Behavioural Consequences and Neural Correlates of Bladder Inflammation in the Laboratory Rat

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Abstract

The treatment and management of pain continues to reflect the complex nature of pain itself. At present, pain cannot be definitively tested for, only inferred through a combination of sensory and self-report measures, such as quantitative sensory testing procedures and validated questionnaires, which survey various aspects of the pain experience in more detail, as well as the impact of pain on daily life at home and in the workplace. Whilst clinical sensory testing outcomes are analogous to *in vivo* evoked sensory data, modelling the impact of pain on quality of life is more challenging.

One approach is to use an ethological paradigm, i.e. one that measures a behaviour that reflects an innate environmental response. Tests such as the open field and burrowing measure how pain affects threat avoidance (thigmotaxis in the open field) and tunnel maintenance (burrowing) in laboratory rats. Studies have shown their efficacy in detecting subtle behavioural deficits in experimental models of pain, including response to analgesics. Using ethological outcome measures also avoids applying clinical terminology to animal behaviour, and should be used in conjunction with classical sensory reflex-driven assays, such as hotplate and von Frey filaments, to yield a global picture of how experimental pain states affect wellbeing and quality of life.

This study examines how acute visceral inflammation, persistent visceral inflammation, and drug-induced neuropathy (d4T - antiretroviral nucleoside) alter the behaviour of adult female Wistar rats in the open field paradigm. It also investigates how the behavioural phenotype of naïve male Wistar rats responds to repeated open field exposure.

Significant increases in thigmotaxis were observed following acute bladder inflammation, and repeated exposure to open field in naïve rats. There was no effect on either open field behaviour or evoked sensory measures in female rats treated with d4T, and acute bladder inflammation failed to alter burrowing behaviours. As d4T has been previously shown to alter thigmotactic behaviour, this data suggests that females show different sensitivity compared to males.

To determine whether visceral inflammation influences behaviour in a dose-dependent manner, bladder tissue from the visceral inflammation groups was assayed for levels of cytokines using an RNA microarray featuring 92 inflammatory cytokines and 4 housekeeping genes. The greatest effect was detected following acute inflammation, with both models showing increased levels of CCL12, CCL7, and IL-1β when compared to naive tissue.

To correlate the changes in behaviour with a neural substrate, c-Fos immunoreactivity in the amygdala was measured in both visceral inflammation groups. No significant activational differences were seen following acute bladder inflammation, whereas persistent visceral inflammation significantly increased c-Fos immunoreactivity in the caudal regions of the capsular central amygdala.

Visceral inflammation is associated with peripheral increases in inflammatory cytokines, and thigmotactic behaviour in the open field. Differences in activation in the amygdala were lower than expected due to high levels of variation associated with inflammatory stimuli at the time-point tested. Significant variation was particularly seen in repeated visceral inflammation, suggestive of the biphasic behavioural response to stress e.g. attack or defend phenotypes. Further examination of these differences at the individual level could shed light on the process of pain chronification, and ultimately help understand why only some individuals develop chronic pain.

Declaration of Originality

I would like to acknowledge the contribution of Tim Pheby for his assistance with measurement of reflex hypersensitivity, burrowing, and immunohistochemical outcomes.

I hereby declare that all other work reported herein was composed by me and originated entirely by me. Information derived from published and unpublished work of others has been cited within the text, with full references to be found at the end of this thesis.

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List of Abbreviations

Abbreviation: Definition, page number of first occurrence

A

AA: Acetic Acid

AAA: Anterior Amygdala Area

ACC: Anterior Cingulate Cortex

AChE: Acetylcholinesterase

ACo: Anterior Cortical Amygdaloid Nucleus

ACTB: Beta Actin

ACTH: Adrenocorticotropic Hormone

AHi: Amygdalohippocampal Transition Area

ANOVA: Analysis of Variance

AO: Anaesthesia Only

APir: Amygdalopiriform Transition Area

ARRIVE: Animal Research; Reporting of In Vivo Experiments

ART: Anti-Retroviral Therapy

ATP: Adenosine Tri-Phosphate

AVP: Vasopressin

B

BAOT: Bed Nucleus of the Accessory Olfactory Tract

BAS: Bed Nucleus of the Anterior Commissure

BCG: bacillus Calmette-Guérin

BDNF: Brain-Derived Neurotrophic Factor

BL: Basolateral Nucleus of the Amygdala

BM: Basomedial Nucleus of the Amygdala

BPS: Bladder Pain Syndrome

C

CCI: Chronic Constriction Injury

CCK: Cholecystokinin

CCL: CC Chemokine Ligand

Ce: Central Amygdala

CeC: Capsular Nucleus of the Central Amygdala

CeL: Lateral Nucleus of the Central Amygdala

CeM: Medial Central Amygdala

CFA: Complete Freund's Adjuvant

CGRP: Calcitonin Gene Regulated Protein

CI: Confidence Interval

CNFD: Corneal Nerve Fibre Density

COCP: Combined Oral Contraceptive Pill

CONSORT: Consolidated Standards of Reporting Trials

COX: Cyclooxygenase

CPP: Conditioned Place Preference

CPSP: Central Post-stroke Pain

CRF: Corticotrophin Releasing Factor

CRPS: Complex Regional Pain Syndrome

CSF: Colony Stimulating Factor

CTLA: Cytotoxic T-Lymphocyte-Associated Protein (AKA RANTES)

CxA: cortex-Amygdala Transition Zone

CXCL: Chemokine (C-X-C motif) Ligand

CYP: Cyclophosphamide

D

d4T: 2',3'-didehydro-2',3'-dideoxythymidine AKA Stavudine

DAB: 3,3'-Diaminobenzidine

DFNS: German Research Network on Neuropathic Pain

DMV4: Diagnostic Manual v4

DPN: Diabetic Peripheral Neuropathy

DRG: Dorsal Root Ganglia

E

EA: Extended Amygdala

EQ-5D: EuroQoL Test 5 D

ERK: Extra-cellular Signal-Regulated Kinase

ESSIC: International Society for the Study of BPS

F

FAAH: Fatty Acid Amide Hydrolase

FC: Fold Change

FDR: False Discovery Rate

FIC: Feline Interstitial Cystitis

fMRI: Functional Magnetic Resonance Imaging

FS: Free-standing Rearing Activity

FSH: Follicle Stimulating Hormone

G

GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase

H

HIV: Human Immunodeficiency Virus

HPRT: Hypoxanthine-guanine phosphoribosyltransferase Gene

I

I: Instrumentation

IASP: International Association for the Study of Pain

IBS: Irritable Bowel Syndrome

IC: Interstitial Cystitis

IC/BPS: Interstitial Cystitis/Bladder Pain Syndrome

ICI: Inter-Contraction Interval

ICN: Intercalated Nuclei

IENFD: Intra-Epidermal Nerve Fibre Density

IHC: Immunohistochemistry

IL: Interleukin

iNOS: Inducible Nitric Oxide Synthase

IQR: Interquartile Range

L

La: Lateral Nucleus of the Amygdala

LH: Luteinizing Hormone

LORETA: Low Resolution Electromagnetic Tomography

LOT: Lateral Olfactory Bulb

LPS: Lipolysaccharide

M

MAGL: Monoacylglycerol Lipase

MCP: Monocyte Chemotactic Protein

MIF: Migration Inhibitory Factor

N

N: Naive

NAc: Nucleus Accumbens

NC3Rs: National Centre for the Replacement, Refinement, and Reduction of Animals in Research

NCF: Nucleus Cuniformis

NGF: Nerve Growth Factor

NIH: National Institutes for Health

NK: Natural Killer Immune Cells

NK1: Neurokinin-1

NMDA: N-Methyl-D-aspartate

NNTRI: Non-nucleotide Reverse Transcriptase Inhibitors

NOS: Nitric Oxide Synthase

NPSI: Neuropathic Pain Symptom Inventory

NRS: Numeric Rating Scale

NRTI: Nucleoside/Nucleotide Reverse Transcriptase Inhibitor

NSAID: Non-Steroidal Anti-Inflammatory

O

OAB: Overactive Bladder

P

PAG: Periaqueductal Grey

PB: Parabrachial Nucleus

PET: Positron Emission Tomography

PFC: Pre-Frontal Cortex

PHN: Post-Herpetic Neuralgia

PLCo: Posteriolateral Cortical Amygdaloid Nucleus

PMCo: Posteriomedial Cortical Amygdaloid Nucleus

PPBP: Pro-Platelet Basic Protein (isoform of CXCL-7)

PROK: Prokinectin

PTGES: Prostaglandin-Endoperoxidase Synthase

PVN: Paraventricular Nucleus

Q

QST: Quantitative Sensory Testing

R

RCT: Randomised Controlled Trial

RM ANOVA: Repeated Measure Analysis of Variance

ROS: Reactive Oxygen Species

rTMS: Repetitive Transcranial Magnetic Stimulation

RVM: Rostroventral Medulla,

S

SAM: Significance Analysis of Microarrays

SIV: Simian Immunodeficiency Virus

SLEA: Sublenticular Extended Amygdala

SNI: Spared Nerve Injury

SNRI: Serotonin-Noradrenaline Re-Uptake Inhibitor

SNT: Spinal Nerve Transection

ST: Bed Nucleus of the Stria Terminalis

T

T: Turpentine

tDCS: Transient Direct Contact Stimulation

TENS: Transcutaneous Electrical Nerve Stimulation

TGF: Transforming Growth Factor

TMS: Transcranial Magnetic Stimulation

TMT: Trimethylthiazoline

TNBS: Trinitrobenzenesulfonic acid

TNF: Tumour Necrosis Factor

TRPM: Transient Receptor Potential Melastatin

TRPV: Transient Receptor Potential Vanilloid

V

V: Vehicle

VAS: Visual Analogue Scale

VTA: Ventral Tegmental Area

VZV: Varicella Zoster Virus

W

WHO: World Health Organisation,

WKY: Wistar Kyoto

WS: Wall-Supported Rearing Activity,

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Thank-you.

