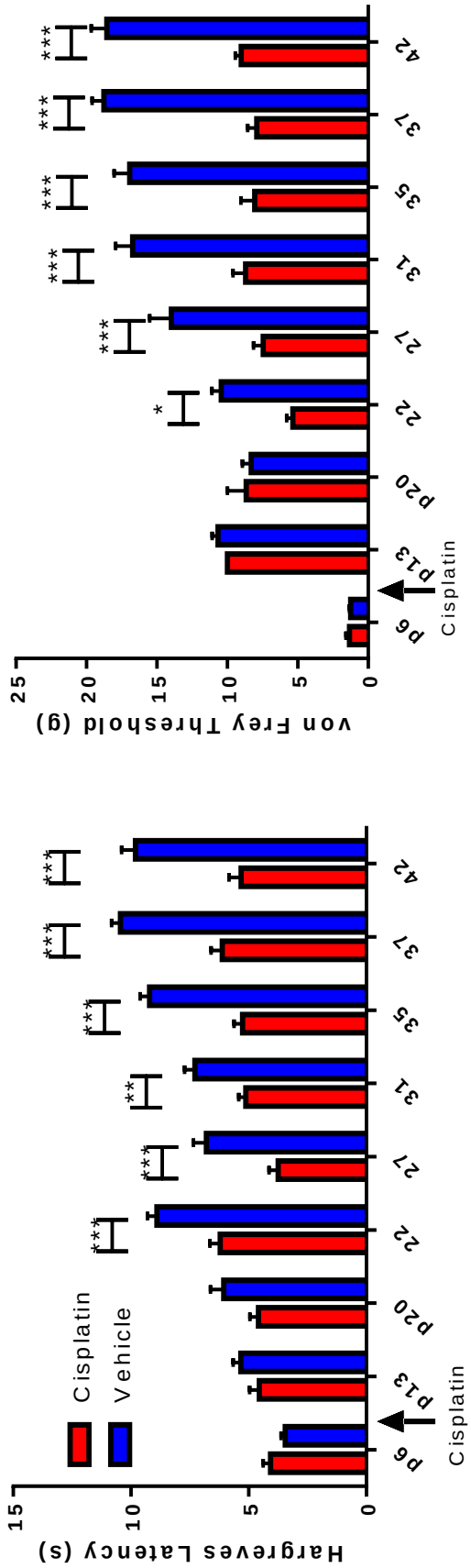


Figure 3: Cisplatin significantly reduces mechanical and thermal withdrawal thresholds for prolonged periods following cessation of treatment

contribute towards external noxious stimuli being detected and transmitted into the CNS where it can be experienced as pain. Also the mechanisms underlying sensitization of nociceptive pathways, which can produce an exaggerated painful response to an already painful stimulus (hyperalgesia) or can provoke a painful response to a normally innocuous stimulus (allodynia) (Schaible 2006).

It is important to differentiate between nociception and pain. Even though they are often used as synonyms, they are subtly different things. Nociception refers to the physical detection of a noxious stimuli in the environment. Whereas pain is the perceived experience of that stimuli in the CNS.

Nociception can be objectively measured and studied, but pain is altered by emotional and historical factors which change person by person, meaning that any analysis becomes subjective.

Briefly, noxious stimuli are detected by specialised receptors in free nerve endings of the periphery. Signals are transmitted up the nociceptive primary afferent, A-delta (Aδ) and C-fibres to the dorsal horn of the spinal cord. In the spinal cord primary afferent fibres synapse with second order neurones which continue the nociceptive signal up a variety spinal tracts, but primarily the spinothalamic tract. Spinal tracts propagate the signal to a number of brain centres, including the thalamus, limbic system, somatosensory cortex and frontal cortex, that localise, categorize and attribute emotion content to the nociceptive signal (Julius and Basbaum 2001).

Peripheral nociception

Nociception has distinct neuronal receptors, pathways and connections to innocuous touch. Noxious stimuli are detected by specialised thermal, mechanical and chemical receptors on free nerve endings of nociceptive fibres in the skin, muscle, viscera and other tissues of the periphery (Almeida, Roizenblatt and Tufik 2004). Receptors can be themselves be an ion channel, altering axonal firing directly or by activating G-protein coupled receptors (GPCR) to influence other ion channels (Julius and Basbaum 2001).

The cell bodies for nociceptive neurones are in the DRG on the dorsal side of the spinal cord. The peripheral axonal processes of the nociceptive neurones extend to the skin and viscera where the nerve endings contain chemical, thermal and mechanical receptors. In the other direction, nociceptors extend axons into the superficial layers of the dorsal horn. When stimulated by mechanical, thermal and/or chemical stimuli, action potentials are generated which are propagated to the dorsal horn of the spinal cord via the DRG (Schaible 2006).

Primary afferent fibres transduce and propagate tactile information from the periphery to the CNS. A-alpha (Aα) and A-beta (Aβ) fibres are large diameter myelinated fibres that detect non-noxious mechanical sensation and are not involved with nociception. Aδ fibres are medium diameter, lightly myelinated fibres, they detect mechanical, thermal and chemical noxious stimuli and are the quicker, sharp acute pain. Aδ fibres are split into 2 types, both types are sensitive to mechanical nociceptive stimulation, but have different thresholds for temperature. Type 1 have a threshold of ~53^{o}c, containing the heat (and capsaicin) gated Transient Receptor Potential Vanilloid-like 1 (TRPV1) receptors, type 2 have a threshold of ~43^{o}c and contain TRPV2 receptors. The final category are C-fibres, these are small diameter unmyelinated fibres, they also detect mechanical, thermal and chemical noxious stimuli but is the slower, dull throbbing pain. C-fibres are also split into 2 groups, however are differentiated by their neurochemistry rather than their nociceptive functionality. The first group are peptidergic neurones which contain the neuromodulators Substance P and calcitonin gene related peptide (CGRP) and express the Tyrosine Kinase A (TrkA) neural growth factor (NGF) receptor. These fibres innervate deeper layers of the skin and other tissues. The second group, non-peptidergic neurones express Isolectin B4 (IB4) and $P2X_3$ adenosine triphosphate (ATP) receptors. These fibres innervate the epidermis of the skin (Schaible 2006).

Spinal nociception

The dorsal horn of the spinal cord is the first stage of somatosensory processing. Peripheral inputs are processed and modulated by a complex, specialised and dynamic network of spinal neurones and glia, before the incoming information is relayed by projection neurones onto higher brain

centres (A. Todd, 2010). The larger ventral horns of the grey matter contain the cell bodies for motor neurones that then exit the spinal cord to innervate muscle. The dorsal horns receive input for all sensory processes including nociception. Rexed (1952) layered the spinal cord into 6 distinct laminae by cellular neuronal histology. The dorsal horn consists of laminae I-VI, the intermediatry zone is laminae VII and X, and the ventral horn is laminae VIII and IX (Rexed 1952). The most superficial laminae, I and II (also known as marginal zone and substantia gelatinosa) are structurally distinct as they are densely packed with small neurones. The laminae are also functionally distinct as different laminae receive information from different neuronal populations concerning different sensory stimuli. Innocuous mechanoreceptive fibres like Aβ fibres synapse in laminae II-V, whereas nociceptive Aδ and C-fibres innervate laminae I and II. Aδ axons also form a synapse in lamina V with efferent motor neurones when part of a flexor withdrawal reflex (A. Todd, 2010).

All primary afferent fibres use the excitatory fast-acting neurotransmitter glutamate neurotransmitter at their synapse with either interneurons or second order fibres. At high frequency firing, in addition to glutamate, peptidergic C-fibres can also release the excitatory slow acting Substance P neuropeptide, ATP and inflammatory cytokines (Gangadharan and Kuner, 2015).

4 types of second order neurones synapse with the primary afferent fibres entering the dorsal horn of the spinal cord; non-nociceptive specific neurones, nociceptive specific neurones, wide-dynamic range neurones and interneurons. Non-nociceptive specific neurones synapse only with Aα and Aβ fibres, responding to innocuous stimuli signals. Nociceptive-specific neurones synapse with Aδ and C-fibres, responding to noxious stimuli signals. Wide dynamic range (WDR) neurones receive inputs from Aβ, Aδ and C-fibres responding to both innocuous and noxious stimuli signals. There are also interneurons, which form an intermediately connection between the primary afferent fibres and ascending spinal second order neurones (Almeida, Roizenblatt and Tufik 2004).

Also present at the synapse between the primary afferent neurones and the second order neurones are inhibitory interneurons, which make up 30-45% of laminae I-III interneurones (Todd and Sullivan,

1990). These cells tonically fire under normal circumstances releasing inhibitory *gamma*-Aminobutyric acid (GABA) neurotransmitter to inhibit the activity of nociceptive pathways to produce baseline hypoalgesia. They can be modulated by supraspinal sites to control nociceptive processing at the spinal level (Yoshimura and Furue 2006).

Ascending tracts

The main ascending spinal nociceptive pathways are the Spinothalamic tract (STT) and the spinoparabrachial tract (SBT), however a number of other spinal tracts are also involved in supraspinal transmission of nociception.

STT neurones originate in laminae I and V of the dorsal horn. It is made up of nociceptive-specific, non-nonciceptive and WDR neurones, receiving input nociceptive signals from Aδ and C-fibres in lamina I and innocuous signals from Aβ fibres in lamina V. The tract ascends the spinal cord (on the contralateral side to the stimulus) to the thalamic ventral posteriorlateral (VPL) nucleus. The thalamus is a midline structure of the brain between then cerebral cortex and the midbrain. It plays a major role in the processing of ascending somatosensory signals and relaying to the somatosensory (S1) cortex where the pain is localised and categorised (Almeida, Roizenblatt and Tufik 2004).

Spinoparabrachial tract neurones receive direct input laminae I and II and consists of nociceptive-specific neurones, thus is a purely nociceptive tract, SBT projects to the parabrachial nucleus. The parabrachial nucleus has two pathways to the brain; the spinoparabrachial amygdaloid pathway to the amygdala and stria terminalis, and the spinoparabrachial hypothalamic pathway to the ventromedial nucleus of the hypothalamus. The SBT appears to play a role in motivational and affective regulation as neuroendocrine responses to pain (Almeida, Roizenblatt and Tufik 2004).

Spinoreticular tract (SRT) neurones originate in laminae V, VII, VIII and X of the spinal cord and consist of nociceptive-specific, non-nociceptive specific and WDR neurones. SRT projects to areas of the brainstem including the medial rhombencephalic reticular formation and the nucleus reticularis

giagantocellularis, then to thalamus and hypothalamus in the limbic system, then frontal cortex. Involved in the emotional and higher functions of pain, it also activates brainstem structures that responsible for descending spinal inhibition (Almeida, Roizenblatt and Tufik 2004).

The spinohypothalamic tract (SHT) originates in laminae I, V and X and is composed of nociceptive-specific, non-nociceptive specific and WDR neurones. Non-nociceptive specific tracts propagate through the trigeminal-hypothalamic tract, while nociceptive-specific tracts propagate along the parallel trigeminal-hypothalamic pathway and the reticular-thalamic pathway. SHT appears to be involved with integration with the autonomic nervous system (Almeida, Roizenblatt and Tufik 2004).

Spinomesencephalic tract (SMT) neurones originate in laminae I, IV, V, VII and VIII and consist of nociceptive-specific, non-nociceptive specific and WDR neurones. SMT projects to the locus coeruleus in the pons and the periaqueductal grey (PAG) in the midbrain and is involved with descending spinal control of pain (Almeida, Roizenblatt and Tufik 2004).

Descending control

Supraspinal sites in the midbrain of mature animals balance inhibition and excitation in the spinal cord through descending monoaminergic control. The primary nucleus for descending control is the PAG in the midbrain, which coordinates noradrenergic and serotonergic descending projections to the dorsal horn, to inhibit or facilitate nociceptive signalling at the synapse of the primary afferent fibre and second order neurone (Ossipov, Dussor and Porreca 2010). The PAG receives input from the periphery along the SMT, as well descending input from the limbic system (in particular the amygdala, thalamus and hypothalamus) and cortical regions in the brain (Schaible 2006).

The PAG projects to the rostral ventromedial medulla (RVM), which contains the, serotonin rich, raphe magnus, the reticularis gigantocellularis pars alpha and the nucleus paragigantocellularis lateralis (Vanegas and Schaible 2004). Neurones from the RVM descend along the spinal dorsolateral funiculus (DLF) to the dorsal horn, RVM descending projections can be off-cells (providing nociceptive inhibition), on-cells (nociceptive activation) or neutral cells (Yoshimura and Furue 2006).

The RVM is rich in endogenous opioid receptors, which activate off-cells and inhibit on-cells (Heinricher, Morgan and Fields 1992), therefore the RVM is an important target area in the clinical management of pain.

The PAG and RVM both communicate with another midbrain nucleus the Locus coeruleus. The Locus coeruleus exerts further inhibition upon the dorsal horn via noradrenergic projections. Release of noradrenaline causes the activation of α_2-adrenergic receptors, inhibiting the release of excitatory neurotransmitters pre-synaptically and membrane hyperpolarisation post-synaptically, inhibiting nociceptive firing (Ossipov, Dussor and Porreca 2010). In addition, activation of α_1-adrenergic receptors causes depolarisation of inhibitory GABAergic interneurons to further inhibit nociceptive pathways (Gassner, Ruscheweyh and Sandkuhler 2009).

Nociceptive development

At birth the nociceptive systems are not fully developed, they finish maturing over the following months and years. In the past this has led to the conclusion that while neonates may show generalised nociceptive reflexes, they do not 'experience' pain (Marchant 2014). For many years this meant that clinicians performed painful procedures, such as heel lances, without any form of anaesthesia. We now know this is demonstrably not true (Simons and Tibboel 2006), nociceptive insults early in life having profound effects of nociceptive and somatosensory development (Hathway, Vega-Avelaira and Fitzgerald 2012), which will be explored in greater detail later. Typically, neonatal pain response is characterised by generalised, uncoordinated and inappropriate responses to a stimulus. For example, in response to the aforementioned heel lance, a neonate would move their entire body, rather than just the affected area, suggestive of disorganisation of sensory/mechano reflexes. Nociceptive thresholds for young animals are also much lower showing a tonic hyperexcitability (Hathway, Vega-Avelaira and Fitzgerald 2012).

Sensory neurones differentiate from the ectoderm in 2 waves early in development. TrkB and TrkC positive non-nociceptive large diameter neurones, followed by small diameter TrkA positive

neurones. Up until birth there is a steady increase in DRG sensory neurones, beyond what is necessary for functional innervation (Kitao, et al. 1996). This over-abundance of free nerve endings in the epidermis means any nociceptive signals are unspecific, explaining the generalised neonatal behaviours. Finite supplies of neurotrophic factors (such as NGF) causes functional pruning of 16% of neurones in the first 5 postnatal days, 'tuning' the nociceptive fields (Coggeshall, Pover and Fitzgerald 1994). This functional tuning happens both in the peripheries and the CNS.

As well as extending dendritic processes to the epidermis, DRG neurones also extend axonal projections to the dorsal horn of the spinal cord. Similar to differentiation of DRG neurones, innervation of the spinal cord occurs in 2 waves, the A-fibres at embryonic day (E)15-17 and C-fibres at E18-20 (Mirnics and Koerher 1995). C-fibres innervate in their adult patterns in laminae I and II from the offset, A-fibres however, initially project in greater range than in the adult, from laminae I to V, and are pruned back by P21 to just laminae III-IV (Fitzgerald, Butcher and Shortland 1994). Initially only the peptidergic (CGRP positive) neurones can be detected in the spinal cord, non-peptidergic (IB4 positive) neurones are present in the DRG from E18 but are not detected in the spinal cord until P5 (Pignatelli, Ribeirp-da-Silva and Coimbra 1989).

The lowered nociceptive threshold seen in neonates are due, at least in part, to immaturity of descending nociceptive control from the midbrain to the spinal cord. In the adult, descending serotonergic fibres from the PAG and RVM, can produce either excitation or inhibition of spinal cord second-order neurones. In neonates, stimulation of the PAG does not produce analgesia (as in the adult) regardless of intensity of stimulation until P21. Serotonergic fibres are present in the grey matter of the spinal cord at birth, however, the adult density and patterns aren't seen until P21, coinciding with the changes in electrophysiology (Van-Praag and Frenk 1991). However, before P21 the PAG-RVM axis does show exclusive powerful facilitation of cord spinal, greater than those seen in the adult (Hathway, Koch and Fitzgerald 2009). This facilitation may be due to noradrenergic processes as opposed to serotonergic, as they appear to mature earlier in the second post-natal week, however this remains to be fully elucidated (Hughes and Barr 1988). Thus P21 appears to be a

critical threshold in descending control of spinal nociceptive processing, where a switch between tonic excitation to inhibition occurs.

Sensitisation

The systems described above can become sensitised to increase neuronal responses to noxious stimuli. This can occur either in the peripheries or in the CNS, sensitisation of both of these areas is fundamental to the development of chronic pain conditions, including that induced by cisplatin and other chemotherapeutic treatment.

Peripheral sensitisation

In the area immediately surrounding any damaged tissue there is a 'red flare' area associated with primary hyperalgesia. This area has increased responses to mechanical and thermal stimuli. Damaged tissue releases a number of inflammatory mediators including; bradykinin, serotonin, prostaglandins, cytokines, and H^+. These mediators can either directly activate the nociceptors to produce action potentials, for example bradykinin can activate receptors to produce signals in Aδ and C-fibres, or can sensitise the receptors so they are more readily activated by other stimuli (Mizumura, et al. 2009).