1 Introduction

"I think about the meaning of pain. Pain is personal. It really belongs to the one feeling it.

Probably the only thing that is your own."

Henry Rollins

1.1 Pain

Pain is a complex experience, dependent on the interplay between sensory aspects and centrally mediated affective, motivational, cognitive, and behavioural elements. With the exception of rare individuals, such as those with congenital insensitivity to pain, for instance Riley-Day syndrome (Norcliffe-Kaufmann & Kaufmann, 2012), everyone has, or will experience pain throughout their lives, and yet it presents one of the most challenging problems in biomedical research, due mainly to its subjective nature.

Fundamentally, pain is a protective mechanism – it acts as a warning system, facilitating an organism's avoidance of interactions and behaviours that lead to pain, as evidenced by reduced life expectancy in those with abnormal pain processing (Verheyen & Castelein, 2007).

Fifty years ago, in 1965, Ronald Melzack and Patrick Wall published their ground-breaking work on Gate Theory (Melzack & Wall, 1965). Until that point, it was generally accepted that pain was a linear process, as described by Descartes, over 300 years previous in 1644 (DeLeo, 2006). Descartes' theory proposed "pain" was a direct signal from the site of injury to the brain, like electricity along a wire.
This idea was appealing for its simplicity and, by the 19th century, had developed into Specificity Theory (Moayedi & Davis, 2013). However, specificity theory was based on the assumption that neurones could be divided into different types, each responding to a specific stimulus modality, and passing information to an equally discrete part of the brain, specialised to respond to that stimulus alone, whereas it is now clear that although some nerves may show comparative modality specialism (e.g. small fibres sensitive to heat), there is a high degree of complexity and overlap between nerve function, in both the central and peripheral nervous system.

Lay definitions of pain tend to be circular - "Pain is that which hurts" - or use words such as "unpleasant" that inadequately convey the pain experience, as many things that are not painful can be unpleasant, and pain can be associated with sensory abnormalities out-with the description of "unpleasant" such as subtle alterations in detection thresholds for mechanical and thermal stimuli. The currently accepted definition of pain was proposed by Merskey in 1979, describing pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or

described in terms of such damage" (Merskey *et al.*, 1979). This was adopted as a formal definition by the International Association for the Study of Pain in 1986 .

Characteristics of Pain

As hinted at above, pain is not a purely sensory condition - it has both affective and cognitive components. Involvement of the amygdala and other limbic regions in the affective component emphasise the highly conserved nature of the responses to pain, and are suggestive of an evolutionary basis in survival by associating threat with fear, facilitating the appropriate protective response (e.g. avoidance, freezing, flight). Whereas the cognitive aspects help maintain context, with increased pre-frontal activity acting to supress medial amygdala activity, correlating with fewer feelings of distress (Lorenz *et al.*, 2003).

Pain has a primarily protective role, signalling that something is damaged and modulating behavioural responses until healing has occurred. How behaviour is affected depends on a number of factors including the type and intensity of pain (e.g. acute pain of a scald compared to a long-term constant dull ache), the disposition of the individual (e.g. catastrophizing compared to resilient psychological phenotypes) and their medical history, in addition to environmental factors such as immediate surroundings, and perceived security.

Clinically, diagnoses of pain involve description of the spatial location (e.g. leg, foot, upper arm), duration (e.g. acute, persistent, or chronic), intensity (often rated using a scale such as the visual analogue scale (VAS) or numeric rating scale (NRS)), and descriptors that convey the quality of the pain (e.g. burning, shooting, dull). Other factors such as previous surgery or injury are generally included, although assessments of mental health and consideration of other co-morbidities are rare in initial consultations. By definition, a diagnosis of neuropathic pain requires the presence of pain associated with nerve injury or dysfunction – this is usually done by a two-stage triage:

A. Documentation of a lesion or disease of the somatosensory system.

Followed by

B. Use of symptom descriptors and pain location to determine whether any pain is likely to be of neuropathic origin.

Diagnosis of visceral pain, on the other hand, involves the presence of pain originating from internal organs, indicated by diffuse pain in relevant direct dermatomal and referred segmental dermatome regions, compared to somatic pain, which is indicated by pain confined to a specific region without

referred pain. Additionally, conditions such as arthritis show a mixed presentation, with somatic elements acting in combination with persistent local inflammation at joints to cause long term pain and progressive damage of affected joints.

The Study of Pain

In pain, the relationship between intensity of experience and severity of damage is not linear – factors such as duration, intensity, and effect on daily activities contribute to the overall experience, meaning a single measure of pain intensity gives little information on prognosis and impact on daily life. This, in combination with a medical focus on curative rather than palliative treatment, has led to pain being neglected, part in due to its heterogeneity, and part because it is seen as an inevitable part of life. Pain is often assumed to have a discrete cause, which when removed will also remove the pain, and any other outcome is dismissed as pain being 'merely symptomatic'. However, pain often lingers long after the presumed cause has been resolved.

Compared to genetic tests capable of predicting the likelihood of developing Alzheimer's disease and imaging techniques ranging from X-ray to fMRI, developed to diagnose and assess conditions from cancer to broken bones, pain remains unquantified in comparison. The primary means of 'detecting' pain is to ask: verbal report, or validated questionnaires such as the McGill Pain Questionnaire (Melzack, 1975), and PainDETECT (Freynhagen *et al.*, 2006) can only relay pseudoquantitative information about the pain. Sensory testing, involving quantitative measurement of evoked responses to a variety of stimuli, is gaining traction as a method of assessing pain in more detail, but is currently used more as a clinical tool than a diagnostic aid (Pfau *et al.*, 2014).

Subjective Assessment of Pain

Quantification of the pain experience is difficult, primarily because diagnosis relies heavily on patient reported outcomes, with no definitive and clinically useful biomarkers, lacking a means of direct quantification in both humans and other organisms. Additional factors such as heterogeneous presentation and involvement of numerous un-related and inter-dependent factors further complicate diagnosis. The most widely used methods are based on communication between the patient and practitioner (patient reported outcome). Visual (VAS) and numeric (NRS) rating scales ask the patient to rate their pain on a finite scale, with the minimum or 'zero' being no pain and the maximum (e.g. 10, 100) being the worst pain you can imagine. These are useful as an indicator of pain intensity as there is some evidence they show intra-individual stability, although without standardisation there is high inter-individual variation. This is to be expected when reducing a personal subjective experience to a number, and scores can also be heavily influenced by external factors such as mood and clinical environment (Carlsson, 1983; Price *et al.*, 1983). Therefore,

questionnaires have been developed to quantify the subjective experience of pain, and are often combined with rating scales, questionnaires covering other aspects of the experience such as catastrophizing, and elements from the Diagnostic Manual v4 (DMV4) allowing comprehensive psychological phenotyping.

The McGill Pain Questionnaire (Melzack, 1975) is a widely validated specialised pain questionnaire (Holroyd *et al.*, 1992), with translations used worldwide (Vanderiet *et al.*, 1987; Boureau *et al.*, 1992; Masedo & Esteve, 2000; Mystakidou *et al.*, 2002; Yakut *et al.*, 2007), but there are many others frequently used, including PainDETECT (Freynhagen *et al.*, 2006), the neuropathic pain symptom inventory (NPSI, Bouhassira *et al.*, 2004) and the EQ-5D (EuroQoL Test 5 D, Torrance *et al.*, 2014) which gives a standardised measure of general health. These are often added to give depth to VAS scores, but as they rely on the patient being able to reliably describe how they feel, they are of limited use with infants, and individuals otherwise unable to communicate their answers.

Quantitative Assessment of Pain: Biomarkers

Ideally, a clinical diagnosis is based on reliable biological phenomena. The search for a pain biomarker has covered targets including autonomic measures such as blood pressure (Saccò *et al.*, 2013), heart rate (Mazurak *et al.*, 2012), and skin conductance (Storm, 2008), in addition to circulating blood corticosterone level and even finger length (de Kruijf *et al.*, 2014). Currently, the most robust markers are associated with quantitative sensory testing (Backonja *et al.*, 2013), and functional imaging studies (Tracey, 2008). However, these currently have more use as research tools than diagnostic criteria, as there is a difference between measurement and quantification of the pain experience and the sensory profiling and blood flow correlates provided by quantitative sensory testing (QST) and fMRI respectively.

Sensory testing concentrates on the underlying mechanisms and takes a more phenotypic approach to pain, focusing on characterising the sensory profile specific to a patient rather than determining a profile to fit all patients with a specific diagnosis. It involves a battery of tests designed to assess the limits and thresholds of mechanical, thermal, and vibration detection, alongside measurement of hypersensitivity, “wind-up”, and paradoxical heat sensations (i.e. mis-matched thermal perception, with cooling stimuli perceived as warm). The results are then compared with data from healthy subjects, and the deviation from normal values calculated for each outcome to produce a visual representation (Z-score), which enables rapid assessment of abnormal sensory responses. One of the most widely used and validated protocols was developed by the German Neuropathic Pain Research Network (DFNS, Pfau *et al.*, 2014).

They defined three types of threshold:

- Sensation - the first point at which something is detected, such as warmth
    - Uniform across populations
- Pain Perception - the point at which a heat stimuli changes from warm to a sharp pain
    - Some cultural influence
- Pain Tolerance - the limit of pain endurance
    - Dependent on individual characteristics and testing environment

Responses to a range of stimuli are assessed, and compiled to produce a Z-score, which indicates deviation from the expected normal population values. Current guidelines suggest that although QST is a reliable and robust method for screening small fibre neuropathy in clinical trials, it is not yet suitable as a diagnostic test for neuropathic pain (Backonja *et al.*, 2013). Additionally, the presence of co-morbidities, particularly other pain conditions, complicates the use of QST as a diagnostic tool, as illustrated by studies looking for evidence of widespread sensitisation in patients with interstitial cystitis/Bladder Pain Syndrome (IC/BPS), where inclusion of patients with both IC/BPS and fibromyalgia showed more widespread hypersensitivity (Clemens *et al.*, 2008; Nickel *et al.*, 2010) than those with IC/BPS alone (Lai *et al.*, 2014).

Functional imaging techniques such as fMRI can be used to monitor alterations in blood flow during the pain experience and highlight functional associations between physical symptoms/experiences and cerebral activation. Such studies have largely confirmed anatomical and functional pre-clinical research indicating areas such as the insula (Wiech *et al.*, 2014), anterior cingulate cortex (ACC; Shackman *et al.*, 2011), thalamus (Baliki *et al.*, 2008), amygdala (Bornhovd *et al.*, 2002), and prefrontal cortex (PFC; Lorenz *et al.*, 2003) are activated during pain. Loss of grey matter has also been detected in these areas in patients with chronic back pain (Apkarian *et al.*, 2004; Schmidt-Wilcke *et al.*, 2006), fibromyalgia (Kuchinad *et al.*, 2007), and osteoarthritis, where it was demonstrated that this decrease is a consequence not cause of pain, as increases in grey matter volume were seen following total hip replacement in these patients (Rodriguez-Raecke *et al.*, 2009). However, there are a number of limitations to interpretation of such data. Firstly, fMRI measures changes in blood oxygenation indicative of increased brain activity with a 6-10 second lag between action potential and altered perfusion. This lag has been shown to change with age, emphasising the importance of accurately stating the age range of subjects (Taoka *et al.*, 1998). Secondly, the precision of data is dependent on the power of MRI scanner used, with precision increasing with

tesla rating (Skouras *et al.*, 2014), highlighting the need for accurate reporting of methods and caution when interpreting results where the resolution of the equipment is unknown. It is also important to be aware that studies discussing activation of small, complex nuclei such as the amygdala may miss the subtle yet important differences in activation arising through differential activation of sub-nuclei with opposing or synergistic actions.

Pain has been associated with sympathetic abnormalities, particularly in conditions such as CRPS (Complex Regional Pain Syndrome) and fibromyalgia (Maletic & Raison, 2009; Borchers & Gershwin, 2014; Rockett, 2014). These manifest as alterations in autonomically controlled phenomenon such as sweating and skin temperature in patients with CRPS (Rockett, 2014), however, these changes are not robust enough to provide a definitive diagnosis, largely due to high heterogeneity.

Thermographic studies have been conducted to investigate whether differences in regulation of skin temperature can be indicative of pain conditions (Karpman *et al.*, 1970), with decreases in pedal thermoregulation noted in IC/BPS patients (Irwin *et al.*, 1993), although as with other autonomic outcomes, this is not robust enough for use as diagnosis.

Loss of small fibres is implicated in neuropathy (Lauria *et al.*, 2012). This was initially documented clinically for the purpose of diagnosing small fibre neuropathies using skin biopsies, measuring the intra-epidermal nerve fibre density (IENFD), with decreases seen in HIV (Shikuma *et al.*, 2012; Phillips *et al.*, 2014), and diabetes (Malik *et al.*, 2011). However, skin biopsy is an invasive technique, with values obtained dependent not only on the presence of neuropathy, but also sampling location. The cornea is an attractive alternative, as it is highly innervated, and can be visualised non-invasively using confocal corneal microscopy (Herrmann, 2008). Decreases in corneal nerve fibre density (CNFD) have been found associated with neuropathy in sarcoidosis (Dahan *et al.*, 2013), Charcot Marie-Tooth disease type 1A (Tavakoli *et al.*, 2012), Fabry's disease (Tavakoli *et al.*, 2009), and diabetes (Quattrini *et al.*, 2007; Tavakoli *et al.*, 2010), with many studies comparing IEFND to CNFD and finding good correspondence between the measures (Quattrini *et al.*, 2007; Tavakoli *et al.*, 2010; Ferrari *et al.*, 2013a; Asghar *et al.*, 2014). Further characterisation of CNFD in other peripheral neuropathies is required, but it offers an attractive means to non-invasively survey nerve fibre density, a potential quantitative biomarker that correlates with pain intensity.

The biomarkers documented to date represent a useful battery of research tools to study clinical pain patient cohorts, with the aim of understanding individual differences in sensory dysfunction, but until high heterogeneity associated with diagnostic imprecision is resolved, accurate identification of patient populations using biomarkers is unfeasible.

Chronic Vs. Acute

A useful method of characterising pain is by duration. Acute pain is a relatively short-lived response to tissue damage. It can vary in intensity (e.g. a cut finger versus appendicitis) but the pain subsides rapidly once the harmful stimulus is withdrawn. Persistent pain is a term sometimes used to describe pain which is of longer duration than acute, yet below the 3-6 month classification of chronic, and is often seen in in post-operative pain, and pain conditions with an inflammatory component (Cregg *et al.*, 2013; Zhang *et al.*, 2014b). Chronic pain, as suggested by the name, is of longer duration. Definitions vary but range from 3 to 6 months as a minimum duration threshold, or alternatively, it can be defined as "pain that extends beyond the expected period of healing", although this excludes conditions such as arthritis where the pain can be present for many months, if not years, in association with unhealed tissue damage due to pathological inflammation. Furthermore, many chronic pain conditions involve neuropathic elements, complicating treatment. When pain is present continuously for three or more months, the affective and cognitive dysfunctions increase leading to further difficulties during treatment (Sternbach, 1974). Physiologically, chronic pain is associated with neurological changes - neurons can become sensitized, firing spontaneously in the presence of continued afferent barrage.

Neuropathic pain refers to pain due to damage or disruption to central or peripheral nerves involved in the generation and control of pain, and is formally defined by IASP as:

Pain caused by a lesion or disease of the somatosensory nervous system

(Jensen *et al.*, 2011)

It can have a variety of causes, including trauma/surgery (e.g. CRPS, post-operative neuropathic pain), infection (e.g. varicella zoster virus/VZV, leprosy), viral (e.g. HIV), and chemical (e.g. chemotherapeutic or anti-retroviral agents), and is also associated with other medical conditions such as diabetes (Bridges *et al.*, 2001; Attal *et al.*, 2008; Jensen *et al.*, 2011).

Other chronic conditions, such as chronic cough share many similarities with chronic pain, such central and peripheral nerve sensitisation (O'Neill *et al.*, 2013), and over-expression of sodium channels implicated in pain, such as Nav1.7-1.9 (Muroi & Undem, 2014).

Affective Aspects

The majority of therapeutic approaches and research strategies concentrate on ameliorating the sensory elements of pain; however, it is clear that when most people talk about pain, they are talking about a category of experiences with a common theme. We are able to distinguish the pain of

toothache from the pain of sunburn - these different pain experiences are characterised more by the emotional component than any identifiable common element. This emotional association is specific to an individual, but is broadly based on anxiety concerning the current situation and future projections of outcome. The behavioural response is not specific to the sensory experience that triggered it, with cognitive elements such as anticipation of a painful stimulus also shown to influence perception (Ploner *et al.*, 2010).

All types of pain are associated with affective symptoms, but due to its prolonged duration, chronic pain in particular is associated with a high burden of psychological symptoms. This can be explained by the duration of pain extending beyond that expected, increasing anxiety, decreasing the patient's sense of control over the situation, and permeating all thoughts about the future. Evidence of this can be seen by the correlation between catastrophizing thought patterns and both duration of pain (Gilliam *et al.*, 2010), and association with prolonged post-operative recovery (Lautenbacher *et al.*, 2010).

Surveys have shown numerous co-morbidities within and between pain syndromes, with anxiodepressive signs, lethargy, catastrophizing, sleep disturbances, and reduced quality of life commonly encountered (Twillman, 2007; Radat *et al.*, 2013; Chung *et al.*, 2014). There is additionally considerable overlap between chronic pain syndromes, especially between somatic and visceral pain conditions such as fibromyalgia, irritable bowel syndrome (IBS), and interstitial cystitis (IC) (Pezzone *et al.*, 2005).

The current translational difficulties in developing effective pain relief are associated in part with a pre-clinical focus on positive sensory signs, whereas the clinical burden of chronic pain comes largely from negative behavioural responses, such as avoiding situations perceived as exacerbating, and a general decrease in quality of life as a result of unpredictable spontaneous pain.

Experimental Pain in Human Subjects

The study of pain in humans relies on the ability to induce transient, standardised episodes of pain without causing underlying damage. Researchers have utilised heat, cold, chemical irritants, and ischaemia to induce pain in healthy volunteers (Staahl & Drewes, 2004; Oertel & Lötsch, 2013). One problem quickly recognised was insensitivity to opioids in methods of inducing pain involving thermal and chemical stimuli. Beecher's constriction model is an exception to this, perhaps due to the indirect method of activating nociceptors, suggesting inherent differences in processing between pain of external or internal origin (Smith *et al.*, 1966).

These models are of limited clinical relevance to chronic pain due to their typically short duration of nociceptor activation, compared to the sustained activation seen in chronic pain conditions, and the fact they tend to model only one aspect of the pain experience and focus on evoked rather than spontaneous pain (Reddy *et al.*, 2012).