Alternatively, inflammatory mediators can upregulate the transcription of new membrane ion channels and receptors. When activated GPCRs can activate membrane bound ion channels to depolarise the cell and produce an action potential, they also initiate a number of intracellular cascades (Rang, Bevan and Dray 1991). MAPK is produced which is involved in the regulation of transcription and translation of DNA, thus altering the synthesis of protein receptors. Adenylate cyclase, is also activated to convert adenosine monophosphate (AMP) to the second messenger cyclic AMP (cAMP) which activates the enzyme protein kinase A (PKA) which in turn activates the transcription factor cAMP response element-binding protein (CREB) which like MAPK control transcription and therefore protein synthesis (Stanley 1991). TRPV1 receptors and tetrodotoxin-resistant (TTX-R) Na^+ channels are examples of receptors that are upregulated by the activation of

GPCRs, increasing the levels of these channels causes sensitisation of the dendrite to noxious stimuli (Cheng and Ji 2008). Aracadonic acid is also produced via activation of the enzyme phospholipase A2 (PLA2), this acts as a secondary messenger to activate inflammatory prostaglandin production (Julius and Basbaum 2001).

The peripheral sensitisation discussed so far has been in response to the presence of a sensitising stimulus, for example tissue injury or inflammation. In the case of nerve injury, as with chemotherapy treatment, damage to the nerves gives rise to neuropathic pain arising from the nerves themselves, separate to external stimulus, which can continue long after the original cause has concluded. Because of this neuropathic pain can be considered a separate condition in its own right, to the cause of the injury (Von Hehn, Baron and Woolf 2012). Spontaneous and ectopic firing of the PAFs is a major driver of sensitisation following nerve injury, producing paresthesia, dysthesia and pain. While the injured neurones directly affected by do under go changes it is thought to be the neighbouring uninjured neurones that exhibit altered firing patterns and drive painful sensations (Von Hehn, Baron and Woolf 2012).

Alterations in the expression of ion channels in neighbouring PAFs drive the increased sensitisation and ectopic firing patterns, particularly alterations in the TRP_{V1}, sodium and potassium channels. As with other peripheral sensitisation, neurotrophins, particularly NGF and cytokines are released at the site of injury, these activate kinase pathways to alter gene expression. TRP_{V1} is upregulated in nociceptive fibres with expression correlating with development of thermal hyper and hypoalgesia (Hong and Wiley 2005), in addition, TRP_{V1} also starts to be expressed in large diameter, non-nociceptive Aβ fibres, changing the phenotypic behaviour of those fibres (Pabbidi, et al. 2008). TRP_{V1} knock out mice, do not demonstrate heat hyperalgesia following treatment with Cisplatin (Ta, et al. 2010). Potassium channels, responsible for maintaining the -70mV resting membrane potential, are decreased following activation of protein kinase C Zeta type (PKMζ) by neurotrophins. TWIK-related spinal cord potassium channel (TRESK) is highly expressed in the DRG. However, following nerve injury there is a 30-40% down-regulation, causing a steady depolarisation of the normal resting

membrane potential in the sensory neurones (Tulleuda, et al. 2011). Influx of sodium ions to the neurones depolarised the cell generating the action potentials. Voltage gated sodium ion channels are expressed differentially by fibre type, during nerve injury expression is altered. Na_V 1.6 is expressed in non-nociceptive PAF, Na_V 1.7 is expressed across all somatosensory fibres, Na_V 1.8 is expressed in nociceptive A and C fibres and Na_V 1.9 in C fibres. There is a downregulation of all of these sodium channels in the soma following nerve injury. However, the normally embryonic form Na_V 1.3, expression remerges in the injured adult PAF. In addition, in the neighbouring undamaged fibres, Na_V 1.3 and Na_V 1.8 are upregulated, supporting that these are the main drivers of neuropathic pain. Together these ion channel alterations serve to produce hyperexcitability in the primary sensory neurones, leading to ectopic firing patterns (Von Hehn, Baron and Woolf 2012).

Central sensitisation

As in the periphery, prolonged nociceptive activation alters the afferent somatosensory network activity and structure, lowering the magnitude of stimulus required to pass the activation threshold, making the (patient) more sensitive to noxious stimuli.

There are two stages of central sensitisation, in the acute phase the sensitisation is activity dependant and will return to normal firing patterns once increased activity from the primary afferent fibres desists. In the chronic stage, there is a change in the transcription of the cell altering the plasticity of the cell making the changes permanent. If the chronic stage persists permanent cellular loss can be seen (Latremoliere and Woolf 2009).

One of the two experimentally determined mechanisms present in acute central sensitisation is wind up of second order neurones by C-fibres. Under normal conditions, firing of the primary afferent fibres causes Ca^{2+} influx through voltage gated ion channels which triggers exocytosis of fast acting glutamate neurotransmitter from the presynaptic membrane. However, when the rate of firing is increased more Ca^{2+} is present in the axon terminal which causes the release of substance P in addition to glutamate (Budai 2000). Because substance P is slow acting, if the stimuli is persistent or

intense, it doesn't get cleared from neurokinin1 (NK1) receptors before the next stimulation arrives, producing short term excitability in the second order neurone. Wind up disappears quickly once the higher rate of stimuli stops (Schaible 2006).

The other acute mechanism is the recruitment of glutamatergic ion channel N-methyl-D-aspartate (NMDA) receptors on the post-synaptic membrane. Under normal conditions, NMDA receptor's channel is blocked by a magnesium plug preventing influx of ions. During high intensity firing depolarisation of the membrane dislodges the magnesium plug allowing influx of Na^+, K^+ and Ca^{2+} increasing the sensitivity of the membrane (Budai 2000). As with wind up, once high intensity firing ends the magnesium block reasserts itself and sensitivity returns to normal (Schaible 2006).

During the chronic phase of central sensitisation permanent changes start to be seen in the cell. The second order neurones start to produce pro-inflammatory mediators such as Prostaglandin E2 (PGE_2), aracadonic acid, nitrogen oxide and cytokines that diffuse back across the synapse to further enhance the action of the primary order neurone (Schaible 2006). These mediators also increase the transcription of membrane receptors and ion channels to further increase the sensitivity of the second order neurones (Julius and Basbaum 2001).

Chronic high intensity firing also causes inhibition of the inhibitory interneurons resulting in disinhibition of the synapse. If this persists for a long time, the influx of Ca^{2+} into the cell activates apoptotic pathways and the cell dies off. As well as the loss of the tonic inhibition to the nociceptive synapse, hard-wiring the hyperalgesia, there is also a loss of modulatory control from the supraspinal centres (Latremoliere and Woolf 2009).

Neurones are not the sole components of the spinal cord, the modulatory signals released from the neurones during central sensitization (as discussed above) also act on glial cells in the surrounding environment. Interactions with glial cells are an important factor in the functioning and modulation of nociceptive pathways, astrocytes and microglia can actively modulate nociceptive pathways by facilitating or attenuating neuropathic pain signals.

Microglia, the neuro-immune glia cells derived from bone marrow, are one of the major glial cell types that contribute to central sensitisation. The increased release of excitatory neurotransmitters as well as pro-inflammatory peptides, cytokines and chemokines, from the sensitised neurone, activate microglial cells in the dorsal horn. Excitatory neurotransmitters including: glutamate, ATP and NO, activate microglia and cause migration to the site of activation (Lively and Schlichter 2013) as well as the release of further ATP from the microglia themselves (Milligan and Watkins 2009). Substance P acts on P2X receptors, cytokines such as IL-1 β and TNF α, act through the toll like receptors (TLRs) to activate intracellular cascades through the extracellular signal-regulated kinase (ERK), c-Jun N-terminal Kinase (JNK), and p38 MAPK pathway (Kaminska 2005) to cause the upregulation and release of further cytokines, which feedback to the neurone to activate similar intracellular transcription controlling pathways and increase ion channel production, thereby increasing the sensitivity of the cell (Latremoliere and Woolf 2009).

Astrocytes encompass the synapse and can directly facilitate or attenuate synaptic transmission. Similar to microglia, astrocytes are activated by pro-inflammatory cytokines and chemokines. The MAPK1 and JNK signalling pathways are activated to upregulate the synthesis of pro-inflammatory factors, IL1β, IL6, tumour necrosis factor α (TNFα), PGE_2, and nitric oxide (NO) from the astrocyte. If activation of the astrocyte is prolonged, glutamate transporters (such as GLT1 and GLAST) by the astrocyte to clean the synapse are downregulated leading to an increase in excitatory glutamate present in the synapse (Milligan and Watkins 2009).

The different types of glia are suggested to be activated and acting during separate stages of central sensitisation, with microglia activated during the initial inflammatory pain, which in turn releases factors causing prolonged activation of astrocytes during neuropathic pain (Milligan and Watkins 2009). However, in juvenile animals (Ikeda, Kiritoshi and Murase 2012) suggested the opposite with microglia involved in neuropathic pain and astrocytes during inflammatory pain.

So far what has been described has been one-on-one interactions between a presynaptic primary afferent neurone and a post synaptic second order neurone. However, in reality many primary afferent neurones can synapse with a single secondary order neurone, and in the case of WDR neurones can synapse with a mix of innocuous Aβ fibres and nociceptive Aδ and C-fibres (Gangadharan and Kuner, 2013). Thus when a WDR neurone become sensitised by high intensity firing from nociceptive stimuli, they are also sensitised to input from innocuous stimuli. This could be one of the cellular mechanisms responsible for producing allodynia.

Early life insults

During early development, particularly before the critical threshold at p21, nociceptive insults can have a profound impact on the development of somatosensory and nociceptive systems, altering adult processing of pain.

The extent and effect of the alterations is dependent on the type of neonatal insult. Mechanical insults from repeated pin-pricks produces thermal hyperalgesia. Irritating the colon mechanically or chemically produces visceral hypersensitivity in the adult (Fitzgerald 2005). Inflammatory insults, can produce a counterintuitive global increase in baseline pain thresholds (hypoalgesia) (Walker, Fitzgerald and Hathway 2015). For example, injection of the inflammatory agent carrageenan to the hind paw in neonates produces an initial thermal and mechanical hyperalgesia in just the first week, then a sustained generalised thermal hypoalgesia into adulthood. However, any further injury to the paw then produces an increased hyperalgesia response (Ren, et al. 2004).

It is rare for neonates with nerve injury to develop neuropathic pain (as happens in adults). Suppression of the inflammatory response suppresses the development of neuropathic pain until adolescence. Following nerve injury in neonates before Postnatal day 25 (P25), an anti-inflammatory response is triggered with the release of interleukin 4 and 10 (IL4, IL10), as opposed to the pro-inflammatory response seen in adults with the release of TNF and brain-derived neurotrophic factor (BDNF). Administration of intrathecal anti- IL10 to nerve injured infant mice produced neuropathic

pain behaviour, suggesting IL10 was suppressing pain hypersensitivity. IL10 does appear to only suppress the pain rather than ablate it completely as a delayed emergence can occur later in adolescence (McKelvey, et al. 2015).

Several potential mechanisms for the developmental alterations to nociceptive thresholds have been proposed, both in the periphery and centrally. In the immediate vicinity of the insult, the inflammatory response to tissue damage causes the release of neurotrophic factors (Constantinou, et al. 1994). This could cause disruption of the normal neuronal pruning that takes place in the first few weeks of life, resulting in activity-independent over-abundance of nociceptive nerve endings in the affected area (De Lima, et al. 1999). Depending on which primary afferent fibres were affected, a strengthening or weakening of nociceptive pathways converging on the spinal cord may occur. Central control may also play a role in the development of hypoalgesia. Following nociceptive insult to the paw there was a resulting increase in descending inhibition from the RVM and a decrease in baseline sensitivity in the adults at 6 weeks post injury. Application of analgesia during the insult prevented this effect (Walker, Fitzgerald and Hathway 2015).

Summary

Cisplatin is a successful treatment for solid-state tumours due to its cytotoxic nature. However, it is this very nature that causes (among other side effects) neuropathic pain, severely detrimental to the quality of life of those affected. Prolonged pain causes fundamental changes to the nociceptive pathways involved. The functionality of the entire system, from the peripheral cells immediately affected, to the descending control in the CNS is affected. The immature nervous systems of children are particularly at risk, as the system is not yet hardwired and pain thresholds can be permanently altered.

In this project the effect of Cisplatin on juvenile nociceptive pathways will be investigated. This includes the peripheral afferent fibres carrying sensory information from the periphery that are directly affected by the Cisplatin, as well as the interneurons and glial cells in the CNS that can modulate incoming signals in the dorsal horn of the spinal cord. Any changes in these systems will be

investigated immediately following Cisplatin treatment for the acute affect and a month later for more long lasting chronic effects.

Hypothesis

The introduction of chemotherapeutic drugs to a juvenile rat will cause long term chronic pain by altering nociceptive neurochemistry in the dorsal horn of the spinal cord.

Methods

Animal model

Experiments were carried out in Sprague Dawley rats. Times mated pregnant dams were bought from Charles River UK, they were received at E17 and allowed to habituate. Experiments were conducted under UK Home Office regulations and in concordance with the Animal (Scientific Procedures) Act (1986) and conformed to the ARRIVE guidelines. Experiments were carried out under Home Office Project Licence 40/3468 and personal licences I627684BE and I56AAD26C. Approval was granted by the University of Nottingham Animal Welfare and Ethical Review Board. Animals were housed in light/dark cycled rooms with access to food and water *ab libitum*. Experimenters were blind to all treatment groups.

Cisplatin induced neuropathy

Pups of mixed gender were treated via IP injection of either 0.01M phosphate buffered saline (PBS) (Sigma, P4417-100TAB) vehicle or cisplatin (1ml/kg, 0.5 mg/ml) (Sigma, P4394-25MG) once a day, for 5 consecutive days, from postnatal (P) day 7 to P day 11. Animal weights and behaviours were monitored regularly for signs of distress. To minimise the chances of rejection by the mother and to reduce maternal and litter distress, the time that pups were absent from the home cage was minimised as much as possible. Additionally, prior to returning to the cage pups were rubbed in bedding material to remove foreign olfactory cues.

Animals were sacrificed at P13 (cisplatin n=8, vehicle n=11), or at P45 (cisplatin n=11, vehicle n=11), via IP injection of sodium pentobarbital (200mg/ml) (Euthatal). Tissues were preserved via transcardiac perfusion with 50ml 0.01M PBS followed by 50ml of 4% paraformaldehyde (Sigma, 441244-1kg) using a 50ml syringe (Fisher Scientific, DC-M0163) as a perfusion pump at a rate of 10-20ml per min. Spinal cords, DRG's and blood were collected for analysis.

Tissue preparations

Spinal cord samples were stored in frozen Optimal cutting temperature compound (OCT) embedding matrix (Fisher Scientific, 23-730-571) at -80^{o}c. Following removal from OCT, spinal cords were thawed over wet-ice, to prevent freeze-thaw tissue damage, and immersed in 0.04% sucrose azide (0.4g sodium azide (Sigma, S8032-25G), 300g sucrose (Sigma, S0389-5KG), and 1L ddH_2O). Spinal cord samples were given 48 hours to fully absorb the sucrose azide solution. Subsequently 40 µm thick sections of the lumbar enlargement were prepared using a Leica SM2010R frozen microtome and placed into a test tube containing fresh Sucrose Azide and stored until needed at 4^{o}C.

DRG samples were stored in frozen OCT at -80^{o}c. While still frozen in OCT, DRG samples were sliced into 10µm thick sections using a Leica CM1900 cryostat and mounted upon Superfrost plus microslides (VWR international, 48311-703). Up to 4 DRG's per animal were bundled and sliced together, with multiple sections of each DRG being taken. Slides were stored until needed at 4^{o}C.

Blood was collected during intercardiac perfusion in P13 rats. Blood was stored with 50 µl of heparin (Wockhardt, PA 1339/9/4) into 0.5ml microtubes (Sarstedt, 72.699). Samples were spun down at 13,000 rpm on a Thermo Scientific Heraeus Pico17 centrifuge for 5 minutes to separate plasma from erythrocytes, leukocytes and platelets. Blood plasma was pipetted out and stored at -80^{o}C.

Immunohistochemistry

Spinal cords

Spinal cord samples were immunostained using free-floating immunohistochemistry protocols. Samples were washed in 0.1% PBS 3x for 5 minutes to remove sucrose azide. Sections were then incubated with 3% blocking solution (0.1M PBS, 3% goat or donkey serum, 0.3% triton X-100 (sigma, X100-100ML)) for one hour at room temperature. Primary antibodies were prepared in the dilutions below in trizma hydrochloride solution (TTBS) (300ul Triton X-100 (Sigma, T9284-500mL), 0.87g NaCl (Fisher Scientific, S/3160/63) and 100ml ddH_2O (pH approximately 7.4)). Primary antibodies, sources, concentrations and incubation times are: Mouse anti-NeuN (Millipore) (1:100), overnight. Mouse

anti-GFAP (Fisher Scientific) (1:100) overnight, co-stained with rabbit anti-IBA1 (WAKO) (1:1000) for 72 hours. IB4-FITC conjugate (Sigma) (1:500) overnight, co-stained with rabbit anti-CGRP (Millipore) (1:100) overnight and goat anti-TrkA (R&D Systems) (1:100), overnight. (followed by Donkey anti-goat biotin (1:500) 2 hours). Mouse anti-NF200 (Sigma) (1:750) 24 hours, co-stained with rabbit anti-VGLUT2 (Synaptic Systems) (1:750) 24 hours.