1.2 Visceral Pain

Visceral pain is defined as pain affecting the internal organs (viscera) and is thought to be the most widely experienced type of pain, with 16-25% of the general population affected by pelvic, abdominal, or thoracic pain (Collett, 2013). Pain originating from an internal organ is characterised by diffuse presentation due to sparse innervation, with less than 10% of afferent inflow to the thoracolumbar spinal cord coming from the viscera (Cervero *et al.*, 1984). Viscera receive a higher level of sympathetic innervation compared to somatic tissue, and both sympathetic and parasympathetic fibres converge from many proximal organs at the spinal cord, leading to referred pain in distal dermatome-relevant sites e.g. arm pain during a myocardial infarction, and lower back pain during urinary tract and kidney infections (Giamberardino, 1999). The low density and increased spacing of sensory fibres results in difficulty pinpointing the exact source of the pain, unlike most other pain complaints involving highly innervated skin and muscle, and it is this inability to locate the pain that underlines increased levels of anxiety and affective disorders associated with visceral pain conditions (McMahon *et al.*, 1995; Jänig, 2010). This relationship is reciprocal, and symptoms often flare up under stress due to activation of sympathetic nerves (Gareau *et al.*, 2008; Larauche *et al.*, 2011). However, it is important to remember that the lower density of fibres is not indicative of a lesser pain experience, as evidenced by not only childbirth, but also conditions such as appendicitis and kidney stones.

Despite being so common, visceral pain is inherently difficult to study in animal models, and this is reflected in the literature. A PubMed search conducted on the 19th November 2014 (search terms: (pain) AND (neuropathic) vs. (pain) AND (visceral) – no limitation on date or language range) revealed 13,562 results for pain with a neuropathic element, compared to 5186 for pain with a visceral component. Of these, 6006 neuropathic studies were conducted in non-humans (filter: other), compared to 1724 for visceral (filter: other). The earliest studies were published in 1966 and 1906 for neuropathic and visceral pain respectively, and although this is a crude method of measuring interest, it highlights the disparity present in the study of different pain conditions, and reflects the difficulties present in the study of visceral pain.

The Urinary Bladder

As the majority of this study focuses on bladder inflammation, I will now discuss the anatomy of the bladder to further understand how it responds to nociceptive stimuli. The human urinary bladder is a hollow, distensible organ located on the pelvic floor with an average volume of 300-400ml (Hinman & Cox, 1967). Micturition, or the process of expelling urine stored in the bladder, occurs when the muscle layers at the bladder neck and sphincter are relaxed. The timing of this is dictated primarily by bladder volume, with volumes of 150ml sufficient to activate stretch receptors, which respond proportionally to the rising volume of urine within the bladder by increasing the sense of 'urgency', however other factors including stress/anxiety and environmental conditions can also influence micturition frequency.

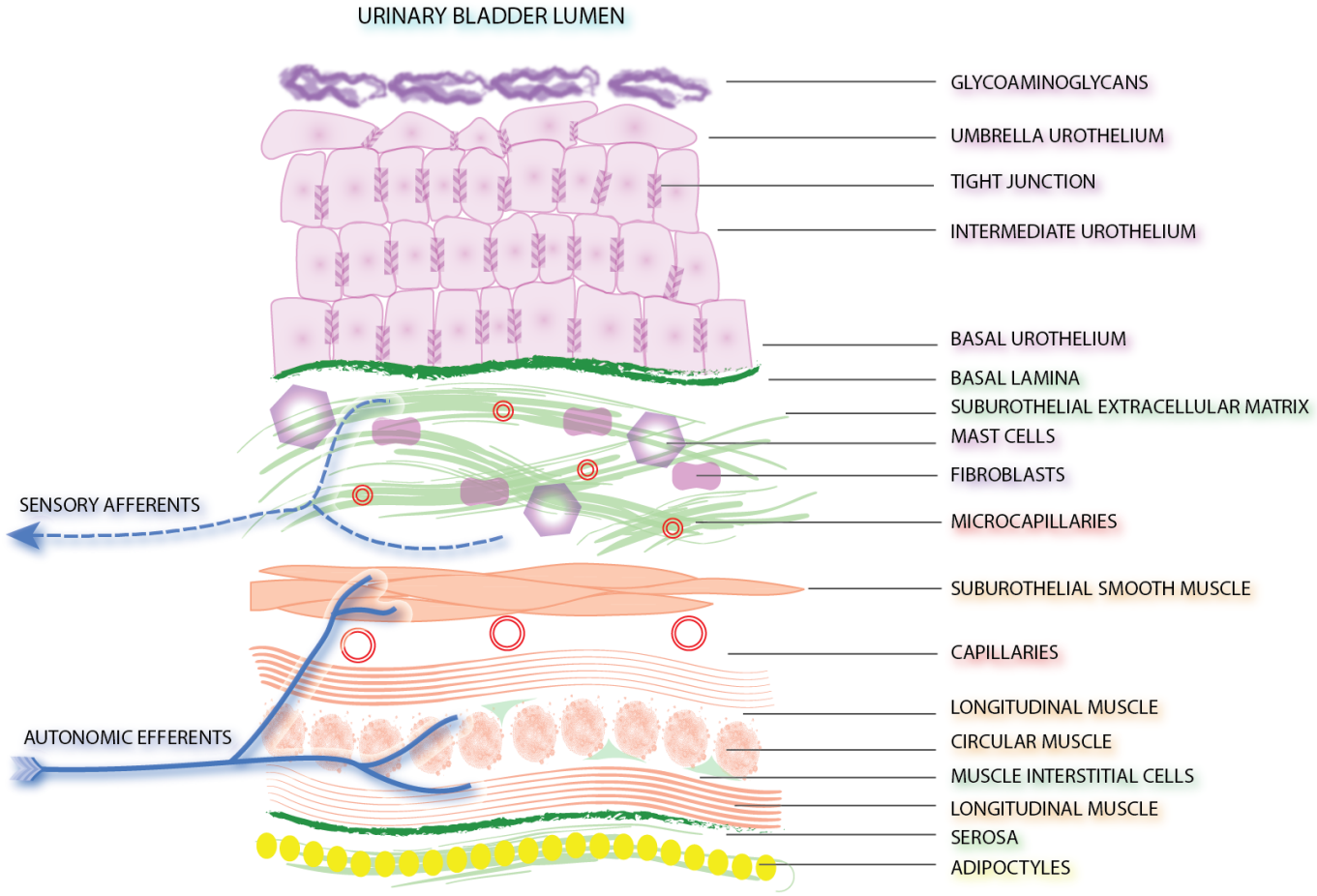


Figure 1-1: Cellular structure of the bladder wall
Illustrating urothelial, suburothelial, and detrusor muscle zones, in addition to main cell types and points of innervation. Adapted from Rahnama'i *et al.*, 2013; Birder, 2014)

The cellular structure of the urinary bladder shown in Figure 1-1 illustrating the complexity of this organ. Sensory afferents detect alterations in the extracellular environment between the urothelium and muscle layers (where autonomic afferents controlling bladder function terminate), and the

presence of mast cells and vascular structures enables rapid inflammatory responses. Glycosaminoglycans act as a first line of defence, coating the inner lumen of the bladder in a protective layer, minimising potential irritation due to contact with bladder contents, in addition to the barrier provided by tight junctions between urothelial cells (Keay *et al.*, 2014; Birder, 2014).

Figure 1-2 illustrates the innervation of the urinary bladder, with motor innervation provided by both the sympathetic (hypogastric plexus and associated nerves; T12-L2 in man, L1-L2 in rats), and parasympathetic (pelvic splanchnic nerves and inferior hypogastric plexus; S2-4 in man, L6-S2 in rats) nervous systems, and sensory information from the superior and inferior aspects of the bladder transmitted along general visceral afferent fibres which follow the sympathetic and parasympathetic fibres respectively (Lukacz *et al.*, 2011). The high levels of convergent innervation in the pelvic region prompts some to suggest an umbrella term of Urologic Chronic Pelvic Pain Syndrome (UCPPS) to describe conditions such as cystitis and prostatitis which share many common features (Tirlapur *et al.*, 2013; Clemens *et al.*, 2014). There are also strong associations between pelvic pain conditions and both chronic pain disorders (Suskind *et al.*, 2013), and a wide range of other non-pain conditions (Keller *et al.*, 2012). Furthermore, increased firing is also noted in the bladder in association with colorectal irritation (Ustinova *et al.*, 2006), and there is a high degree of overlap between chronic visceral conditions in general, such as that between IC/BPS and IBS (Clemens *et al.*, 2012) emphasising the links between pelvic innervation.

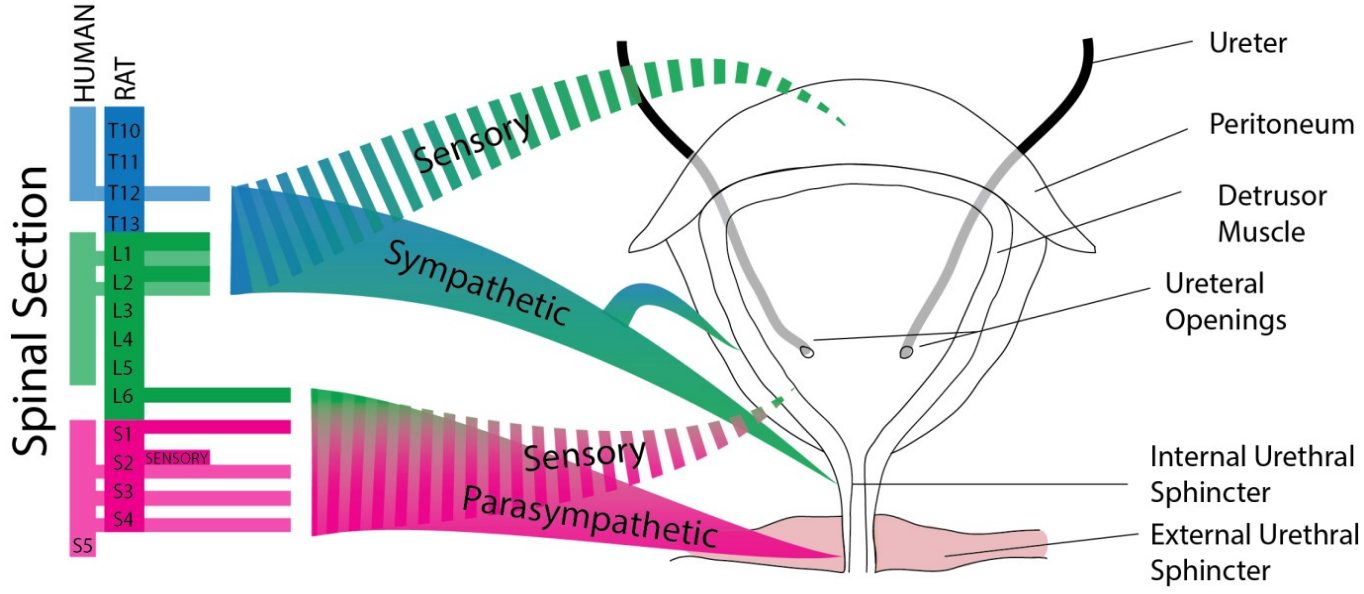


Figure 1-2: Innervation of the bladder
Sympathetic, parasympathetic, and sensory innervation in both man and the laboratory Rat, illustrating the differences and similarities between species, with thoracic innervation involved in humans but not rats

Cystitis

The term 'cystitis' comes from the Latin/Greek for 'bladder' and 'disease characterised by inflammation', and is widely used to describe inflammatory diseases of the bladder. Interstitial cystitis (IC) is a sub-type defined by a diagnosis of exclusion which shows considerable overlap with other bladder conditions such as overactive (OAB) and bladder pain syndrome (BPS) (Diaz, 2014). The term IC/BPS is frequently used as an umbrella term, covering bladder syndromes featuring persistent suprapubic pain with or without increased urinary frequency and urgency, and may include the presence of Hunner's ulcers (Hanno *et al.*, 2011; Killinger *et al.*, 2013), although these are only seen in 5-10% of IC/BPS cases (Logadottir *et al.*, 2012).

Clinical biomarkers such as anti-proliferative factor (Kim & Freeman, 2011), GP51 (Byrne *et al.*, 1999), and increased nerve fibre density (Christmas *et al.*, 1990) have also been associated with a diagnosis of IC/BPS. These new fibres show up-regulation of a number of factors implicated in pain including substance P and CGRP (Pang *et al.*, 1995; Schnegelsberg *et al.*, 2010), TRPV1 and NGF (Liu *et al.*, 2014), cyclooxygenase-2 (COX-2) (Klinger *et al.*, 2007) and CB1 (Mukerji *et al.*, 2010), with levels correlating with pain scores.

Putative causes of IC/BPS include autoimmune, allergic, and genetic, with stress and anxiety playing a role in common with other visceral pain disorders (Chung *et al.*, 2014).

Referred hyperalgesia in IC/BPS and other bladder pain conditions has been documented.

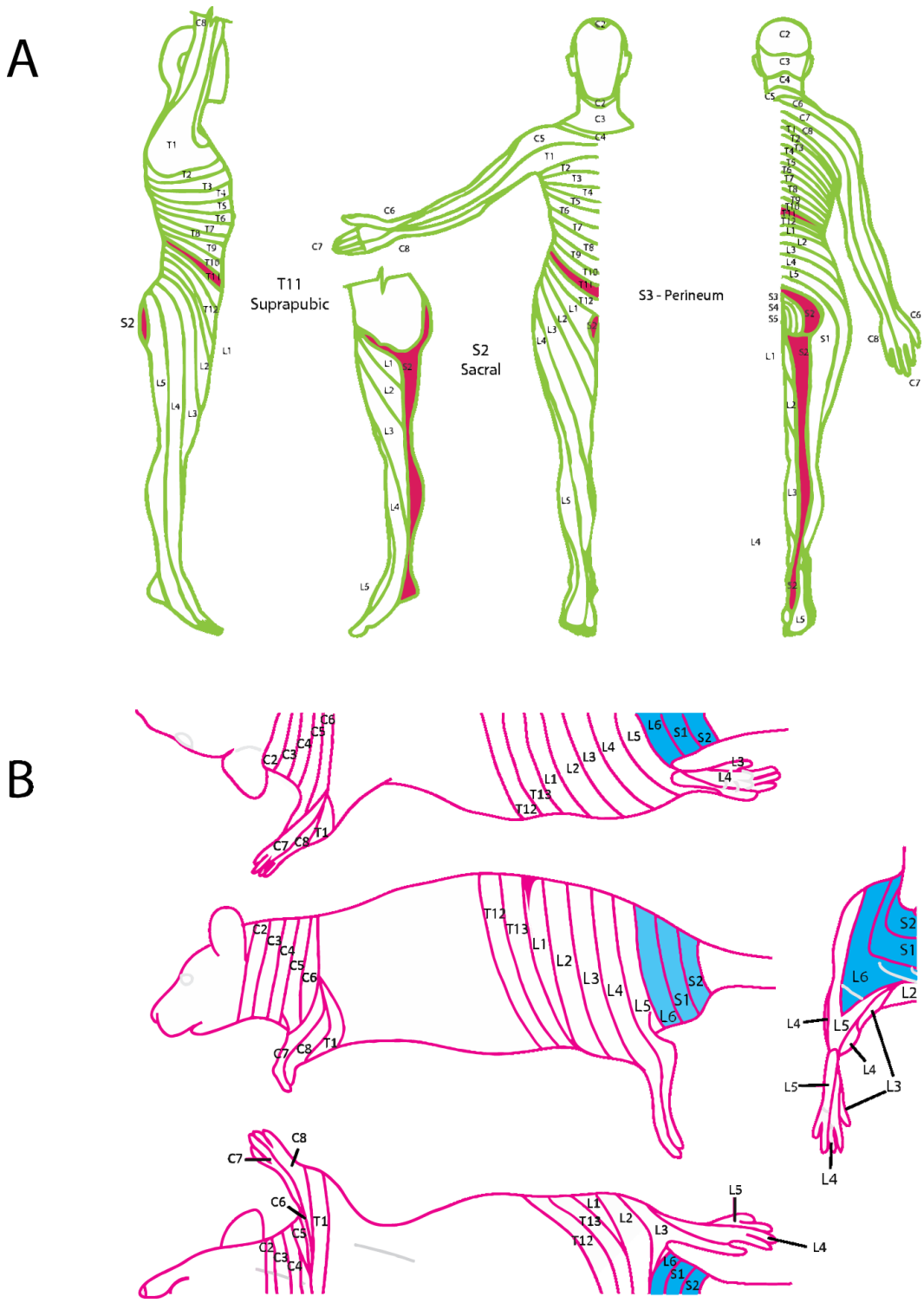


Figure 1-3: Segmented referral dermatomes for the bladder in man and the laboratory rat
A) IC/BPS: Suprapubic Area (T11), Sacral (S2), Perineum (S3), (based on an original image from Maitland, 1968b)
B) Rat Bladder somatic referral regions (adapted from Takahashi & Nakajima, 1996)

Figure 1-3 shows the segmental referral dermatomes associated with bladder pain: The suprapubic area (T11), sacrum (S2), and perineum (S3). A recent study demonstrated increased VAS ratings to T11 pressure pain stimuli in patients with IC/BPS and no co-morbidities (Lai *et al.*, 2014), in contrast with previous studies which found evidence of wider general sensitisation to pressure stimuli in

IC/BPS, although these patients also had a number of co-morbidities, and exhibited increased variation in heat thresholds, suggestive of the presence of different sensory phenotypes within the group (Ness *et al.*, 2005).

1.3 Current Therapeutic Approaches

Pain represents a serious economic concern in terms of both working days lost and productivity (Harman & Ruyak, 2005; Beckett *et al.*, 2014), emphasising the need for more effective analgesic treatments.

NSAIDs and Paracetamol

For mild to moderate acute pain, non-steroidal anti-inflammatory (NSAID) drugs, such as aspirin and ibuprofen are widely used and generally available over the counter. These drugs act primarily in the periphery, by counteracting inflammatory processes and reducing activation of peripheral sensory nerve endings through inhibition of pro-inflammatory enzymes such as COX (Björkman, 1995). COX-2 is expressed on endothelial tissue, whereas COX-1 predominates in the gastrointestinal tract – NSAIDs which can specifically target COX-2 have fewer side-effects such as ulceration (Patrignani & Patrono, 2014). Cardiovascular complications (hypertension and atherothrombosis) are associated with increasing dosage (Bruno *et al.*, 2014) meaning such drugs are not suitable for treating patients with existing heart conditions. Additionally, non-specific COX-inhibitor NSAIDS are contraindicated in individuals with asthma and cardiovascular complaints, which is why COX-2 specific drugs are the preferred choice. Acetaminophen (paracetamol) is another commonly used analgesic, classified as an aniline analgesic and recommended as a first line treatment for osteoarthritis, with contraindications compared with classic NSAIDs (Jóźwiak-Bebenista & Nowak, 2014). It is comparable with NSAIDs in terms of efficacy, but has low anti-inflammatory activity, and has central activity via TRPV1 receptors (Mallet *et al.*, 2010) in a fatty acid amide hydrolase (FAAH-dependent manner, Barrière *et al.*, 2013), emphasising the role of the endocannabinoid system in nociception (Rice *et al.*, 2002; Rea *et al.*, 2007).

Opioids

Morphine and other opioids are the first line therapeutic approach for severe acute pain, such as emergencies characterised by sudden onset of severe pain, post-operative analgesia and during labour to make prolonged but finite pain more bearable, and in the final stages of treatment for many terminal diseases (Finnerup *et al.*, 2015). Opioids act on the descending control pathways of the spinal cord, activating rich seams of endogenous opioid receptors, and causing the suppression of signals returning from the periphery (Sandkühler, 1996). In addition to the matter of tolerance and addiction, and side effects such as cognitive impairment, concerns regarding the social and

ethical issues associated with morphine and other opioids have negatively influenced prescription and use (Flemming, 2010).