Table 1: Primary antibodies used in Spinal immunohistochemistry

PRIMARY ANTIBODY	COMPANY	DILUTION	INCUBATION TIME
MOUSE ANTI-NEUN	Millipore	1:100	Overnight
MOUSE ANTI-GFAP	Fisher Scientific	1:100	Overnight
RABBIT ANTI-IBA1	WAKO	1:1000	72 Hours
IB4-FITC CONJUGATE	Sigma	1:500	Overnight
RABBIT ANTI-CGRP	Millipore	1:100	Overnight
GOAT ANTI-TRKA	R&D systems	1:100	Overnight
DONKEY ANTI-GOAT BIOTIN	*Jackson*	*1:500*	*2 Hours*
MOUSE ANTI-NF200	Sigma	1:750	24 Hours
RABBIT ANTI-VGLUT2	Synaptic systems	1:750	24 Hours

The samples were then washed in PBS 5x for 10 minutes. Life technologies secondary antibodies used were; Goat anti-mouse 488 (1:500), goat anti-mouse 555 (1:500), goat anti-rabbit 488 (1:500), goat anti rabbit 555 (1:500), goat anti-rabbit 568 (1:500), streptavidin 405 (1:1000) and donkey anti-rabbit 568 (1:500). Secondary antibodies were prepared in the dilution above in TTBS. Samples were incubated with secondary antibodies for 2 hours at room temperature in the dark, before washing in PBS 5x for 10 minutes. Sections were mounted on gelatinised slides from University of Nottingham AMU, and allowed to dry overnight in the dark. Slides were cover slipped (22x50mm) using flouromount (sigma, F4680-25ML), sealed and stored in the dark at 4°c until needed.

Table 2: Secondary antibodies used for spinal immunhistochemistry

SECONDARY ANTIBODY	COMPANY	DILUTION	INCUBATION TIME
GOAT ANTI-MOUSE 488	Life technologies	1:500	2 Hours
GOAT ANTI-MOUSE 555	Life technologies	1:500	2 Hours
GOAT ANTI-RABBIT 488	Life technologies	1:500	2 hours
GOAT ANTI-RABBIT 555	Life technologies	1:500	2 Hours
GOAT ANTI-RABBIT 568	Life technologies	1:500	2 Hours
STREPTAVIDIN 405	Life technologies	1:1000	2 Hours
DONKEY ANTI-RABBIT 568	Life technologies	1:500	2 Hours

DRG's

DRG samples were stained using slide-mounted immunohistochemistry. Slides were washed in 0.1% PBS 3x for 5 minutes to remove sucrose azide and were then incubated with 3% blocking solution (0.1M PBS, 5% bovine serum albumin (BSA), 10% fetal bovine serum (FBS), 0.2% triton X-100 (sigma, X100-100ML)) for one hour at 4^{o}C. Primary antibodies, sources, concentrations and incubation times are: Mouse anti-NeuN (Millipore) (1:100), co-stained with rabbit anti-CGRP (sigma) (1:1000) and anti-IB4 (1:500) overnight in the dark at 4^{o}c (followed by donkey anti-goat biotin (1:500) for 2 hours in the dark at 4^{o}c). Rabbit anti-PGP (millipore) (1:500) co-stained with mouse anti-NF200 (Sigma) (1:750) and goat anti-TrkA (R&D Systems) (1:100), all overnight at 4^{o}C. (followed by donkey anti-goat biotin (1:500) for 2 hours in the dark at 4^{o}c).

Table 3: Primary antibodies used for DRG immunohistochemistry

PRIMARY ANTIBODY	COMPANY	DILUTION	INCUBATION TIME
MOUSE ANTI-NEUN	Millipore	1:100	Overnight
RABBIT ANTI-CGRP	Sigma	1:1000	Overnight
GOAT ANTI-IB4		1:500	Overnight
DONKEY ANTI-GOAT BIOTIN	*Jackson*	*1:500*	*2 Hours*
RABBIT ANTI-PGP	Millipore	1:500	Overnight
MOUSE ANTI-NF200	Sigma	1:750	Overnight
GOAT ANTI-TRKA	R&D systems	1:100	Overnight
DONKEY ANTI-GOAT BIOTIN	*Jackson*	*1:500*	*2 Hours*

The samples were then washed in PBS 5x for 10 minutes. Life technologies secondary antibodies used were; goat anti-mouse 488 (1:500), goat anti-mouse 555 (1:500), goat anti-rabbit 488 (1:500), goat-anti rabbit 568 (1:500), and streptavidin 405 (1:1000). Secondary antibodies were prepared in the dilution above in PBS. Slides were incubated with secondary antibodies for 2 hours in the dark at 4°c, before washing in PBS 5x for 10 minutes. Slides were allowed to dry overnight in the dark. Slides were cover-slipped (22x50mm) using flourosave (Millipore, 345789), sealed and stored in the dark at 4°C until needed.

Table 4: Secondary antibodies used for DRG immunohistochemistry

SECONDARY ANTIBODY	COMPANY	DILUTION	INCUBATION TIME
GOAT ANTI-MOUSE 488	Life technologies	1:500	2 Hours
GOAT ANTI-MOUSE 555	Life technologies	1:500	2 Hours
GOAT ANTI-RABBIT 488	Life technologies	1:500	2 hours
GOAT ANTI-RABBIT 568	Life technologies	1:500	2 Hours
STREPTAVIDIN 405	Life technologies	1:1000	2 Hours

Microscopy

Slides were imaged using DMRB fluorescent (for P45) or Leica SPE confocal microscope (for P13 and DRG's) due to availability. An image was taken at 10x magnification of the right dorsal horn of each spinal cord section, or the entire section of DRG. Some spinal cord samples were too fragile to be successfully sectioned and stained, due to their age and the cisplatin treatment, therefore n=4 were imaged for both cisplatin and vehicle groups. For DRG's n=8 were imaged for both cisplatin and vehicle groups. Cisplatin and vehicle groups were imaged in the same session using the same settings.

IL6 ELISA kit

Blood plasma samples were tested using Rat interleukin 6 (IL6) ELISA kit from Eagle Biosciences (IL621-K01). N=6 for both cisplatin and vehicle groups. The general ELISA protocol outlined by Eagle Biosciences was followed include details. A standard serial dilution of a known concentration of IL6 was also tested to create a standard curve. The samples were analysed using a Sigma labtech

FLOUstar Omega Atmospheric control unit to find the optical density (OD) of the samples was given. The standard sample OD results were entered into http://www.elisaanalysis.com/ to create a 4 parameter logistic (4-PL) ELISA curve for concentration. The OD's from the animal samples were compared against the 4PL curve to give the sample concentration. Cisplatin and vehicle concentrations were compared and analysed for statistical significance using GraphPad Prism (version 6.07) using an unpaired T-test with Welch's correction. Graphs of serum IL6 concentration, were presented as the mean of concentrations across all animals, with standard error of the mean (SEM) bar.

Statistical Analysis

In the spinal cord stains: NeuN, GFAP and IBA1, cells counts were taken from five 100µm x 100µm regions of interest (ROI) for lamina I, II and V of the dorsal horn, using the ROI manager plugin on ImageJ (version 1.43m). Results for each group, were averaged to give a single cell count for each lamina, and then for the entire dorsal horn section. Results were statistically analysed using GraphPad Prism (version 6.07), results were tested for parametric normality with D'Agostino and Pearson omnibus normality test. Cisplatin and vehicle cell counts were compared and analysed for statistical significance using an unpaired T-test or a Mann-Whitney test. Graphs of cell counts, are presented as the mean of cell counts across all animals, with standard error of the mean (SEM).

In the spinal cord stains: CGRP, IB4, TrkA, NF200 and vGLUT2, 4x lines of interest were taken to measure grey value (AU) intensity using the plot profile tool on ImageJ. Lines extended 600µm, from the outer surface, down to lamina V of the dorsal horn, following the outer curve of the dorsal horn. This is a similar method as previously used by (Lorenzo, et al. 2008)**.** Results for each group were averaged to give a single intensity profile for cisplatin and vehicle groups. The area under the curve (AUC) was taken for the averaged intensity profile to create a figure for the entire dorsal horn. The averaged results were statistically analysed using GraphPad Prism (version 6.07), results were tested for parametric normality with D'Agostino and Pearson omnibus normality test. Cisplatin and vehicle

cell counts were compared and analysed for statistical significance using an unpaired T-test with Kolmogotov-Smirnov post-hoc test. Graphs for intensity lines, were presented as the mean of area under the curve (AUC), across all animals with SEM bar.

In the DRG stains: IB4, CGRP, NF200 and TrkA, cell counts were taken for the entire section and standardised as a percentage of the neuronal cell (NeuN or PGP) count. Results for each group, were averaged to give a single cell count for DRGs in each group. Results were statistically analysed using GraphPad Prism (version 6.07), results were tested for parametric normality with D'Agostino and Pearson omnibus normality test. Cisplatin and vehicle cell counts were compares and analysed for statistical significance using an unpaired T-test with Welch's correction. Graphs of cell counts, were presented as the mean of cell counts, across all animals with SEM bar

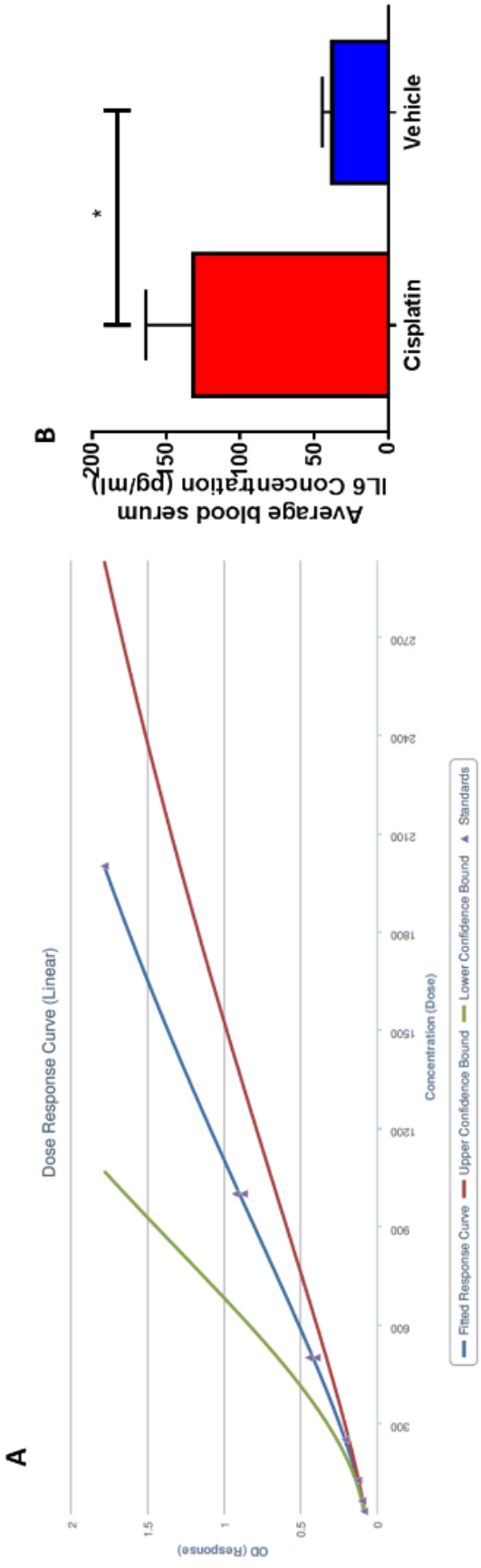


Figure 4: **Increase in blood plasma IL6 concentrations in P13 cisplatin animal model** (**A**) 4-PL ELISA standard concentration curve from http://www.elisaanalysis.com/ (**B**) P13 blood serum concentrations of IL6 (cis n=6, veh n=6) significant difference found (cis>veh) *p<0.05

Results

Juvenile Blood Plasma IL6 Concentration

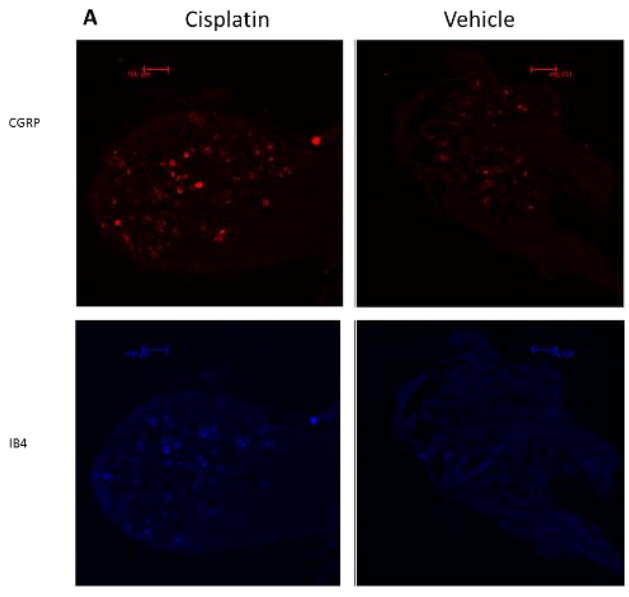
A statistically significant (p=0.0039) increase in IL6 was shown in the blood plasma of the cisplatin treated animals at P13 (131.9±31.91 pg/ml) compared to the vehicle animals (38.00±6.748 pg/ml) (Figure 4b), suggestive of systemic inflammation and possible neprotoxic side effects from the cisplatin treatment.

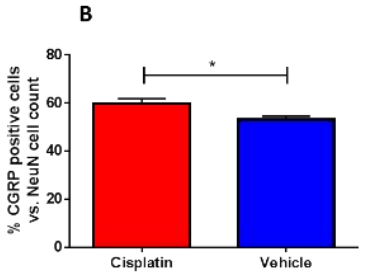
Juvenile DRG Immunohistochemisty

On postnatal day 16 (immediately following the end of cisplatin treatment at p11) in the rat dorsal root ganglion, cisplatin treatment only showed alterations in peptidergic neuronal number, after normalisation against the neuronal cell count.

There were no significant differences (p=0.0527) in IB4 positive non-peptidergic neurones with similar cell counts found between the cisplatin (56.86±2.625) and PBS vehicle (49.53±2.528) treatment groups (Figure 5c). Cisplatin (55.91±1.394) and PBS vehicle (51.58±1.850) treatment groups also showed no significant difference (p=0.0703) for neurofilament (NF200) positive markers in the DRG Figure 6b). Likewise, DRG TrkA positive NGF receptor markers did not show a significant difference (p=0.3031) between the cisplatin (74.53±1.732) an PBS vehicle (71.03±2.867) treatments (Figure 6c).

However, the cisplatin treated animal group (59.72±2.092) did show a significant (p=0.0152) increase in CGRP positive peptidergic neurones in the compared with the vehicle group (53.10±1.468) (Figure 5b).





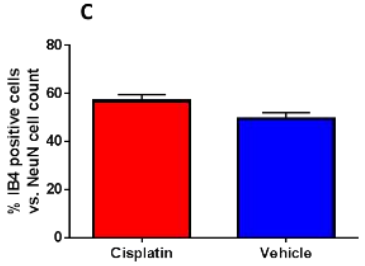
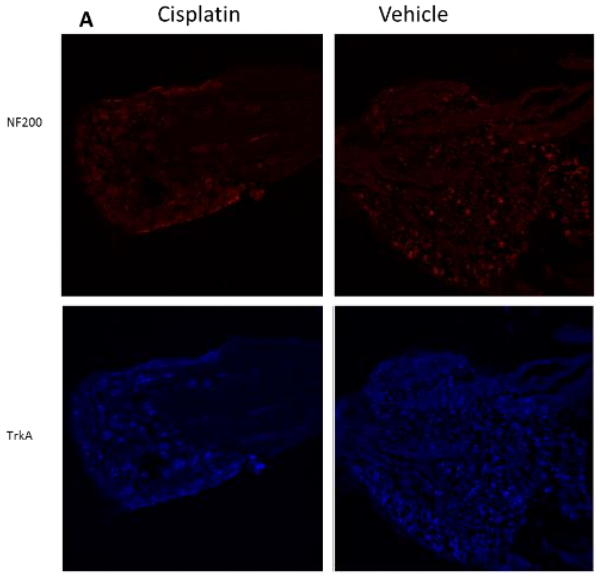
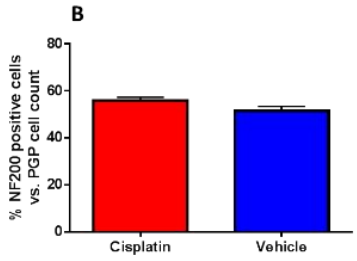


Figure 5: **CGRP cell counts increased in P16 DRGs, No statistical difference for IB4 positive cell counts** (**A**) immunohistochemistry of P16 DRG with CGRP and IB4 (**B**) Averaged CGRP cell counts normalised against NeuN cell count, across all animals (cis n=2, veh n=2), significant difference shown (cis>veh) *P<0.05 (**C**) Averaged IB4 cell counts normalised against NeuN cell count, across all animals (cis n=2, veh n=2), no significant differences shown.





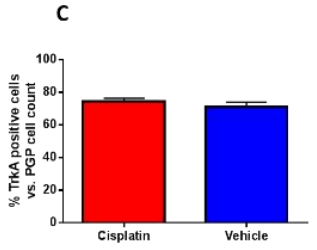


Figure 6: **No statistical difference for NF200 or TrkA positive cell counts in P16 animal DRGs** (**A**) immunohistochemistry of P16 DRG with NF200 and TrkA (**B**) Averaged NF200 cell counts normalised against PGP cell count, across all animals (cis n=2, veh n=2), no significant differences shown (**C**) Averaged TrkA cell counts normalised against PGP cell count, across all animals (cis n=2, veh n=2), no significant differences shown

Juvenile Spinal Cord Immunohistochemistry

On postnatal day 13, immediately following the end of cisplatin treatment at p11, Cisplatin treatment caused significant acute histological changes to occur in the spinal cord of treated animals compared to their PBS treated counterparts.