Anti-Epileptics and Anti-Depressants

Since the early 60s, drugs originally developed to treat other neurological disorders, such as epilepsy and major depression, have been adopted as first-line treatments for pain conditions with a neuropathic element (Finnerup *et al.*, 2015).

Drugs such as Gabapentin and pregabalin are examples of gabaergic drugs with origins as anti-convulsants that act by decreasing neural activity, whereas duloxetine and amitriptyline are anti-depressants, which act via actions on serotonin and noradrenaline re-uptake inhibition. As detailed below, both categories of drugs are now recognised as having analgesic properties.

Gabapentin ("Neurontin") was originally developed by Pfizer and was approved as an anticonvulsant for the treatment of epilepsy in 1993 (Graves & Leppik, 1993; Goa & Sorkin, 1993; Moore *et al.*, 2011). It targets the α2δ-1 accessory subunit of voltage-gated calcium channels, and inhibits nerve injury-induced trafficking of α1 pore-forming units, in addition to reducing movement of α2δ-1 subunits from dorsal root ganglia (DRG) to the dorsal horn (Kukkar *et al.*, 2013). It was subsequently found effective in the treatment of various chronic and neuropathic pain conditions, and is now a recommended treatment for diabetic peripheral neuropathy (DPN; Attal *et al.*, 2010), post-herpetic neuralgia (PHN; Segal & Rordorf, 1996; Wiffen *et al.*, 2013), and neuropathic pain originating from damage to the central nervous system (Finnerup, 2008). Gabapentin shows some efficacy in phantom limb pain (Abbass, 2012), and has been suggested as a surgical pre-treatment to reduce the incidence of post-surgical chronic pain (Clarke *et al.*, 2012). It was shown more effective than amitriptyline in the treatment of chronic pelvic pain (Cheong *et al.*, 2014), and has some efficacy in chemotherapy-induced neuropathy (Bar Ad, 2010), and fibromyalgia (Moore *et al.*, 2014), although other studies have suggested pregabalin is superior for the treatment of fibromyalgia (Wiffen *et al.*, 2013). It is not effective for all pain conditions, and has low efficacy in post-surgical pain, HIV-associated peripheral neuropathy, CRPS, and migraine (Moore *et al.*, 2014). Other similar drugs include pregabalin ("Lyrica") and gabapentin enacarbil, which was approved for PHN in 2012 (Thomas & Farquhar-Smith, 2013).

Duloxetine ("Cymbalta") is a balanced serotonin-noradrenaline re-uptake inhibitor (SNRI, developed by Eli Lilly (Bellingham & Peng, 2010), and licensed for DPN and major depressive disorder in 2004 (Lunn *et al.*, 2014). In 2008 it was also licensed for the treatment of fibromyalgia (Lunn *et al.*, 2009), and in 2010, osteoarthritis and musculoskeletal pain were added as approved indications (Citrome &

Weiss-Citrome, 2012; Myers *et al.*, 2014b). Its efficacy in fibromyalgia is thought to be due largely to amelioration of affective symptoms (Lunn *et al.*, 2009).

Amitriptyline ("Elavil") was developed by Merck, and is one of the most widely prescribed tricyclic anti-depressants (Bryson & Wilde, 1996; Mika *et al.*, 2013). It was licensed in 1961 and is featured on the WHO list of essential medicines (WHO, 2013). In common with duloxetine, amitriptyline also acts as an SNRI, although is associated with high inter-patient variation in metabolism, making it difficult to accurately determine an effective dose applicable to all patients (Bryson & Wilde, 1996). Amitriptyline is now indicated for major depressive disorder, generalised anxiety disorder, attention deficit hyperactivity disorder, bipolar disorder, migraine, PHN, insomnia, fibromyalgia, and IBS (Bryson & Wilde, 1996). As it has been prescribed for many years, numerous systematic reviews have been conducted to further investigate the efficacy of amitriptyline in neuropathic and chronic pain disorders – second tier efficacy for DPN was noted (Bryson & Wilde, 1996; Moore *et al.*, 2012), with fibromyalgia presenting both as treatable (Häuser *et al.*, 2011)and unaffected by amitriptyline treatment (Nishishinya *et al.*, 2008). It is a third tier treatment for phantom limb pain (McCormick *et al.*, 2014), and recommended for IC/BPS (Offiah *et al.*, 2013). Interestingly, there is evidence that amitriptyline has topical efficacy (Kopsky & Hesselink, 2012), although the quality of this evidence is poor, as noted in a later systematic review of topical analgesics (Argoff, 2013).

Topical Analgesics

The use of topical treatments for pain is well established – historical accounts of applying poultices to painful areas have been documented for centuries (Arnstein, 2013). Topical application of analgesics is attractive as it minimises side effects associated with systemic dosing, and allows targeted treatment of superficial painful areas (Stanos & Galluzzi, 2013). Compounds applied topically can be divided into NSAIDs (e.g. ibuprofen, diclofenac, and keptoprofen), local anaesthetics (primarily lidocaine), and counter-irritants (salicylates, menthol, camphor, and capsaicin) that activate and then desensitise peripheral afferents (Barkin, 2013). Topical NSAIDs are routinely recommended as treatment for arthritis (Altman & Barthel, 2011), whereas capsaicin and lidocaine are more effective in neuropathic pain (Gilron *et al.*, 2006), including conditions such as PHN (Wolff *et al.*, 2011)and CRPS (Hsu, 2009).

Topical capsaicin exerts its effects by desensitising C-fibres via defunctionalisation of TRPV1 receptors (Sharma *et al.*, 2013), and induces die-back of affected nociceptive fibres, resulting in decreased epidermal nerve fibre densities (Gibbons *et al.*, 2010), and it has been shown that higher concentrations of capsaicin, whilst associated with increased management for clinicians, are most effective in treatment of chronic neuropathic pain (Derry *et al.*, 2013).

Lidocaine, on the other hand, acts primarily via blockade of sodium channels (Barkin, 2013), and also activates TRPV1 and TRPA1 receptors, and studies into the use of 5% transdermal patches have shown results in a similar reduction in epidermal nerve fibre density after 42 days of treatment, which is associated with decreased mechanical sensitivity, whilst leaving pressure, thermal, and cold responses relatively unaffected (Wehrfritz *et al.*, 2011). It has been shown efficacious for various neuropathic conditions including PHN (Attal *et al.*, 2010; Mick & Correa-Illanes, 2012), although studies into other conditions such as post-herniorrhaphy pain have so far been inconclusive (Bischoff *et al.*, 2013), emphasizing the need for high quality, large-scale clinical trials.

Non-Pharmaceutical Approaches

Non-pharmaceutical treatments such as transcranial magnetic stimulation, acupuncture (and associated acupressure based treatments) are also widely used to treat pain, with varying degrees of efficacy and specificity (Dworkin *et al.*, 2013).

Transcranial magnetic stimulation (TMS) is a non-invasive technique that uses electromagnetic induction of weak electric currents to depolarize or hyperpolarize neurons. The magnetic field has a similar strength to that used in MRI. It has a penetration of approximately 5cm, meaning targeted stimulation by positioning the stimulator over relevant brain areas, such as the primary motor cortex. It can take the form of direct, constant stimulation (tDCS), or short repetitive bursts (rTMS), and both are associated with analgesic effect in fibromyalgia (Marlow *et al.*, 2013), and other pain conditions with a rheumatic element (Pérocheau *et al.*, 2014), as well as neuropathic pain associated with spinal cord injury (Nardone *et al.*, 2014). In a recent set of guidelines, rTMS was recommended as an analgesic when applied to the primary motor cortex contralateral to the site of pain (Lefaucheur *et al.*, 2014), and a systematic review of the animal literature on non-invasive analgesic therapy conducted in 2012 found analgesic efficacy in studies investigating both tDCS and rTMS (Volz *et al.*, 2012).

Transcutaneous electrical nerve stimulation (TENS), as the name suggests, involves application of electrical stimulation via electrodes attached to the skin. The pulse width, frequency, and intensity of electrical stimulation can be varied dependent on requirements – low frequency is associated with muscle contraction, whereas higher frequencies activate sensory nerves. It has been suggested as an effective adjunct therapy in the treatment of chronic pain, although the majority of systematic reviews have been inconclusive (Nnoaham & Kumbang, 2008; Khadilkar *et al.*, 2008; Rutjes *et al.*, 2009; Robb *et al.*, 2009). Efficacy has been suggested in treatment of DPN (Dubinsky & Miyasaki, 2010), and pre-clinical studies have demonstrated TENS decreases microglial activation clinically when applied immediately following surgery (Matsuo *et al.*, 2014), and ameliorates Randall-Selitto

sensitivity in carrageenan-induced pain *in vivo* in an opioid-dependent manner system (Sabino *et al.*, 2008). However, to enable valid assessment of TENS-induced analgesia, it is acknowledged that the quality of clinical research needs improvement, particularly regarding standardisation of dosing and outcomes (Bennett *et al.*, 2011).

Acupuncture has been used medicinally in China for over 3000 years (Zhuang *et al.*, 2013). It is a therapeutic technique involving insertion of fine needles at specified points along meridian lines on the body, with point corresponding to almost every ailment imaginable (Yang *et al.*, 2011), including pain (Wang *et al.*, 2008). Numerous studies have been conducted to understand how and when acupuncture is effective as an analgesic – in the last 5 years, 1896 articles looking at "(Acupuncture) AND Pain", were published, and while the majority were in English (1464), or Chinese (374), the remainder were in 15 different languages, highlighting the worldwide interest in the topic, and the potential for 'lost data' when reviewing the literature related to acupuncture. Systematic reviews have been cautiously positive (Lam *et al.*, 2013b; Manyanga *et al.*, 2014; Yang *et al.*, 2014), but acknowledge the high bias present due to difficulties using double blind protocols with traditional acupuncture techniques. Electro-acupuncture, a technique involving the application of electrical stimuli to traditional acupuncture pressure points, is used as an alternative to traditional needle acupuncture and involves activation of neurotransmitter systems including serotoninergic, opioidergic, and GABA-ergic (Kim *et al.*, 2013). Pre-clinical studies have demonstrated electro-acupuncture is as effective as celecoxib in providing analgesia following spinal nerve ligation (Lau *et al.*, 2010), and is also effective in inflammatory models of pain such as CFA (Lee *et al.*, 2012; Du *et al.*, 2014), and carrageenan (Yoo *et al.*, 2011). It is also worth noting that the increasing use of electro-acupuncture has to potential to increase study quality as it would theoretically be easier to conduct high quality trials into as it permits double blinding through use of automated electro-acupuncture techniques (Lam *et al.*, 2013b).

Surgery

All the treatments discussed so far are largely modulatory in mechanisms, associated with minor anatomical alteration (with the exception of capsaicin-induced denervation). In the early 19th century, neurosurgery was commonly used to treat intractable pain. Many neurosurgical interventions, such as lesions of the spinothalamic tract, showed initial promise, only for the analgesia to subside and be replaced with pathological sensations ranging from numbness and slight tingling to constant lancinating pain worse than the original complaint. Furthermore, surgery performed on individuals who were misdiagnosed would result in permanent nerve damage without the potential for benefit (Giller, 2003). The apparent initial success of these approaches may be due

to a placebo effect associated with surgery itself, however modern treatment advises against surgical intervention in all but the most intractable and distressing pain conditions. However, modern techniques of neuroablation show promise for conditions including CRPS and failed lower back surgery syndrome (Raslan *et al.*, 2007). Neuroablation involves the temporary insertion of an electrode into neural tissue and generation of a high frequency alternating current which heats and de-activates surrounding tissue. Techniques involving extremely low temperatures, cryogenic neuroablation (or cryosurgery) to similarly 'burn' target tissue have also shown some benefit in lower back pain (Stelzer *et al.*, 2013), although the quality of this evidence is low, as evidenced by the single Cochrane review into the efficacy of neuroablation, which found only one high quality study, showing no significant benefits associated with sympathectomy (Straube *et al.*, 2013).

Development of Future Analgesic Therapies

One of the major obstacles to development of effective analgesics is high heterogeneity within patient populations. Resistance to opioids is common, with fentanyl effective in only 50-60% of the population (Landau *et al.*, 2008). Therefore, strategies such as stratified and personal medication are becoming more widely used (Baron *et al.*, 2012; Demant *et al.*, 2014). These aim to use predictors that identify individuals who will benefit from a particular treatment (e.g. high efficacy, low toxicity), and those for whom other options should be pursued (e.g. low efficacy, high toxicity).

1.4 Detecting Nocifensive Responses in Animals

The problems associated with assessing and studying pain in non-human organisms are due to the fact that primary clinical assessments of pain are verbal (e.g. VAS rating scales). Not all patients are able to adequately communicate (e.g. coma patients, young children and babies, and the elderly), thus it is essential we are able to detect and characterise pain when we are unable to ask the patient to describe how they feel.

Pain is not unique in this respect - any condition with a strong neurological component is subject to problems when translating from the clinical situation to *in vivo* modelling. Further understanding of how behaviour changes, and identification of related biomarkers benefits both basic researchers, striving to improve existing models and accurately interpret what they observe, and clinicians by providing an objective means of diagnosis.

The first hurdle is to define pain in non-human species. To do this, one must identify what signs an animal displays when injured, such as changes in posture, attending to the injured area, or vocalising. Many of these are also recognisable as human responses to pain, and are emotionally driven behaviours that can be classified as protective or defensive. Due to the involvement of

emotional and cognitive elements, it is important to think ethologically when translating such behaviours into animal models. Identification of innate behaviours observed in the model animal, awareness of their wild behaviours and of how they react to naturalistic stimuli under laboratory conditions is key to development of robust behavioural measures capable of detecting nociceptive changes. Paradigms designed to detect these behavioural patterns are largely observational, and measure interaction with a test environment using outcomes such as burrowing activity, exploration, and thigmotaxis.

Compared to behavioural changes, sensory nociceptive alterations perhaps associated with pain are much easier to investigate in animal models, and form the basis of pre-clinical research into understanding and treatment of pain. Sensitivity to mechanical, thermal, and cold stimuli is still the most common outcome measures, used both in basic research to determine nocifensive phenotype, and in pre-clinical drug trials to detect analgesic effect.

Terminology is important to avoid misinterpretation of findings – use of words such as “pain”, “allodynia”, and “anxiety/depression” implies knowledge of internal conscious experience. Instead, words such as nociceptive/neuropathic, hypersensitivity, and affective changes are more objective when applied to what is seen in animal models, and avoids the assumption that what is seen in the animal is directly equivalent to that seen in man.

Evoked Sensory Reflexes

Detecting sensory abnormalities, and responses to temperature (heat/cold), pressure, and touch, rely on measuring the latency to respond, or response threshold, defined as point of withdrawal from the stimulus.

These evoked measures of experimental nociception approximate the sensory testing of QST used clinically (Pfau *et al.*, 2014). Von Frey filaments are used to assess mechanical withdrawal thresholds - these consist of a set of calibrated hairs or glass fibres, which produce a defined pressure on contact. They are sequentially applied to the affected area (most commonly hind-paw) following an "up-down" protocol (Chaplan *et al.*, 1994). Electronic devices, such as that illustrated in Figure 1-4, can be used in place of von Frey hairs, and apply ramped pressure to a point on the hindpaw, measuring the point of withdrawal. Measurements are typically repeated 3-5 times, for both hind-paws, and the average withdrawal threshold for each calculated. However, in models involving a unilateral neuropathy, effects have been documented contralateral to the site of nerve injury (Seltzer *et al.*, 1990), although not always in the early post-operative stages (Kim & Chung, 1992), mimicking the situation seen clinically (Konopka *et al.*, 2012); therefore it is important to always

compare observations with those seen in naïve and sham surgery animals, rather than using the 'uninjured' side in animals with neuropathy.

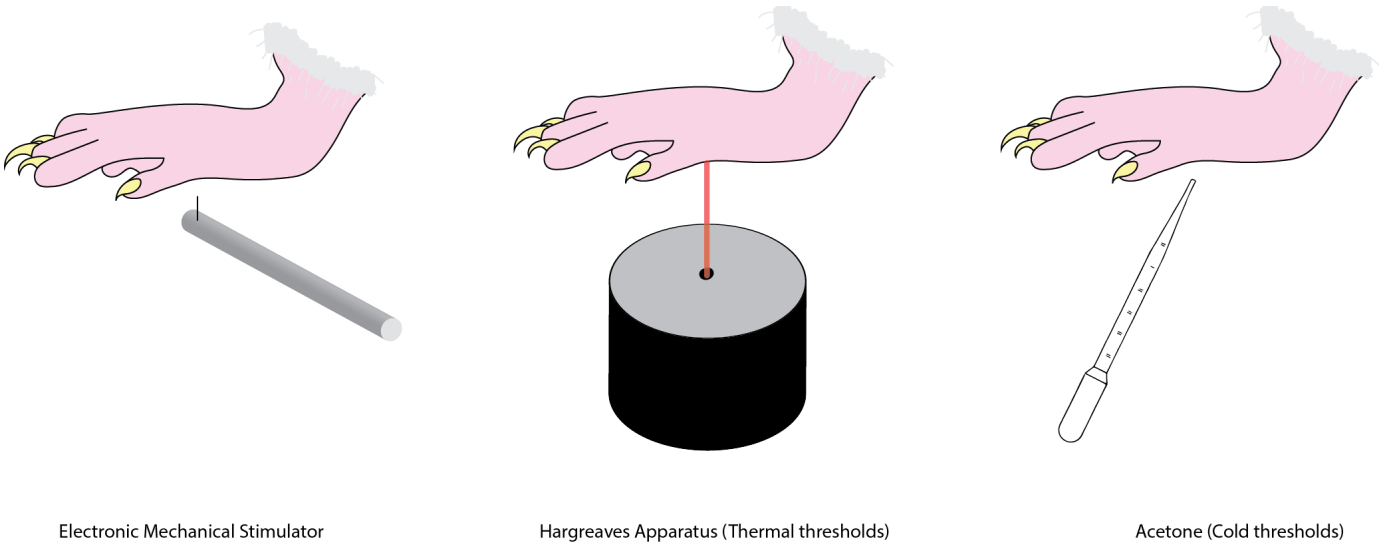


Figure 1-4: Measurement of evoked nociceptive outcomes
From left to right: electronic mechanical stimulator for measurement of mechanical thresholds; Hargreaves Apparatus for measurement of thermal thresholds; Acetone drops used to test cold thresholds

However, mechanical sensitivity exhibits location-associated variation due to differences in innervation density, skin thickness and hairiness, and also circadian variations, with significantly higher thresholds noted between 3pm and 7pm (Minett *et al.*, 2014). Additionally, there are multiple nerves innervating the surface of the hind-paw; the peroneal nerve innervates the dorsal hind-paw, with the ventral paw receiving innervation from the saphenous, tibial, and sural nerves as shown in Figure 1-5 (Kambiz *et al.*, 2014). Therefore, it is important to match test location with the target of the nerve(s) affected.

Thermal sensitivity can be assessed using radiant heat (e.g. Hargreaves) or contact (e.g. Tail-flick and hot-plate) and is typically recorded as latency to withdrawal. Thermal thresholds are thought to be highly conserved – nociceptive fibres respond to a range of temperature stimuli through activation of Transient Receptor Potential Vanilloid (TRPV) family receptors as summarised in table 1-1.