At the acute time point immediately following the end of cisplatin treatment there was a significant change in some cellular markers in the dorsal horn of the spinal cord. The central terminals of CGRP-positive DRG cells were significantly increased ($P<0.0001$) in cisplatin treated animals (52.06±0.7) compared with the PBS vehicle injected animals (39.65±0.5) (Figure 8c). IB4-positive non-peptigergic DRG cell terminals were also showed a significant increase ($p<0.0001$) in cisplatin animals (17.22±0.3) vs. PBS vehicle animals (13.08±0.1) Figure 8e). These results indicate that at a cellular level there are changes in both primary afferent termination within the dorsal horn.

However, there was no significant differences ($p=0.5678$) in NeuN positive non-specific neuronal cells, cisplatin average cell counts were 18.58±0.7002 compared with 19.18±0.7913 in PBS vehicle treated animals (Figure 7b). Similarly, Cisplatin treated animals (6.749±0.1491) compared PBS vehicle treated animals (7.079±0.1241) showed no significant change ($p=0.0913$) in GFAP positive astrocyte glial cells (Figure 7d). This indicates that during the acute phase immediately following cisplatin treatment, there were no changes in the number of neurones or states of reactive gliosis in the dorsal horn. Interestingly however, significant decreases can be seen in these cellular markers in particular laminae. NeuN positive neurones only in lamina 1 did show a significant decrease (0.0016) in cell counts in cisplatin treatment (24.85±0.3273) compared with PBS vehicle treatment (26.24±0.2507) (Figure 7c). GFAP positive astrocyte cell counts showed a significant decrease ($p=0.0026$) only in lamina 2 in the cisplatin group (5.727±0.1542) vs the PBS vehicles (6.5±0.1824) (Figure 7e). This is suggestive that any acute changes to afferent input are specific to each lamina.

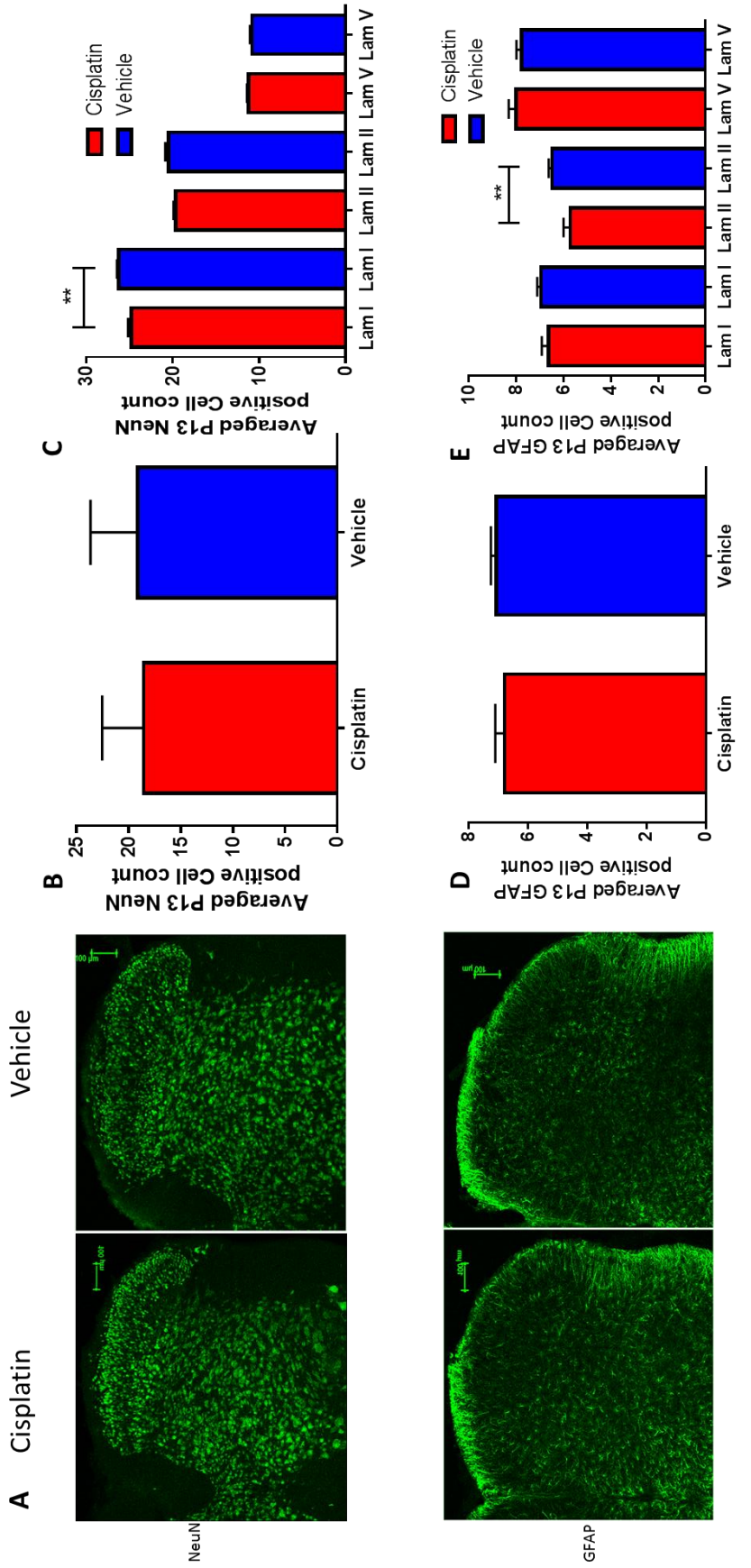


Figure 7: **No statistical difference for NeuN or GFAP positive cell counts in P13 spinal cord dorsal horn (A)** immunohistochemistry of P13 rat spinal cord with NeuN and GFAP positive cells (**B**) Averaged NeuN positive cell count across all animals (cis n=4, veh n=4) and all laminae of the dorsal horn with SEM, no overall significant difference found (**C**) Averaged NeuN positive cell count across all animals (cis n=4, veh n=4) for each laminae of the dorsal horn with SEM significant difference found only in lamina 1 (cis<veh) *p<0.05 (**D**) Averaged GFAP positive cell count across all animals (cis n=4, veh n=4) and all laminae of the dorsal horn with SEM, no overall significant difference found (**E**) Averaged GFAP cell count across all animals (cis n=4, veh n=4) for each laminae of the dorsal horn with SEM significant difference found only in lamina 2 (cis<veh) *p<0.05

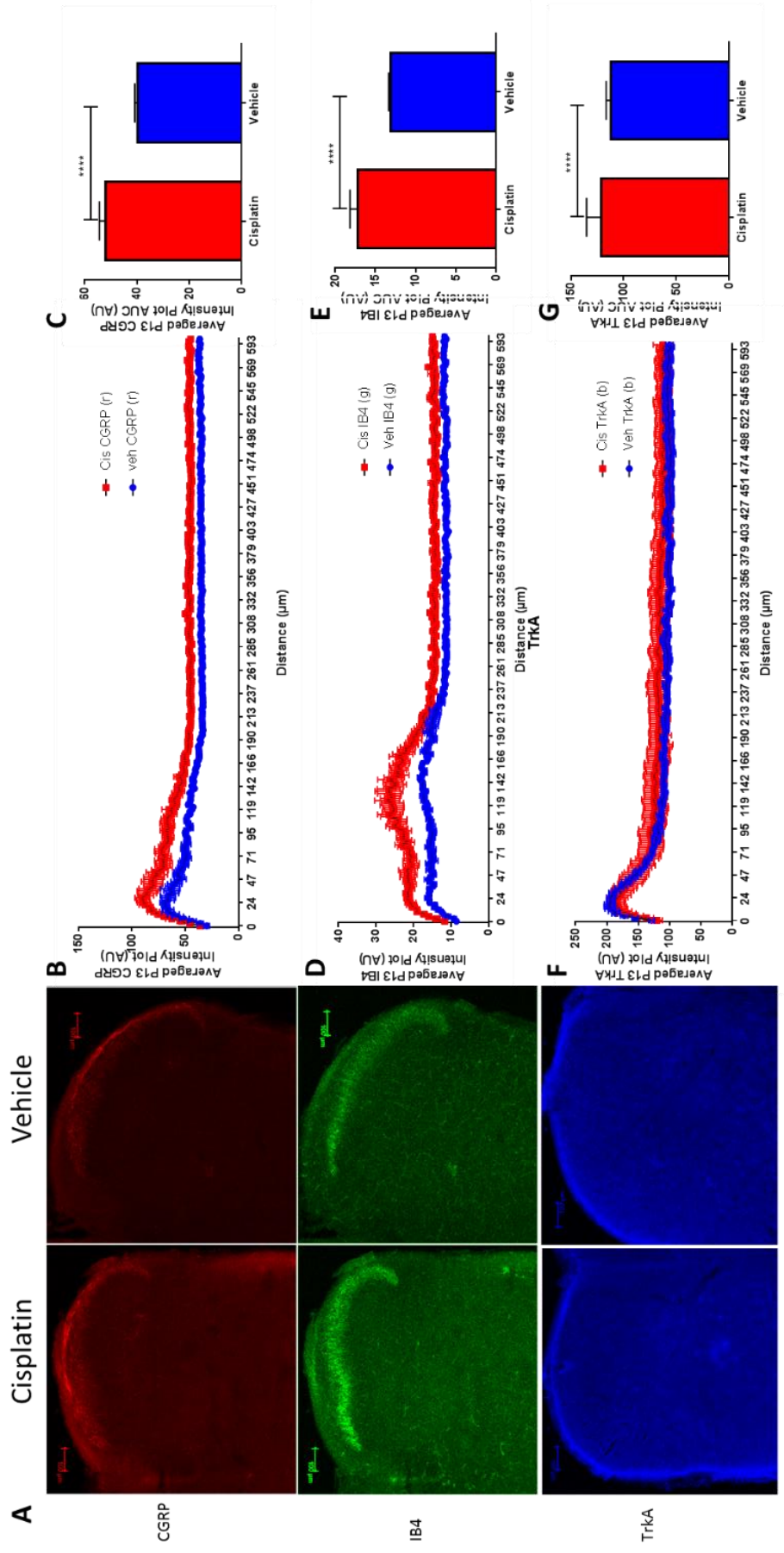


Figure 8 : **CGRP, IB4 and TrkA expression increased in P13 spinal cord dorsal horn (A)** immunohistochemistry of P13 rat spinal cord with CGRP, IB4 and TrkA (**B**) Averaged CGRP positive intensity 600µm line across all animals (cis n=4, veh n=4) (**C**) AUC for CGRP averaged across all animals with SEM, significant difference shown (cis>veh) *p<0.05 (**D**) Averaged IB4 intensity 600µm line across all animals (cis n=4, veh n=4) (**E**) AUC for IB4 averaged across all animals with SEM significant difference shown (cis>veh) *p<0.05 (**F**) Averaged TrkA intensity 600µm line across all animals (cis n=4, veh n=4) (**G**) AUC for TrkA averaged across all animals with SEM significant difference shown (cis>veh) *p<0.05

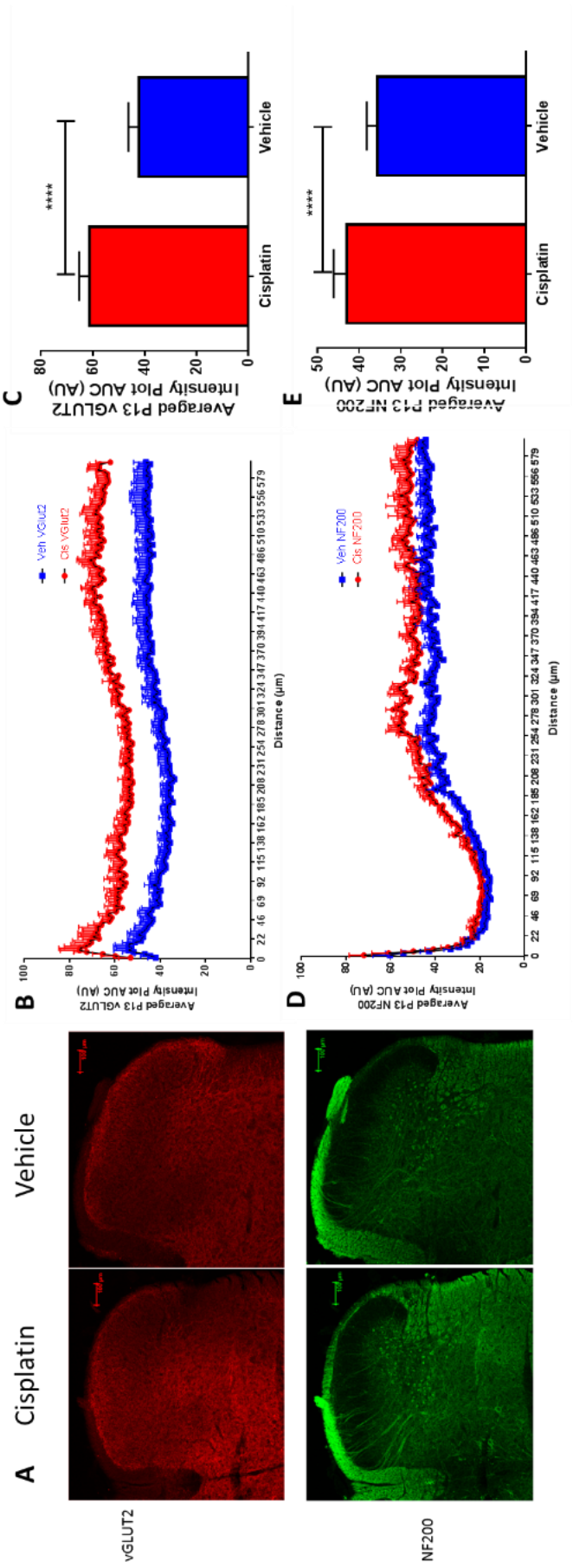


Figure 9 : **vGLUT2 and NF200 expression increased in P13 spinal cord dorsal horn (A)** immunohistochemistry of P13 rat spinal cord with vGLUT2 and NF200 **(B)** Averaged vGLUT2 intensity 600µm line across all animals (cis n=4, veh n=4) **(C)** AUC for vGLUT2 averaged across all animals with SEM significant difference shown (cis>veh) * p<0.05 **(D)** Averaged NF200 intensity 600µm line across all animals (cis n=4, veh n=4) **(E)** AUC for NF200 averaged across all animals with SEM significant difference shown (cis>veh) * p<0.05

There are also changes in intercellular receptor markers immediately following cisplatin treatment. NGF receptor TrkA markers showed a significant increase ($p<0.0001$) in intensity across the dorsal horn in cisplatin treated animals (121.0±1.0) compared with PBS vehicle treated animals (112.1±1.4) (Figure 8g). Intensity for excitatory vascular glutamate receptor (vGLUT2) markers also was significantly increased ($p<0.0001$) in cisplatin treated group (61.32±0.4) vs the PBS vehicle treated group (42.26±0.3) (Figure 9c). Finally, intensity for NF200 markers found a significant increase ($p<0.0001$) in the cisplatin treated group (43.03±0.8) compared to the PBS vehicle treated group (35.67±0.6) (Figure 9e). This data is indicative of acute upregulation of receptors and markers for neuronal sprouting in the DRG terminals of the dorsal horn of the spinal cord.

Adult Spinal Cord Immunohistochemistry

On postnatal day 45, 34 days after the end of cisplatin treatment at p11, Cisplatin treatment caused significant chronic histological changes to occur in the spinal cord of treated animals compared to their PBS treated counterparts.

At the chronic time point long after the end of cisplatin treatment there was a significant change in some cellular markers in the dorsal horn of the spinal cord. IB4 positive non-peptidergic neuronal markers showed a significant increase ($p=0.0001$) in intensity across the dorsal horn in cisplatin treated animals (27.9±1.2) compared with PBS vehicle treated animals (23.6±0.9) (Figure 11e). A significant decrease ($9=0.0001$) was seen in cisplatin treated animals (6.6±0.1) compared with PBS treated vehicle animals (9.2±0.1) for IBA1 positive microglial cell counts (Figure 10c). This suggests long-term cell type specific changes in dorsal horn innervation and depletion of CNS immune cells.

There were no significant differences ($p=0.3$) between cisplatin (17.8±0.6) and vehicle (16.8±0.7) treatment groups in NeuN positive non-specific neuronal cell at the chronic time point (figure 10b). CGRP positive peptidergic neurones also showed no significant differences ($p=0.3$) between cisplatin (43.7±3.1) and vehicle (42.9±2.6) treatment groups (Figure 11c). Over the entire dorsal horn GFAP

positive astrocyte glia did not show any significant changes (p=0.1) between cisplatin (3.2±0.1) and vehicle (2.9±0.1) treatment groups (Figure 10f). However, in laminae V specifically, there was an increase in GFAP in cisplatin treated rats (2.7±0.2) compared with their PBS vehicle (2.2±0.1) treated counterparts (Figure 10g).

Changes in intercellular receptor markers long after the end of cisplatin treatment were also shown. There were significant increases (p=0.0001) in the NGF receptor TrkA intensity, in animals treated with cisplatin (93.54±41.8) vs those treated with PBS vehicle (59.2±2.8) (Figure 11g). Cisplatin treatment (43.8±0.2) also showed significant increases (p=0.0001) of intensity for the excitatory glutamate receptor vGLUT2 markers compared to PBS vehicle treatment (26.7±0.1) (Figure 12c). Intensity of NF200 markers in cisplatin treated animals (36.3±0.7) were significantly increased (p=0.0001) when compared against PBS vehicle treated animals (18.1±0.3) (Figure 12e). This data is indicative of chronic, long-term upregulation of receptors and markers for neuronal sprouting in neurones of the dorsal horn of the spinal cord.

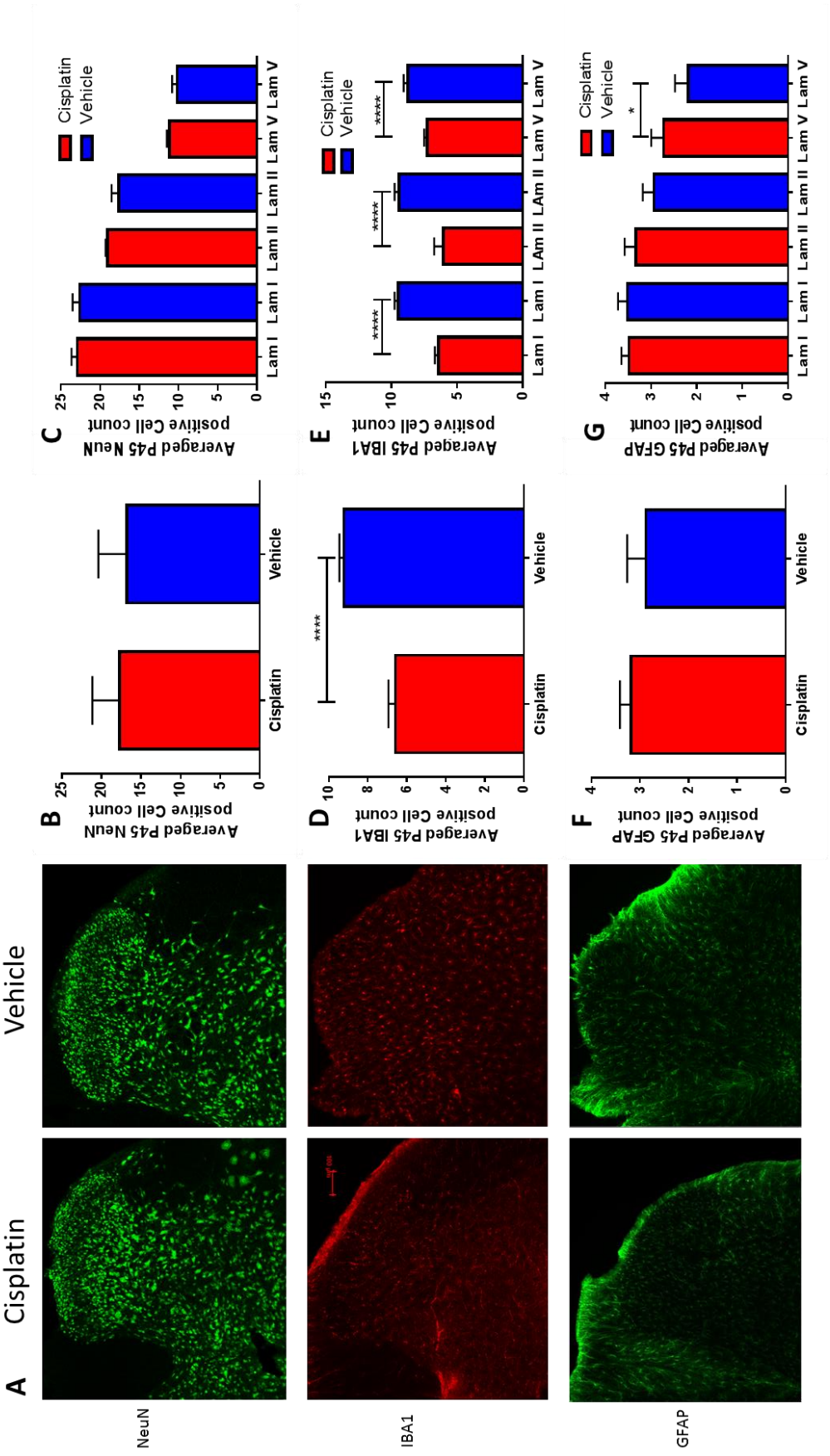


Figure 10 : **IBA1 cell count decreased in P45 spinal cord dorsal horn, no statistical difference for NeuN or GFAP cell counts** (**A**) immunohistochemistry of P45 rat spinal cord with NeuN, IBA-1 and GFAP (**B**) Averaged NeuN positive cell count across all animals (cis n=4, veh n=4) and all laminae of the dorsal horn with SEM, no significant differences shown (**C**) Averaged NeuN positive cell count across all animals (cis n=4, veh n=4) for each laminae of the dorsal horn with SEM *p<0.05 (**D**) immunohistochemistry of P45 rat spinal cord with NeuN, IBA-1 and GFAP (**E**) Averaged IBA-1 cell count across all animals (cis n=4, veh n=4) significant difference shown (cis>veh) *P<0.05 (**C**) AUC for IBA-1 averaged across all animals with SEM, significant difference shown in all laminae (cis>veh) *p<0.05 (**F**) Averaged GFAP intensity 600µm line across all animals (cis n=4, veh n=4) no significant difference shown (**G**) AUC for GFAP averaged across all animals with SEM, significant difference shown in laminae 5 (cis>veh) *p<0.05

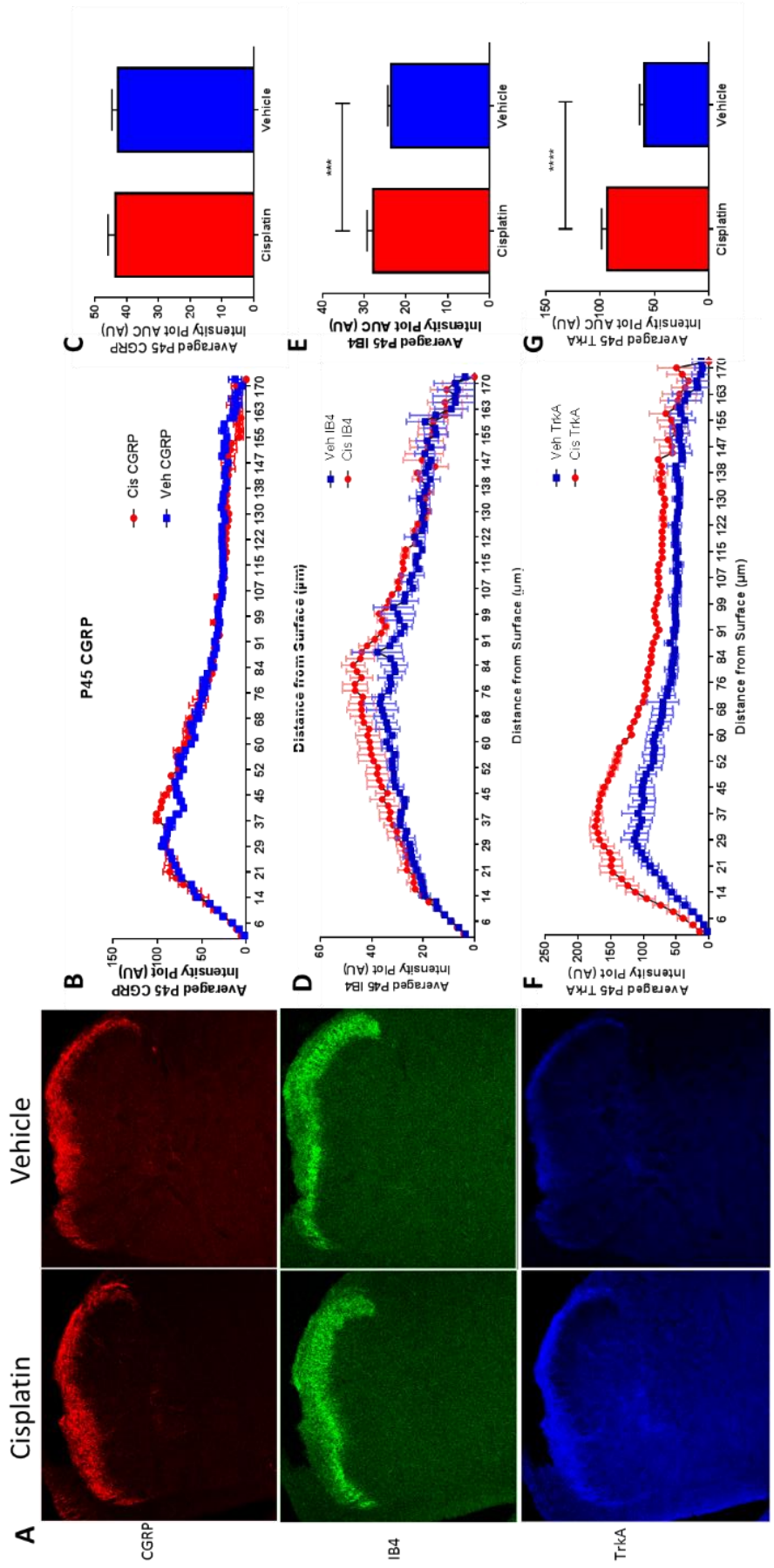


Figure 12: **IB4 and TrkA expression increased in p45 spinal cord dorsal horn, no statistical difference for CGRP expression** (**A**) immunohistochemistry of P45 rat spinal cord with CGRP, IB4 and TrkA (**B**) Averaged CGRP intensity 600µm line across all animals (cis n=4, veh n=4) (**C**) AUC for CGRP averaged across all animals with SEM, no significant differences shown (**D**) Averaged IB4 intensity 600µm line across all animals (cis n=4, veh n=4) (**E**) AUC for IB4 averaged across all animals with SEM, significant differences shown (cis>veh) *p<0.05 (**F**) Averaged TrkA intensity 600µm line across all animals (cis n=4, veh n=4) (**G**) AUC for TrkA averaged across all animals with SEM, significant differences shown (cis>veh) *p<0.05

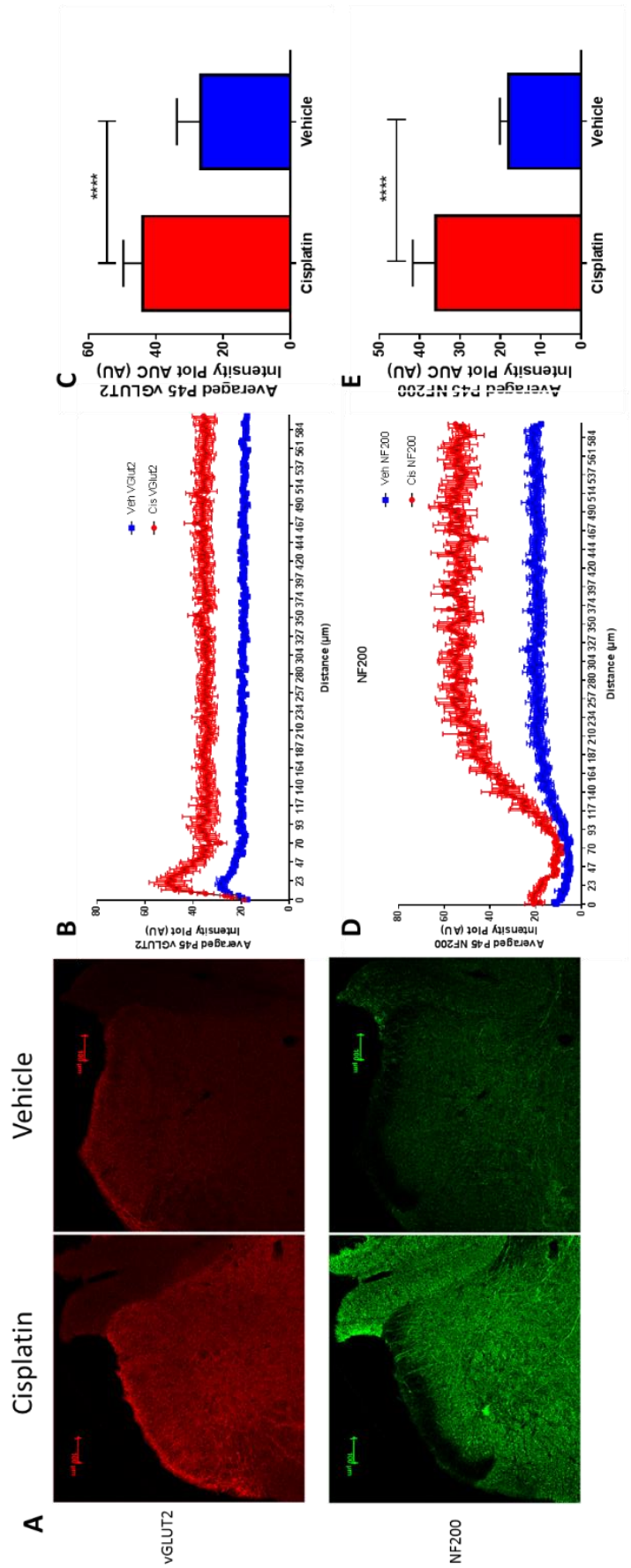


Figure 13: **vGLUT2 and NF200 expression increased in P45 spinal cord dorsal horn** (**A**) immunohistochemistry of P45 rat spinal cord with vGLUT2 and NF200 (**B**) Averaged vGLUT2 intensity 600µm line across all animals (cis n=4, veh n=4) (**C**) AUC for vGLUT2 averaged across all animals with SEM, significant differences shown (cis>veh) *P<0.05 (**D**) Averaged NF200 intensity 600µm line across all animals (cis n=4, veh n=4) (**E**) AUC for NF200 averaged across all animals with SEM, significant differences shown (cis>veh) *p<0.05

Discussion

6% of paediatric cancer patients are now surviving for at least 10 years or more (Cancer Research UK 2016). Therefore, we now have to look beyond the immediate survival of the children affected by cancer and at the long term impacts of the chemotherapeutic drugs given to sensitive developing systems. In this study we have shown that treatment with the cancer chemotherapeutic Cisplatin during the second postnatal week had significant effects on several components of the nociceptive system in both the peripheral and central nervous systems. These changes occurred both immediately following treatment at P13 (juvenile time point) and at P45, 34 days after the end of treatment.

Cisplatin induced peripheral sensory alterations

Chronic pain usually arises following the sensitisation of peripheral afferent nociceptive and non-nociceptive fibres to noxious stimuli. The cell bodies for these fibres lie in the DRG with peripheral processes to the skin and viscera and central processes in the dorsal horn of the spinal cord (Julius and Basbaum 2001). Inflammatory mediators (such as bradykinins, interleukins, growth factors, as well as others), released by damaged tissue and white blood cells, sensitise these fibres either by direct activation or the upregulation of expression of membrane bound receptors (Mizumura, et al. 2009). This leads to greater responses to noxious stimuli (hyperalgesia) or a pain reaction to a normally innocuous stimuli (allodynia) (Schaible 2006). We have previously shown that administration of Cisplatin to a neonatal rat pup produces both thermal and mechanical allodynia that is sustained long after Cisplatin treatment has ended (Figure 3). The histological changes in A- and C-fibres primary afferents in the DRG and spinal cord were investigated to elucidate any potential mechanisms that underlie this behavioural change.

In the DRG an increase in peptidergic C-fibres was seen at the juvenile time point. Peptidergic C-fibres are central to the development of central sensitisation via the release of substance P (Budai 2000) and the recruitment and sustained activation of NMDA receptors (Budai 2000). As well as the recruitment of NMDA receptors, peripheral sensitisation also causes the activation of GPCRs during

central sensitisation. GPCR activation induces an upregulation in protein synthesis, including membrane receptors and neuropeptides (including the vasodilator CGRP (Brain, et al. 1985)). Protein upregulation hard-wires activity-dependant changes in neuron function so they become permanent and activity independent (Schaible 2006). Thus, increased CGRP positive cell counts in the DRG indicates an upregulation of peptidergic gene expression. This concurs with previous research by Barajon *et al.,* who found neuropeptides CGRP and galanin were increased by Cisplatin treatment and concluded that DRG cells were the primary target of Cisplatin neurotoxicity (Barajon, et al. 1996). However the N number for this experiment was very low, durther DRG stains would be required to confirm this finding. DRG levels of NF200 and IB4 at the juvenile time point showed no difference between the treatment groups. This suggests that Aβ and non-peptidergic C fibres are not effected by cisplatin treatment. It might be therefore that Cisplatin's effects are limited to peptidergic C-fibres immediately following treatment. A small increase in the NF200 and IB4 cell counts was seen, this was statistically insignificant. However, this could be due to the low N number and may be altered with further samples. If significant this would concur with the increase in intensity seen in the dorsal horn terminals of the spinal cord.

Previous unpublished research by the University of Nottingham Developmental Neurobiology lab, also investigated further aspects of Cisplatin's paediatric effect. At P45 an increase in TrkA positive neuronal processes was seen in the DRG, as well as increases in PGP9.5-positive neuronal cells and CGRP-positive peptidergic neurones in the plantar skin. It was suggested that Cisplatin causes an increase in NGF/TrkA signalling causing an increase in peptidergic C-fibres in the DRG and signalling peripheral nerve growth. While this experiment saw an increase in TrkA signalling at both the juvenile and adult time points, the peptidergic C-fibre CGRP positive cells only showed a Cisplatin induced increase at the juvenile time point, however, this was seen both in the DRG as well as the dorsal horn.

As well as an increase in the DRG, Cisplatin treatment also caused an increase in juvenile peptidergic C-fibre terminations in the dorsal horn, however by the later adult time point, there was no

significant difference between the vehicle and Cisplatin treatment groups. This may suggest that Cisplatin only has a short term, acute effect on peptidergic C-fibres. We know that peptidergic C-fibres are important for the initial development of central nociceptive sensitisation in chronic pain states. The production of substance P, activates NK1 and NMDA receptors leading to sensitisation during high intensity firing. If spinal sensitisation is sustained, further alterations in the structure and functioning of spinal nociceptive pathways occur to hard-wire in the sensitisation (Schaible 2006). Munglani *et al.* found changes in C-fibre peptide expression persist 100-120 days' post injury in a chronic constriction injury (CCI) model. However, the changes they saw were peptide and location specific. Persistent decreases in the neuropeptides substance P and Galanin in laminae I and II and an increase in substance P in laminae III and IV. They also found no change in either CGRP or Neuropeptide Y levels in laminae I or II, which concurs with the findings of this study (Munglani, et al. 1996). These peptide specific alterations in gene expression in the spinal dorsal horn mean that an increase in CGRP alone doesn't confirm alterations in peptidergic C-fibre gene expression as a whole. We've already shown that the expression CGRP and NGF differs in long term pain, therefore further investigation into other C-fibre peptide expression is required to build a more complete picture of how chemotherapeutics effect peptidergic C-fibres long term.

The high affinity NGF receptor TrkA was also significantly increased at both time points. TrkA is preferentially expressed by peptidergic nociceptive neurones, as well as by projection neurons in the spinothalamic and spinoreticular tracts (Pezet, et al. 1999). It is involved with (among other functions) the regulation of gene expression for membrane receptors and the enzymes synthesising neurotransmitters, including substance P, CGRP and $TRPV_1$. Thus upregulation of TrkA results in an increase of neurotransmitters available for release (Berse, Lopez-Coviella and Blusztajn 1999). Upregulated expression of TrkA is triggered as a result of the increased presence of its main ligand NGF. NGF plays an important role in neuronal plasticity, promoting growth, survival and proliferation of neurones, in the spinal cord NGF is released by astrocytes and microglia (Watkins, et al. 1997). NGF may also directly provoke nociceptive responses through further spinal mechanisms, including

directly activating the noxious-heat and ligand gated TRP_{v1} receptor (Eskander, et al. 2015). High levels of NGF could upregulate expression of TrkA causing the increase seen. However other factors including retinoic acid (Holst, et al. 1995), interferon-γ (Lucarelli, Kaplan and Thiele 1996) and nicotine (Li, et al. 2005) have been shown to increase TrkA expression sensitising the cell to NGF. Interferon-γ is known to be released in the dorsal horn during central sensitisation, being involved with disinhibition of synaptic activity of primary afferent fibres (Vikman, et al. 2003), thus is a strong candidate for the increased expression of TrkA. Whatever causes the increase in TrkA, the result is that the neurone becomes sensitive to further NGF release, which increases production of neurotransmitters available to release, sensitising spinal nociceptive pathways. TrkA expression and by extension the sensitivity of spinal nociceptive pathways, by Cisplatin, appears to be cumulative over time. However, during normal development TrkA expression is downregulated in 37% of dorsal horn neurones by P21 (Molliver and Snider 1997). Thus, it could be the case that expression of TrkA decreases over time in the vehicle group but is elevated initially by the Cisplatin treatment then maintained, at least until P45, long after Cisplatin has been excreted from the subject. This would concur with the behavioural data previously shown, with allodynia being evident at P22 and plateauing until the end of the experiment.

In non-peptidergic C-fibre terminations a similar initial Cisplatin-induced increase in signal intensity was seen. However, unlike the peptidergic C-fibres, the increase was sustained to the later adult time point. This could suggest increased nociceptive signalling to the spinal cord by these fibres.

NF200 is a cyctoskeletal marker, used as a marker of A-fibre neurones (Ruscheweyh, et al. 2007). At both time points NF200 was absent in the superficial layers of the dorsal horn (50-95µm at p13 and 35-91µm at p45), reaching a peak and plateau in deeper layers (280µm at p13 and 230µm at p45). There is no definitive guide to the depth of laminae, however, NF200 appears to be absent from laminae I and II and present in laminae III or IV (Wu, et al. 2010). An increase in NF200 indicates Cisplatin causes an increase in Aβ fibre termination to the dorsal horn, and could suggest sprouting of innocuous PAF axons. This structural reorganisation could recruit Aβ fibres to nociceptive

networks (Woolf and Doubell 1994), underlying the behavioural allodynia produced by Cisplatin. The sprouting caused by the Cisplatin treatment also appears to be cumulative over time. The difference in average intensities between Cisplatin and vehicle treatment groups is greater at the adult time point than the juvenile (Figures 9 and 12). The excess of A-fibre innervation early in life, causes generalised and non-specific nociceptive fields. During maturation in the first few post-natal weeks, nociceptive pathways become more efficient by pruning unnecessary terminations in an activity dependant manner (Beggs, et al. 2002). In the neonate Aβ fibres can be found in the superficial dorsal horn laminae, but retract to just the deeper laminae as the dorsal horn matures. Application of an NMDA antagonist prevents this structural reorganisation (Beggs, et al. 2002), the increased activity from the Cisplatin could be having similar structural effects. In the superficial laminae (first 150µm) (Figures 9 and 12) the average difference in signal intensity between vehicle and Cisplatin treatments appeared to be greater at the adult time point than the juvenile, suggesting Cisplatin interferes with the normal developmental structural reorganisation of Aβ-fibres. Unfortunately it is not possible to make definitive comparisons between the juvenile and adult age groups as they were imaged separately using different microscopes (juveniles on the Leica SPE confocal microscope and the adults on the DMRB fluorescent, due to availability). Therefore while the immunohistochemistry intensity values for the age groups have internal validity, comparing between age groups is not possible.

Cisplatin induced central sensory alterations

The sensitisation of peripheral afferent fibres affects more than just the fibres themselves, the increased firing effects the dorsal horn synapse with ascending interneurons and second order neurones (Wu, et al. 2010). Hard-wiring of central sensitisation in the dorsal horn synapses, causes a transition from peripheral activity dependant to independent sensitivity. We investigated histological alterations to neuronal and glial cells in the dorsal horn of the spinal cord to elucidate the possible mechanisms underlying altered pain thresholds in the experimental groups.

NeuN is a nucleic marker, thus the neurones stained in the dorsal horn are second order and interneurons arising from the SVZ, rather than peripheral afferent fibres, who's nuclei are in the DRG. Given the known neurotoxicity of Cisplatin, the consistency of overall neuronal cell counts at both juvenile and adult time points suggests that the concentration of Cisplatin crossing the BBB into the CNS isn't high enough to induce direct apoptotic cellular death (Jacobs, et al. 2010). This isn't to say that Cisplatin has no CNS effects on neurones in the spinal cord, Andres *et al. (*2014) showed that sub-lethal doses of Cisplatin can injure dendritic processes and destroy synapses in hippocampal neurones without causing apoptosis (Andres, Gong, et al., 2014). It's possible a similar effect is occurring in this case, further research into the neuronal processes and network connectivity may be necessary to fully understand Cisplatin's neuronal and glial effects beyond just cellular death. As we've previously stated Cisplatin shows selective toxicity between cell types (Dietrich, Han, et al., 2006), NeuN is a general non-specific neuronal stain, thus it's possible that neuronal subpopulations, such as interneurons or second order neurones, are selectively affected by Cisplatin, in a way that is masked by the general population.

At both the juvenile and adult time points an increase was seen in vGLUT2 glutamate transporter. vGLUT transporters removes glutamate (the main excitatory neurotransmitter released from nociceptive fibres) from the synaptic cleft and packages into vesicles ready for release (Seal 2016). vGLUT2 transporters are expressed on excitatory interneurons in laminae I and II of the dorsal horn (deeper laminae express vGLUT1), and the terminals of myelinated Aδ afferents in laminae I of the dorsal horn (Todd, et al. 2003). An increase in vGLUT2 transporters suggests that increased removal of glutamate from the synapse is required (Seal 2016). Increased levels of glutamate in the synapse indicates increased excitability in laminae I and II of the dorsal horn.

Signalling between neurones doesn't happen in isolation, astrocytes form part of the tripartite synapse and provide synaptic modulation. Including the release of gliotransmitters (Santello and Volterra 2009), which can excite or inhibit both the pre and post synaptic neurones. Thus astrocytes very much have an active role in neuronal signalling and disruptions can have profound effects on

synaptic efficiency (Araque, et al. 1999). Similarly, to neuronal cells, astrocytes also showed no change in cell number when treated with Cisplatin at either the juvenile or adult time point. The stem cells that produce astrocytes (glial precursor cells) are protected in the sub-ventricular zone behind the BBB, which effectively limits the crossing of Cisplatin (Jacobs, et al. 2010). As with neurones, it's possible that the Cisplatin is having a sub-lethal effect, altering astrocytic processes (and affecting synaptic efficiency (Panatier, et al. 2011)) without causing cellular death (Andres, et al. 2014) in a way that can't be seen by simple cell counts.

At the adult time point a significant decrease was shown in the cell counts of microglia in the dorsal horn of the spinal cord. Microglia are the immune cells of the CNS. Along with blood and muscle cells, microglia are mesodermal in origin, this is distinct from other CNS neurones and glial cells that arise from the ectodermal layer of the germ line (Graeber and Streit 2010). In the adult, microglia originate from bone marrow progenitor cells (Ginhoux, et al. 2013) which as with other rapidly proliferating tissues, Cisplatin has been shown to have a toxic effect (Norrgren, et al. 2006) potentially explaining the decrease seen. Microglia are distributed throughout the CNS in two structurally distinct forms: ramified resting state and circular activated state. In the resting state microglia perform an immune surveillance role in the CNS, using motile processes that activate in response to subtle changes in the environment (Nimmerjahn, Kirchhoff and Helmchen 2005). Once activated microglia undergo a number of changes in their function, structure, and gene expression[1]. The ramified processes are withdrawn and thickened and there is an upregulation of gene expression for membrane-bound receptors and pro-inflammatory cytokines, sensitising the microglia and surrounding neurones (Tsuda, Inoue and Salter 2005). When in a non-pathological state, the population of microglia is very stable (Gehrmann and Banati 1995), however, at day 3 of activation, in a nerve injury pain model, there was a peak in the proliferation of activated microglia, which returned to homeostatic baseline by day 60 (Gehrmann and Banati 1995). As Cisplatin toxicity is

[1] It should be noted this explanation of microglia activation is an oversimplification, further details can be found in this review (Hanisch and Kettenmann 2007)

specific to mitotic cells (Dasari and Tchounwou 2014), the decrease in microglia seen at the adult time point could be due to this proliferation of activated microglia, opening the (normally stable population) microglia up to Cisplatin insult.

Cisplatin induced systemic inflammation

Along with other inflammatory mediators, cytokines play a key role in peripheral sensitisation of nociceptive fibres, directly activating Aδ and C-fibres and upregulating membrane channels and receptors via GPCRs, and the recruitment of microglia, resulting in sensitisation of the peripheral nociceptive fibres (Rang, Bevan and Dray 1991). As well as their role in the inflammatory response, cytokines also play an important role in the development of the CNS. Cytokines are involved in the regulation and differentiation of neurogenesis and gliogenesis from neuroepithelial cells and (by acting as neurotrophic factors) in the survival, migration and innervation of neurones and glia (Deverman and Patterson 2009). Increased prenatal cytokines due to maternal infection has been shown to increase the risk of chronic health conditions including, schizophrenia, autism and cerebral palsy (Ellman and Susser 2009).

Increased IL6 concentrations were shown in the blood plasma of Cisplatin treated rats immediately following Cisplatin treatment, indicative of a systemic inflammatory response. It also an indication of possible nephrotoxicity, a common side effect to Cisplatin (Dasari and Tchounwou 2014). Kossmann *et al.* (1996) showed that release of cerebrospinal IL6 following brain injury, triggers NGF production from astrocytes (Kossmann, et al. 1996). Cytokines, including IL6 can cross the BBB into the CNS (Banks, Kastin and Broadwell 1995), therefore it is possible increased levels of IL6 could elevate spinal NGF levels inducing nociceptive sensitisation. Future experiments could investigate the cerebrospinal fluid concentrations of IL6 to see if they are elevated.

Human Comparison

In biomedical research, the use of animal models is necessary for investigating many disease aspects, including (among others): development, functional histochemical networks, toxicology, immunology. Animal models allow for precise control and analysis of extraneous variables, while still for allowing

for any unexpected outcomes arising from being a complete system to occur. However, care must be made before extrapolating any results to humans. Non-human animal species, including the Sprague Dawley rats used in this experiment show individual development, physiology and behaviours, meaning direct comparisons cannot be made as they are not simply small humans.

Because this experiment focuses on the effect of chemotherapy during development, it is important that the age of the animal model equates as closely as possible to the affected human paediatric patients. However, directly comparing ages between species proves incredibly difficult. Measures are often generic and unspecific, not taking into account developmental differences or the emergence of behaviours at different ages. There is also little consensus within the scientific community, with age comparisons being specific to the model and condition being studied. Simplified measures compare stages of life (pre-weaning, sexual maturity, adulthood and sexual senescence) between humans and rats. Using this measure the key time points from this study are as follows: Cisplatin dosing from P7 to P11 becomes 60 days (2 months) to 95 days (3 months) in humans. The juvenile time point at P13 becomes 112 days (3-4 months) for humans. The adult time point at P45 is around 12.5 years old in humans (Sengupta 2013). However, this measure doesn't address how aspects of development present at different ages in humans and rats. At birth human motor functions are far less developed that in other species (rats are fully mobile before the end of weaning) but nervous systems are far more developed (comparable to P12-P13 rat) (Clancy, et al. 2007). Therefore, in this study, in terms of CNS development the dosing regimen could be comparable to a pre-natal human. By the nociceptive threshold at P21, the volumes of the brains and spinal cord in the rat models are similar to those seen in the adult, therefore, are considered to be adolescent (Koch and Fitzgerald 2014). Currently there is no precise comparison between the ages of different species, but we can estimate that the Cisplatin dosing and juvenile time point takes place early in life during weaning thus is comparable the first 6 months in humans, and by adult time point rat models are at least adolescent if not full adults.

The dose and regimen of Cisplatin treatments in humans varies considerably depending on the type and severity of cancer being treated. However, 250-350 mg/m^2 of Cisplatin reliably produces onset of peripheral neuropathy, almost all patients show peripheral neuropathy after a cumulative dose of 500-600 mg/m^2 Cisplatin (Argyriou, et al. 2012), however this dose of this magnitude would not be ethical or feasible. The Sprague Dawley rat strain used in this study were from post-natal day 7-11, with a weight range from 17-25.5g using a rat conversion factor of 5.9 to convert weight to surface area (Freireich, et al. 1966) it becomes 0.00286-0.004322m^2. As the dose was 1mg/kg this converts to individual doses of 5.9mg/m^2 and a cumulative dose of 29.44mg/m^2. This dose is the same or similar to previous Cisplatin studies that have reliably produced pain behaviours (Han and Smith 2013)

A factor that limits human comparison is that this study gave Cisplatin to otherwise healthy animals, whereas this obviously is not the case for humans receiving the same treatment. One way the presence of cancer in patients could alter the effect of Cisplatin treatment is through the role of neurotrophins. NGF, one of the major neurotrophins is particularly important for the survival and differentiation of paediatric cancers (Baker, et al. 1991) and is released by some cancers (reference). This study showed that expression of the NGF receptor TrkA was upregulated in the spinal cord both at both the juvenile and adult time points, sensitising the peptidergic C-fibres that express TrkA to any further NGF. If levels of NGF are already elevated by the cancer and then TrkA expression is upregulated by the Cisplatin, the sensitisation of peptidergic C-fibres would be greater than Cisplatin treatment than in healthy animals given Cisplatin.

Limitations and Improvements

Several problems were encountered during the experiment, the biggest issue concerned the weight of the juvenile phase Cisplatin treated animals. Cisplatin treated rats failed to gain weight at the same speed as their littermate controls (although they did continue to gain weight). This was an anticipated side-effect of the treatment but raises concerns from an animal welfare point of view for

future study. Several explanations for this were raised including injection of cisplatin into the viscera and competition for food by vehicle treated siblings.

Cisplatin is known to have detrimental effects on the gastrointestinal (GI) tract (Boussios, et al. 2012) causing nausea, vomiting and diarrhoea, limiting the appetite of the treated animals. In addition, direct injection of Cisplatin could damage the gut wall (Peterson and Cariello 2004) and have an antibiotic effect diminishing the gut bacteria (Joyce, et al. 2010) disrupting the absorption of nutrients from the viscera, limiting available supplies necessary during a critical period of growth (Fish and Winick 1969). Due to the size of the neonatal animals, it's possible that the IP injections were made too deep into the viscera injecting Cisplatin into the stomach or intestine, having detrimental effects on food intake at an energy intensive period of development.

In order for the results to be sibling matched, the litters were mixed with both vehicle and cisplatin animals housed together. Another possible explanation for the animals failing to gain weight at the same rate is that the cisplatin treated animals were out competed for the maternal food source by their healthier siblings. Human patients often experience fatigue and lack of motivation, in addition to the lack of appetite. The smaller, weaker and less energetic animals might be less able to fight to gain access to the mother (Hudson and Trillmich 2008).

To address this problem several improvements to the methods were suggested for any future experiments. The route of administration could be changed from IP injection in the flank to subcutaneous (SC) injection in the nape of the neck. The pharmacokinetics of cisplatin administered by IP or SC injection have been shown to be very similar (Tamura, et al. 2002) and this would eliminate the possibility of accidentally injecting the GI tract. In addition to remove the issue of competition for maternal food source, separate litters are to be kept for Cisplatin treated animals and vehicle treated animals. These improvements were approved by the University of Nottingham, biomedical support unit's named animal welfare officer and the home office inspector.

In addition to affecting the health of the animals, the slower growth rate would mean a smaller spinal cord in the Cisplatin treated animals. This could potentially confound results as the fixed size region of interest being analysed would cover a greater percentage of the dorsal horn, similarly the line of interest would reach deeper laminae. For the lines of interest, the peaks and troughs in intensity line up between the cisplatin and vehicle groups suggesting the laminae are similar depths into the dorsal horns. In the future it might be better to perform a cell count on the entire dorsal horn, rather than smaller 100x100µm regions of interest. This would be too time consuming to be performed manually as in this experiment, but could be automated using the ImageJ Analyse Particles tool (Foley 2013). Automating the process would remove any investigator bias and could speed up analysis. However, in the higher laminae where the cells are densely packed could underestimate the number of cells, this could be addressed using the ImageJ watershed tool to separate out cells.

A casual observation seen during tissue collection was that the hearts of the Cisplatin treated animals were noticeably smaller than their PBS vehicle treated sibling counterparts. This was not an expected effect so the equipment required to collect and preserve the tissue wasn't on hand and this tissue was unable to be collected. Cisplatin is known to have a cardio-toxic effect, linked to cardiac events including; arrhythmias, myocarditis, cardiomyopathy and congestive heart failure (Patane 2014). Cardiac tissue's high metabolic rate makes it particularly susceptible to mitochondrial damage leading to the generation of ROS and oxidative stress (El-Awady, et al. 2010). From an animal welfare point of view, if the experiment is repeated in the future, it would be beneficial to collect the cardiac tissue to monitor the systemic health effects of Cisplatin.

For the juvenile time point a cell death marker, cleaved caspase 3 (CC3) was used to investigate if there was any localisation with either neurones or astrocytes in the spinal cord. Unfortunately, CC3 used a rabbit primary antibody, the IBA1 microglial antibody likewise uses a rabbit primary antibody, thus GFAP, CC3 and IBA1 could not be co-stained together. Given the limited supply of spinal cord tissue, the decision was made to exclude the microglial stain for the juvenile time point. For any

future experiments it would be useful to investigate the immune role in establishing juvenile spinal cord, it might also shed some light on the microglial depletion seen at the adult time point.

Future Directions

This research suggests several possible directions that could be further investigated in the future.

This study only investigated microglia numbers at a single time point, therefore it isn't possible to extrapolate any findings to see a general trend in Cisplatin's effect on microglia. Future research could investigate the juvenile time point to see if there are any indicators for the cause of the decrease seen later on. An initial increase followed by a chronic decrease would suggest proliferation creates an opportunity for Cisplatin toxicity, whereas a general decrease would suggest Cisplatin toxicity in the bone marrow.

It has been previously demonstrated that Cisplatin treated animals show pain behaviour and we have found multiple changes in their spinal nociceptive histology. To investigate if there is a direct correlation between these two findings, cisplatin treated animals could be ranked by the degree of severity of pain behaviours shown, then compare this to the extent of spinal histological alterations.

We've suggested that increases in peptidergic nociceptors play an important role the acute development of Cisplatin induced spinal nociceptive sensitisation. Future research could investigate the extent of this claim. Since the NGF/TrkA pathway appears to be a possible mechanism for activation, an anti-NGF or anti-TrkA therapy could be utilised to attempt to curtail acute sensitisation before the changes become hard wired. However due to neuronal plasticity, especially during development, both NGF and TrkA play important roles (including cell survival) outside of nociception, therefore blocking their action may cause profound side effects limiting any potential benefits.

As previously mentioned, Cisplatin shows tissue dependant toxicity, the most affected being precursor stem cells for neurones and glia and oligodendrocytes, with less of an effect on fully differentiated and developed neurones and glia (Dietrich, Han, et al., 2006). The NeuN and GFAP

stains (for neurones and astrocytes respectively) used in this study, are only expressed by fully developed cells. Further investigations could be made into Cisplatin's effect on precursor cells such as; DCX for neuronal precursor cells, A2B5 for oligodendrocyte precursor cells (Dietrich, Han, et al., 2006) and A3B10 for astrocyte precursor cells (Yamamote, et al. 2009). If there is a correlation between the extent of pain behaviour and any changes to precursor cell number, or given the toxicity to oligodendrocytes the myelination of ascending neuronal tracts.

Impact statement

Improved treatments for paediatric cancers mean that more patients are surviving than ever before. Unfortunately, the systemic and aggressive treatments, such as Cisplatin, come with a myriad of harmful side effects. Prevalent among them is the development of chronic pain that can persist for decades beyond the end of treatment severely impacting quality of life. It is important to understand the underlying histological changes to nociceptive pathways and networks that result in long-term alterations in pain perception, in order to improve existing therapies or develop preventative treatments.

Conclusion

In conclusion, this study has shown that paediatric Cisplatin treatment causes significant chronic changes in PNS and CNS nociceptive histology, potentially underlying the chronic pain behaviours that Cisplatin has previously been shown to produce. Further research is required to fully establish the link between changes in cellular histology and animal behaviour and to understand the full extent of Cisplatin's effect on the CNS.

References

Almeida, Tatiana, Suely Roizenblatt, and Sergio Tufik. 2004. “Afferent pain pathways: a neuroanatomical review.” *Brain research* 40-56.

Andres, Adrienne, Xing Gong, Kaijun Di, and Daniela Bota. 2014. “low-doses of cisplatin injure hippocampal synapses: a mechanism for 'chemo' brain?” *experimental neurology* 137-144.

Araque, Alfonso, Vladimir Parpura, Rita Sanzgiri, and Philip Haydon. 1999. “Tripartite synapses: glia, the unacknowledged partner.” *Trends in Neuroscience* 208-215.

Argyriou, Andreas, Jordi Bruna, Paola Marmiroli, and Guido Cavaletti. 2012. “Chemotherapy-induced peripheral neurotoxicity (CIPN): and update.” *Critical reviews in oncology/hematology* 51-77.

Arnesano, Fabio, Maurizio Losacco, and Giovanni Natile. 2013. “An Updated View of Cisplatin Transport.” *European Journal of Inorganic Chemistry* 2701–2711.

Baker, David, Willemina Molenaar, John Trojanowski, Audrey Evans, Alonzo Ross, Lucy Rorke, Roger Packer, Virginia Lee, and David Pleasure. 1991. “Nerve growth factor receptor expression in peripheral and central neuroectodermal tumors, other pediatric brain tumors, and during development of the adrenal gland.” *The American Journal of Pathology* 115-122.

Banks, WA, AJ Kastin, and RD Broadwell. 1995. “Passage of cytokines across the blood-brain barrier.” *neuroimmunomodulation* 241-248.

Barajon, Isabella, Maurizio Bersani, Marina Quartu, Marina Del Fiacco, Guido Cavaletti, Jens Holst, and Giovanni Tredici. 1996. “Neuropeptides and morphological changes in ciplatin-induced dorsal root ganglion neuropathy.” *experimental neurology* 93-104.

Beggs, Simon, Carole Torsney, Liam Drew, and Maria Fitzgerald. 2002. “The postnatal reorganisation of primary afferent input and dorsal horn cell rreceptive fields in the rat spinal cord is an activity-dependant process.” *European Journal of Neuroscience* 1249-1258.

Berse, Brygida, Ignacio Lopez-Coviella, and J Blusztajn. 1999. “Activation of TrkA by nerve growth factor upregulates expression of the cholinergic gene locus but attenuates the response to ciliary neurotrophic growth factor.” *Biochemical journal* 301-308.

Boussios, Stergios, George Pentheroudakis, Konstantinos Katsanos, and Nicholas Pavlidis. 2012. “Systemic treatment-induced gastrointestinal toxicity: incidence, clinical presentation and management.” *Ann Gastroenterol.* 106-118.

Brain, S, T Williams, J Tippins, H Morris, and I MacIntyre. 1985. “Calcitonin gene-related peptide is a potent vasodilator.” *Letters to Nature* 54-56.

Brinkman, TM, L Zhu, CJ Recklitis, C Kimberg, N Zhang, AC Muriel, M Stovall, DK Srivastava, LL Robison, and KR Krull. 2013. “Longitudinal patterns of psychological distress in adult survivors of childhood cancer.” *British Journal of Cancer* 1373-1381.

Budai, Denes. 2000. “Neurotransmitters and receptors in the dorsal horn of the spinal cord.” *Acta Biologica Szegediensis* 21-38.

2007. “Cancer Drug Resistance.” Edited by Beverly Teicher, 287. Springer Science & Business Media.

Cancer Research UK. 2016. *Cancer Statistics for the UK.* 06 07. Accessed 2016. http://www.cancerresearchuk.org/health-professional/cancer-statistics#heading-Zero.

—. 2016. *Children's cancer statistics.* 06 07. Accessed 2016. http://www.cancerresearchuk.org/health-professional/cancer-statistics/childrens-cancers#heading-Zero.

—. 2014. *Great Britain Cancer Incidence (1996-2005) Summary - Children,.* Febuary. Accessed 07 2016. http://publications.cancerresearchuk.org/downloads/Product/CS_DT_CHILDHOOD.pdf.

Cheng, Jen-Kun, and Ru-Rong Ji. 2008. “Intracellular Signaling in Primary Sensory Neurons and Persistent Pain.” *Neurochemical research* 1970-1978.

Clancy, Barbara, Barbara Finlay, Richard Darlington, and KJS Anand. 2007. “Extrapolating brain development from experimental species to humans.” *Neurotoxicology* 1-7.

Coggeshall, Richard, Carolyn Pover, and Maria Fitzgerald. 1994. “Dorsal root ganglion cell death and surviving cell numbers in relation to the development of sensory innervation in the rat hindlimb.” *Developmental Brain research* 193-212.

Constantinou, J, ML Reynolds, CJ Woolf, B Sefieh-Garabedian, and M Fitzgerald. 1994. “Nerve growth factor levels in developing rat skin: upregulation following skin wounding.” *Neuroreport* 2281-2284.

Dasari, Shaloam, and Paul Tchounwou. 2014. “Cisplatin in cancer therapy: molecular mechanisms of action.” *European Journal of Pharmacology* 364-378.

De Lima, J, D Alvares, DJ Hatch, and M Fitzgerald. 1999. “Sensory hyperinnervation after neonatal skin wounding: effect of bupivacaine sciatic nerve block.” *British Journal of Anaesthesia* 662-664.

Deverman, Benjamin, and Paul Patterson. 2009. “Cytokines and CNS development.” *Neuron* 61-78.

Dietrich, Joerg, Ruolan Han, Yin Yang, margot Proschel, and Mark Noble. 2006. “CNS progenitor cells and oligodendrocytes are targets of chemotherapeutoc agents in vitro and in vivo.” *Journal of Biology* 1-23.

Drugs.com. 2016. *Cisplatin Dosage.* 6 July . Accessed July 2016. https://www.drugs.com/dosage/cisplatin.html.

El-Awady, El-Sayed, Yasser Moustafa, Dina Abo-Elmatty, and Asmaa Radwan. 2010. “Cisplatin-induced cardio-toxicity: Mechanisms and cardioprotective strategies.” *European Journal of Pharmacology* 335-341.

Ellman, Lauren, and Ezra Susser. 2009. “The promise of epidemiologic studies: neuroimmune mechanisms in the etiologies of brain disorders.” *Neuron* 25-27.

Eskander, Michael, Shivani Ruparel, Dustin Green, Paul Chen, Elaine Por, Nathaniel Jeske, Xiaoli Gao, Eric Flores, and Kenneth Hargreaves. 2015. "Persistent Nociception Triggered by Nerve Growth Factor (NGF) Is Mediated by TRPV1 and Oxidative Mechanisms." *the journal of neuroscience* 8593-8603.

Fish, Irving, and Myron Winick. 1969. "Effect of malnutrition on regional growth of the developing rat brain." *Experimental Neurology* 534-540.

Fitzgerald, Maria. 2005. "The development of nociceptive circuits." *Nature Reviews Neuroscience* 507-520.

Freireich, EJ, EA Gehan, DP Rall, LH Schmidt, and HE Skipper. 1966. "Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man." *Cancer Chemotherapy reports* 219-244.

Gangadharan, Vijayan, and Rohini Kuner. 2013. "Pain Hypersensitivity mechanisms at a glance." *Disease Model Mechanisms* 889-895.

Gangadharan, Vijayan, and Rohini Kuner. 2015. "Unravelling spinal circuits of pain and mechanical allodynia." *Neuron* 673-675.

Gassner, Matthias, Ruth Ruscheweyh, and Jurgen Sandkuhler. 2009. "Direct excitation of spinal GABAergic interneurones by noradrenaline." *Pain* 204-210.

Gehrmann, Jochen, and Richard Banati. 1995. "Microglia turnover in the injured CNS: activated microglia undergo delayed DNA fragmentation following peripheral nerve injury." *journal of neuropathology and experimental neurology* 680-688.

Gilchrist, Laura. 2012. "Chemotherapy-Induced Peripheral Neuropathy in Pediatric Cancer Patients." *Seminars in Pediatric Neurology* 9-17.

Ginhoux, Florent, Shawn Lim, Guillauma Hoeffel, Donovan Low, and Tara Huber. 2013. "Origin and differentiation of microglia." *Frounters in cellular neuroscience* 1-14.

Graeber, Manuel, and Wolfgang Streit. 2010. "Microglia:biology and pathology." *Acta Neuropathologica* 89-105.

Gutiérrez-Gutiérrez, Gerardo, Maria Sereno, Ambrosio Miralles, Enrique Casado-Saenz, and Eduardo Gutiérrez-Rivas. 2010. "Chemotherapy-induced peripheral neuropathy: clinical features, diagnosis, prevention and treatment strategies." *Clinical Translational Oncology* 81-91.

Han, Yaqin, and Maree Smith. 2013. "Pathobiology of cancer chemotherapy-induced peripheral neuropathy (CIPN)." *Frountiers in Pharmacology* 6-22.

Hanisch, Uwe-Karsten, and Helmut Kettenmann. 2007. "Microglia: active sensor and versatile effector cells in the normal and pathologic brain." *Nature Neuroscience* 1387-1394.

Hathway, G, S Koch, and M Fitzgerald. 2009. "The changing balance of brainstem–spinal cord modulation of pain processing over the first weeks of rat postnatal life." *The Journal of Physiology* 2927–2935.

Hathway, Gareth, David Vega-Avelaira, and Maria Fitzgerald. 2012. "A critical period in the supraspinal control of pain: opioid-dependent changes in brainstem rostroventral medulla function in preadolescence." *Pain* 775-783.

He, Qing, Cynthia Liang, and Stephen Lippard. 2000. "Steroid hormones induce HMG1 overexpression and sensitize breast cancer cells to cisplatin and carboplatin." *PNAS* 5768-5772.

Heinricher, MM, MM Morgan, and HL Fields. 1992. "Direct and indirect actions of morphine on medullary neurones that modulate nociception." *Neuroscience* 533-543.

Hershman, DL, C Lacchetti, RH Dworkin, EM Lavoie Smith, J Bleeker, G Cavaletti, C Chauhan, et al. 2014. "Prevention and management of chemotherapy-induced peripheral neuropathy in servivors of adult cancers: Americal Society of Clinical Oncology clinical practice guideline." *Journal of Clinical Oncology* 32 (18): 1941-67. doi:10.1200/JCO.2013.54.0914.

Holst, Alexander, Alfredo Rodriguez-Tebar, Jean-Jacques Michaille, Danielle Dhouailly, Anders Backstrom, Ted Ebendal, and Hermann Rohrer. 1995. "Retinoic Acid-Mediated Increase in TrkA Expression Is Sufficient to Elicit NGF-Dependent Survival of Sympathetic Neurons." *Molecular and Cellular neuroscience* 185-198.

Hudson, Robyn, and Fritz Trillmich. 2008. "Sibling competition and cooperation in mammals: challenges, developments and prospects." *Behavioural ecology and sociobiology* 299-307.

Hughes, H, and G Barr. 1988. "Analgesic effects of intrathecally applied noradrenergic compounds in the developing rat: differences due to thermal vs mechanical nociception." *Brain Research* 109-120.

Jacobs, Shana, Cynthia McCully, Robert Murphy, John Bacher, Frank Balis, and Elizabeth Fox. 2010. "Extracellular fluid concentration of cisplatin, carboplatin, and oxaliplatin in brain, musle and blood measured using microdialysis in nonhuman primates." *Cancer chemotherapeutics Pharmacology* 65: 817-824. doi:10.1007/s00280-009-1085-7.

Joyce, Kellie, Shruti Saxena, Amanda Williams, Christopher Damurjian, Neil Auricchio, Sabrina Aluotto, Heather Tynan, and Arnold Demain. 2010. "Antimicrobial spectrum of the antitumor agent, cisplatin." *the journal of antibiotics* 530-532.

Julius, David, and Allan Basbaum. 2001. "Molecular mechanisms of nociception." *Nature* 203-210.

Kaley, Thomas, and Lisa DeAngelis. 2009. "Therapy of chemotherapy-induced peripheral neuropathy." *British Journal of Haematology* 3-14.

Kitao, Y, B Robertson, M Kudo, and G Grant. 1996. "Neurogenesis of subpopulations of rat lumbar dorsal root ganglion neurones including neurones projecting to the dorsal column nuclei." *Journal of Comparative Neurology* 249-257.

Koch, Stephanie, and Maria Fitzgerald. 2014. "The selectivity of rostroventral medulla descending control of spinal sensory inputs shifts postnatally from A fibre to C fibre evoked activity." *Journal of Physiology* 1535-1544.

Kossmann, Thomas, Volkmar Hans, Hans-Georg Imhof, Otmar Trentz, and Maria Morganti-Kossmann. 1996. "Interleukin-6 released in human cerebrospinal fluid following traumatic brain injury may trigger nerve growth factor production in astrocytes." *brain Research* 143-152.

Kuhlmann, M, G Burkhardt, and H Kohler. 1997. "Insights into potential cellular mechanisms of cisplatin nephrotoxicity and their clinical application." *Nephrology Dialysis Transplantation* 2478-2480.

Larson, Christopher, Brian Blair, Roohangiz Safaei, and Stephen Howell. 2009. "The Role of the Mammalian Copper Transporter 1 in the Cellular Accumulation of Platinum-Based Drugs." *Molecular Pharmacology* 324-330.

Latremoliere, Alban, and Clifford Woolf. 2009. "Central Sensitization: A Generator of Pain Hypersensitivity by Central Neural Plasticity." *The Journal of Pain* 895-926.

Li, Xinyu, Esperanza Arias, Ramamohana Jonnala, Shyamala Mruthinti, and Jerry Buccafusco. 2005. "Effect of amyloid peptides on the increase in TrkA receptor expression induced by nicotine in vitro and in vivo." *Journal of Molecular Neuroscience* 325-336.

Lin, Zinjian, Tsuyoshi Okuda, Alison Holzer, and Stephen Howell. 2002. "The Copper Transporter CTR1 Regulates Cisplatin Uptake in Saccharomyces cerevisiae." *Molecular Pharmacology* 1154-1159.

Lorenzo, Louis-Etienne, Michele Ramien, Manon St Louis, Yves De Koninck, and Alfredo Ribeiro-Da-Silva. 2008. "Postnatal changes in the rexed lamination and markers of nociceptive afferents in the superficial dorsal horn of the rat." *The Journal of Comparative Neurology* 592-604.

Lu, Qian, Kevin Krull, Wendy Leisenring, Jason Owen, Toana Kawashima, Jennie Tsao, Bradley Zebrack, et al. 2011. "Pain in long-term survivors of childhood cancers and their siblings: a report from the childhood cancer survivor study." *Pain* 2616-2624.

Lucarelli, Enrico, David Kaplan, and Carol Thiele. 1996. "Selective regulation of TrkA and TrkB receptors by retinoic acid and interferon-y in human neuroblastoma cell lines." *The journal of biological chemistry* 24725-24731.

Marchant, Amy. 2014. "'Neonates do not feel pain': a critical review of the evidence." *Bioscience Horizons* 1-9.

Medscape. 2016. *Cisplatin (Rx) Dosing & Uses.* 13 March. Accessed August 19, 2016. http://reference.medscape.com/drug/platinol-aq-cisplatin-342108.

Melli, Giorgia, Michela Taiana, Francesca Camozzi, Daniela Triolo, Paola Podini, Angelo Quattrini, Franco Taroni, and Giuseppe Lauria. 2008. "Alpha-lipoic acid prevents mitochondrial damage and neurotoxicity in experimental chemotherapy neuropathy." *Experimental Neurology* 276-284.

Mirnics, Karoly, and Richard Koerher. 1995. "prenatal development of rat primary afferent fibres: II. Central projections." *Journal of Comparative Neurology* 601-614.

Mizumura, Kazue, Takeshi Sugiura, Kimiaki Katanosaka, Ratan Banik, and Yasuko Kozaki. 2009. "Excitation and sensitization of nociceptors by bradykinin: what do we know?" *Experimental brain research* 1814-1815.

Molliver, Derek, and William Snider. 1997. "Nerve growth factor receptor TrkA is down-regulated during postnatal development by a subset of dorsal root ganglion neurones." *The Journal of Comparative Neurology* 428-438.

Moore, Rhonda, and Hunter Groninger. 2014. "Chemotherapy-induced Peripheral Neuropathy in Pediatric Cancer Patients." *PMC* 1-17.

Morgan, Ryan. 2010. "Rationally Engineering Chemo." *American Scientist* 466. Accessed July 2016. http://chemistry-reference.com/q_compounds.asp?CAS=15663-27-1.

Munglani, R, Harrison S, G Smith, C Bountra, P Birch, P Elliot, and S Hunt. 1996. "Neuropeptide changes persist in spinal cord despite resolving hyperalgesia in a rat model of mononeuropathy." *Brain Research* 102-108.

Nakagawa, H, Toshiaki Fuita, Shigeki Kubo, Koji Tokiyoshi, Masanobu Yamada, Takuji Kanayama, Yasushi Hagiwara, Hiroshi Nakanomyo, and Masaki Hiraoka. 1996. "Difference in CDDP penetration into CSF between selective intraarterial chemotherapy in patients with malignant glioma and intravenous or intracarotid administration in patients with metastatic brain tumor." *Cancer chemotherapy and Pharmacology.* 37 (4): 317-326.

Nimmerjahn, Axel, Frank Kirchhoff, and Frirjof Helmchen. 2005. "Resting Microglial Cells Are Highly Dynamic Surveillants of Brain Parenchyma in Vivo." *Science* 1314-1318.

Norrgren, Kristina, Maria Sjolin, Sven Bjorkman, Johan Areberg, Anders Johnsson, L Johansson, and Soren Mattsson. 2006. "Comparative Renal, Hepatic, and Bone Marrow Toxicity and Radioactive Cisplatin (191Pt) in Wistar Rats." *Cancer biotherapy and radiopharmaceuticals* 528-534.

Oeffinger, Kevin, Ann Mertens, Charles Sklar, Toana Kawashima, Melissa Hudson, Anna Meadows, Debra Freidman, et al. 2006. "Chronic health conditions in adult survivors of childhood cancer." *The New England Journal of Medicine* 1572-1582.

Ossipov, Michael, Gregory Dussor, and Frank Porreca. 2010. "Central Modulation of pain." *The Journal of Clinical Investigation* 3779-3787.

Panatier, Aude, Joanne Vallee, Michael Haber, Keith Murai, Jean-Claude Lacaille, and Richard Robitaille. 2011. "Astrocytes Are Endogenous Regulators of Basal Transmission at Central Synapses." *Cell* 785-798.

Patane, Salvatore. 2014. "Cardiotoxicity, Cisplatin and long-term cancer survivors." *international Journal of cardiology* 201-202.

Peterson, Douglas, and Anna Cariello. 2004. "Mucosal damage: a major risk factor for severe complications after cytotoxic therapy." *Seminars in Oncology* 35-44.

Pezet, Sophie, Brigitte Onteniente, Gael Grannec, and Bernard Calvino. 1999. "Chronic pain is associated with increased TrkA immunoreactivity in spinoreticular neurones." *the journal of neuroscience* 5482-5492.

Pignatelli, D, A Ribeirp-da-Silva, and A Coimbra. 1989. "Postnatal maturation of primary afferent terminations in the substantia gelatinosa of the rat spinal cord. An electron microscopic study." *Brain Research* 33-44.

Ramchandren, Sindhu, Marcia Leonard, Rajen Mody, Janet Donohue, Judith Moyer, Raymond Hutchinson, and James Gurney. 2009. "Peripheral neuropathy in survivors of childhood acute lymphoblastic leukemia." *Journal of the peripheral nervous system* 184-189.

Rang, HP, S Bevan, and A Dray. 1991. "Chemical activation of nociceptive peripheral neurones." *British Medical Bulletin* 534-548.

Recklitis, Christopher, Lisa Diller, Xiaochun Li, Julie Najita, Leslie Robison, and Lonnie Zeltzer. 2009. "Suicide Ideation in Adult Survivors of Childhood Cancer: A Report From the Childhood Cancer Survivor Study." *Journal of Clinical Oncology* 655-661.

Reddel, RR, RF Kefford, JM Grant, AS Coates, RM Fox, and MH Tattersall. 1982. "Ototoxicity in patients receiving cisplatin: importance of dose and method of drug administration." *Cancer Treatment Reports* 19-23.

Rexed, Bror. 1952. "The cytoarchitectonic organization of the spinal cord in the cat." *The Journal of Comparitive neurology* 415-495.

Ruscheweyh, Ruth, Liesbeth Forsthuber, Doris Schoffnegger, and Jurgen Sandkuhler. 2007. "Modification of classical neurochemical markers in identified primary afferent neurones with Aβ, Aδ and C-fibres after chronic constriction injury in mice." *the journal of comparative neurology* 325-336.

Rybak, Leonard, Debashree Mukherjea, Sarvesh Jajoo, and Vickram Ramkumar. 2010. "Cisplatin Ototoxicity and Protection: Clinical and Experimental Studies." *The Tohoku journal of experimental medicine* 177-186.

Santello, M, and A Volterra. 2009. "Synaptic modulation by astrocytes via Ca2+-dependent glutamate release." *Neuroscience* 253-259.

Schaible, HG. 2006. "Peripheral and central mechanisms of pain generation." *Handbook of Experimental Pharmacology* 3-28.

Schultz, Kris Ann, Kirsten Ness, John Whitton, Christopher Recklitis, Brad Zebrack, Leslie Robinson, Lonnie Zeltzer, and Ann Mertens. 2007. "Behavioral and Social Outcomes in Adolescent Survivors of Childhood Cancer: A Report From the Childhood Cancer Survivor Study." *Journal of Clinical Oncology* 3649-3656.

Seal, Rebecca. 2016. "Do the distinct synaptic properties of VGLUTs shape pain." *Neurochemistry International* 82-88.

Sengupta, Pallav. 2013. “The laboratory rat: relating its age with human's.” *International journal of preventive medicine* 624-630.

Siddik, Zahid. 2003. “Cisplatin: mode of cytotoxic action and molecular basis of resistance.” *Oncogene* 7265-7279.

Simo, Marta, Xavier Rifa-Ros, Antoni Rodriguez-Fornelles, and Jordi Bruna. 2013. “Chemobrain: A systematic review of structural and functional neuroimaging studies.” *Neuroscience and Biobehavioural reviews* 1311-1321.

Simons, Sinno, and Dick Tibboel. 2006. “Pain perception development and maturation.” *Seminars in Fetal & Neonatal Medicine* 227-231.

St. Jude Children's Reseach Hospital. 2016. *The Childhood Cancer Survivor Study.* Accessed 07 2016. https://ccss.stjude.org/learn-more.html.

Stanley, McKnight. 1991. “Cyclic AMP second messenger systems.” *Current Opinion in Cell Biology* 213-217.

Strumberg, D, S Brugge, MW Korn, S Koeppen, J Ranft, G Scheiber, C Reiners, C Mockel, S Seeber, and ME Scheulen. 2002. “Evaluation of long-term toxicity in patients after cisplatin-based chemotherapy for non-seminomatous testicular cancer.” *Annals of Oncology* 13 (2): 229-236.

Tamura, Takashi, Jun Imai, Akihiro Matsumoto, Masahiko Tanimoto, akira Suzuki, Yuji Horikiri, Takehiko Suzuki, Hiroyuki Yoshino, and Osamu Ike. 2002. “Organ distribution of cisplatin after intraperitoneal administration of cisplatin-loaded microspheres.” *European journal of pharmaceutics and biopharmaceutics* 1-7.

Todd, A, D Hughes, E Polgar, G Nagy, M Mackie, O Ottersen, and D Maxwell. 2003. “The expression of vasicular glutamate transporters VGLUT1 and VGLUT2 in neurochemically defined axonal populations in the rat spinal cord with emphasis on the dorsal horn.” *European journal of neuroscience* 13-27.

Todd, Andrew. 2010. “Neuronal circuitory for pain processing in the dorsal horn.” *Nature reviews neuroscience* 823-836.

Todd, Andrew, and Anne Sullivan. 1990. “Light microscope study of the coexistence of GABA-like and glycine-like immunoreactivities in the spinal cord of the rat.” *The Journal of Comparative Neurology* 496-505.

Tofthagen, C. 2010. “Patient perceptions associated with chemotherapy-induced peripheral neuropathy.” *Europe PMC* 22-28.

Tsuda, Makoto, kazuhide Inoue, and Michael Salter. 2005. “Neuropathic pain and spinal microglia: a big problem from molecules in 'small' glia.” *Trends in Neuroscience* 101-107.

Vanegas, Horacio, and Hans-Georg Schaible. 2004. “Descending control of persistant pain: inhibitory or facilitatory?” *Brain Research Reviews* 295-309.

Van-Praag, Henriette, and Hanan Frenk. 1991. “The development of stimulation-produced analgesia (SPA) in the rat.” *Developmental brain Research* 71-76.

Vikman, Kristina, Russell Hill, Eva Backstrom, Brita Robertson, and Krister Kristensson. 2003. “Interferon-γ induces characteristics of central sensitization in spinal dorsal horn neurons in vitro.” *Pain* 241-251.

Walker, Suellen, Maria Fitzgerald, and Gareth Hathway. 2015. “Surgical Injury in the neonatal rat alters the adult pattern of descending modulation from the rostoventral medulla.” *Anesthesiology* 1390-1400.

Watkins, L, D Martin, P Ulrich, K Tracey, and S Maier. 1997. “Evidence for the involvement of spinal cord glia in subcutaneous formalin induced hyperalgesia in the rat.” *Pain* 225-235.

Windebank, Anthony, and Wolfgang Grisold. 2008. “Chemotherapy-induced neuropathy.” *Journal of the peripheral nervous system* 27-46.

Woolf, Clifford, and Tim Doubell. 1994. “The pathophysiology of chronic pain — increased sensitivity to low threshold Aβ-fibre inputs.” *Current Opinion in Neurobiology* 525-534.

Wu, Sheng-Xi, Wen Wang, Hui Li, Ya-Yun Wang, Yu-Peng Feng, and Yun-Qing Li. 2010. “The synaptic connectivity that underlies the noxious transmission and modulation within the superficial dorsal horn of the spinal cord.” *Progress in Neurobiology* 38-54.

Yamamote, M, K Yoshimura, M Kitada, J Nakahara, C Seiwa, T Ueki, Y Shimoda, A Ishige, K Watanabe, and H Asou. 2009. “A new monoclonal antibody, A3B10, specific for astrocyte-lineage cells recognizes calmodulin-regulated spectrin-associated protein 1 (Camsap1).” *journal of neuroscience research* 503-513.

Yoshimura, Megumu, and Hidemasa Furue. 2006. “Mechanisms for the Anti-nociceptive Actions of the Descending Noradrenergic and Serotonergic Systems in the Spinal Cord.” *Journal of Pharmacological Sciences* 107-117.

Zebrack, Brad, James Gurney, Kevin Oeffinger, John Whitton, Roger Packer, Ann Mertens, Norman Turk, et al. 2004. “Psychological Outcomes in Long-Term Survivors of Childhood Brain Cancer: A Report From the Childhood Cancer Survivor Study.” *Journal of Clinical Oncology* 999-1006.

Zhen-Zhong, Xu, Kim Yong Ho, Bang Sangsu, Yi Zhang, Berta Temugin, Wang Fan, Bae Oh Seog, and Ji7 Ru-Rong. 2015. “Inhibition of mechanical allodynia in neuropathic pain by TLR5-mediated A-fibre blockade.” *Nature medicine* 1326-1331.

Zhou, Wenjun, Annemieke Kevelaars, and Cobi Heijnen. 2016. “Metformin prevents cisplatin-induced cognitive impairment and brain damage in mice.” *PLOS one.*

Appendix 1

Abbreviations

A-α	A-alpha fibres
A-β	A-beta fibres
A-δ	A-delta fibres
ATP	Adenosine triphosphate
ATR	Ataxia-telagiectasia-mutated related protein
AMP	Adenosine monophosphate
AU	Arbitrary units
AUC	Area under the curve
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
cAMP	Cyclic Adenosine monophosphate
CC	Corpus collosum
CC3	Cleaved caspase 3
CCSS	Childhood cancer survivor study
CDDP	Cis-diamminedichloroido platinum(II)
CGRP	Calcitonin gene related peptide
CIPN	Chemotherapy Induced Peripheral Neuropathy
CNS	Central nervous system
CREB	cAMP response element-binding protein
CSF	Cerebrospinal Fluid
CTR1	High affinity copper uptake protein 1
ddH_2O	Double distilled H_2O
DG	Dentate gyrus
DLF	Dorsolateral funiculus
DRG	Dorsal root ganglion
E#	Embryonic day #
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
GABA	Gamma-Aminobutyric acid
GI	Gastrointestinal
GPCR	G-protein coupled receptor
GRP	Glial restricted precursor cell
HMG	High-mobility group
IB4	Isolectin B4
IP	Intraperitoneal
IL4	Interleukin 4
IL6	Interleukin 6
IL10	Interleukin 10
JNK	c-Jun N-terminal Kinase
MAPK	Mitogen-activated protein kinase
MMR	Mismatch repair
NGF	Neural growth factor
NF200	Neurofilament 200
NK1	Neurokinin 1
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NSC	Neuroepithelial stem cells
NRP	Neurone restricted precursor cell
O-2A	Oligodendrocyte-type-2 astrocytes
OCT	Optimal cutting temperature compound
OD	Optical Density
P#	Postnatal day #
PAG	periaqueductal grey
PBS	Phosphate Buffer Solution
PGE_2	Prostaglandin E2
PKA	Protein kinase A
PLA2	Phospholipase A2
PNS	Peripheral Nervous System
RCT	Randomised control trials
ROS	Reactive Oxygen Species
RVM	rostral ventromedial medulla
SEM	Standard error of the mean
SC	Subcutaneous
SI	Suicide ideation
SBT	Spinoparabrachial tract
SHT	Spinohypothalamic tract
SMT	Spinomesencephalic tract
SNRI	Serotonin-noradrenaline reuptake inhibitor
SRT	Spinoreticular tract
STT	Spinothalamic tract
SVZ	Sub ventricular zone
TLR5	Toll like receptor 5
TNF	**T**umor necrosis factor
TrkA	Tyrosine kinase A
TrkB	Tyrosine kinase B
TrkC	Tyrosine kinase C
TRP_{V1}	Transient receptor potential vanilloid-like 1
TRP_{V2}	Transient receptor potential vanilloid-like 2
TTBS	Trizma hydrochloride solution
TTX-R	Tentrodotoxin-resistant
TUNNEL	Terminal deoxynecleotidyl transferase-mediated dUTP nick-end labelling
vGLUT2	Vascular glutamate 2 receptor
WDR	Wide dynamic range
4-PL	4 parameter logistics