Table 1-1: Activation thresholds for selected TRP receptors involved in temperature sensation (Benham *et al.*, 2003; Bevan & Andersson, 2009; Babes *et al.*, 2011)

	TRPV1	TRPV2	TRPV3	TRPV4	TRPM8	TRPA1
°C	>43	>53	>31	>24	<25	0-15

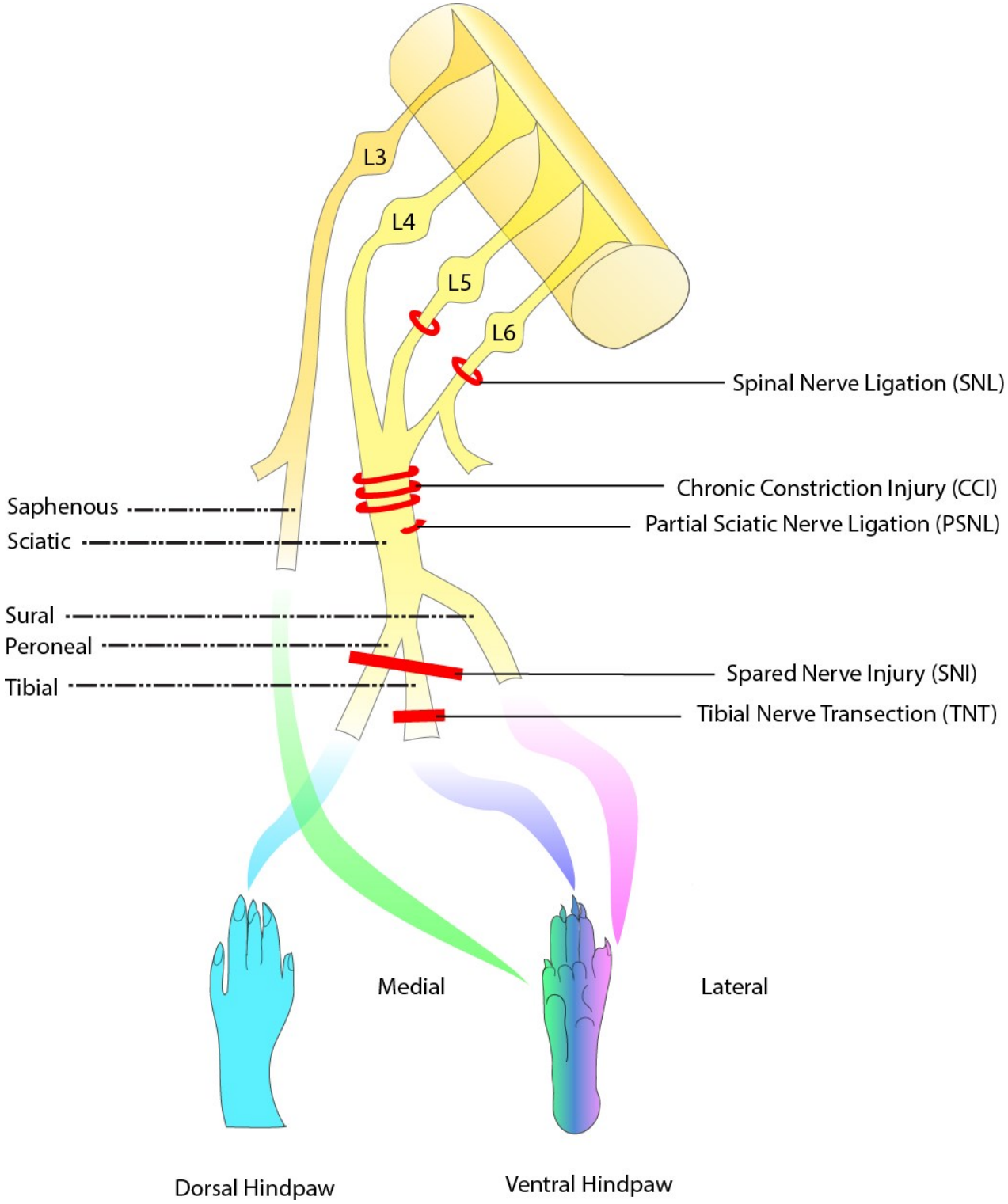


Figure 1:.5: Innervation of the Rat Hindpaw

Illustrating the major points of surgery involved in models of peripheral neuropathy and the different regions of the hindpaw affected

Sensitivity to cold can be detected through use of cold plate (Yoon *et al.*, 1994), or through topical application of compounds such as acetone (Walczak & Beaulieu, 2006), which cools skin via evaporation, methanol (Zhao *et al.*, 2012) which activates TRPA1 receptors in the skin, or icilin, a specific TRPM8 super agonist (Dhaka *et al.*, 2007).

Deep mechanical and pressure-associated pain can be measured using the Randall-Selitto test (Randall & Selitto, 1957; Falk *et al.*, 2014), which involves graded application of pressure to a limb or the tail, with latency to struggle taken as an endpoint. However, this test requires restraint of the animal, which can be associated with stress-induced analgesia (Imbe *et al.*, 2006).

One of the primary concerns with basing pharmaceutical investigations of pain on the ability to reverse mechanical or thermal hypersensitivity is that chronic pain patients show both hyposensitivity (lack of sensation and other negative signs) and hypersensitivity (Maier *et al.*, 2010). Therefore developing methods to detect the other aspects of the pain experience have the potential to increase translation between basic research and drug development.

Behavioural Alterations Associated with Pain

Behavioural assessment of pain is one of the earliest methods used in the study of pain due to its simplicity. Observation of behaviour following application of an irritant compound, such as CFA (Fehrenbacher *et al.*, 2012) or i.p. acetic acid (AA; Depoortère *et al.*, 2011), can reveal characteristic behavioural signs which follow a predictable time-course. Behavioural observation in these models involves counting the frequency of behaviours such as licking (CFA) and writhing (AA) at defined time points.

More complex behavioural assays can be used to reveal subtle changes in behaviour associated with the affective and cognitive elements of a nociceptive experience. Much has been learnt from research on psychiatric disorders, such as anxiety and depression (Walsh & Cummins, 1976; Lister, 1990; Cryan & Holmes, 2005), and it is now routine to use models originally developed to detect behavioural alterations associated with these conditions in pain research.

Rats and mice, the most commonly studied experimental animals, are also prey animals. This is associated with an inherent tendency to minimise behaviours that alert predators to a vulnerable state (Barnett, 2001). Although humans are not prey animals, they still display an analogous modulation of pain signs dependent on situation, and often showing a social component (Craig *et al.*, 1991). To detect these subtle changes in behaviour, we must first understand how the animal interacts with their environment, and how they exist in the wild. For example, rats are social creatures, which live in colonies numbering into the hundreds (Barnett, 2001) and would find being housed individually a stressful experience (Gambardella *et al.*, 1994; Brown & Grunberg, 1995; Okura *et al.*, 2009), whereas mice are comparatively solitary, and are frequently housed individually with

comparatively little effect (Jirkof *et al.*, 2012), although sex-related differences in this have been noted, with solitary housing decreasing exploration in female mice, with the reverse observed in males (Palanza, 2001).

The Open Field Paradigm

The open field paradigm was developed in 1934 (Hall, 1934) to detect emotionality in rats. The original paradigm involved a large circular arena (1.2m diameter) and featured food placed within the arena to test how hunger status influenced exploration. It was noted that animals that had eaten showed increased ambulation, but not all animals ate, and those that didn't eat showed decreased inner zone activity and increased defecation. This increased defecation, and associated decrease in digestive transit time was seen as an indication of increased emotionality, and for a long time, defecation rates in the open field, in combination with general measures of ambulation, were the primary outcomes measures, with high defecation and low ambulation taken as indicative of increased emotionality (Walsh & Cummins, 1976). Thigmotaxis, as defined by movement in parallel with the walls, was evident in this early study, but not suggested as a valid outcome measure until much later. Other outcomes measures highlighted as being robust and useful included rearing activity (Ivinskis, 1968), although the measurement of rearing fell out of favour with the advent of automated scoring systems, which are adept at quantifying horizontal movement, but less accurate on 'vertical activity' such as rearing. These early studies often involved a multi-factorial factor design, and although the sample sizes are small and not justified by sample size calculations, significant differences attributable to environmental enrichment (McCall *et al.*, 1969), illumination levels (Valle, 1970), and early life experiences (Whimbey & Denenberg, 1967a; Whimbey & Denenberg, 1967b)were detected. Repeated exposure was also investigated, with decreases in ambulation observed over time (Hall, 1934), and during the course of each trial (Lát & Gollová-Hémon, 1969; Oldham & Morlock, 2013).

An alternative method of interpreting open field activity was proposed by Lát and Gollová-Hémon in 1969, who suggested a continuum of arousal, with rearing representing the peak of arousal, and sleeping/immobility at the lower end as indicated in Table 1-2, corresponding with characteristic hippocampal slow wave activity (Lát & Gollová-Hémon, 1969).

Table 1-2: Arousal continuum for rat behaviour in the open field
(Lát & Gollová-Hémon, 1969)

High Arousal					Low Arousal
Rearing	Locomotor Activity	Sniffing	Cleaning	Sitting	Sleeping

Automated scoring of open field behaviour was introduced in the 1970s, with the aim of reducing observer bias (Delbarre *et al.*, 1970), and is now widespread. As alluded to earlier, this may have skewed the selection of relevant behaviours by biasing towards those most simple to detect such as spatial and temporal patterns of ambulation, at the expense of more complex behaviours such as sniffing and grooming. This initial study of automation utilised a beam-breaking method, allowing quantification of vertical activity (i.e. rearing), although the majority of systems used currently focus on 2D video capture of horizontal movement, with rearing activity often ignored due to difficulties defining rearing activity associated with overhead image capture techniques such as Ethovision (Noldus *et al.*, 2001). The main advantage of such video tracking software is that minimal specialist equipment is required, compared to more sophisticated, and therefore most costly options such as bespoke open field systems which utilise infra-red beam-break detection of vertical movement (Tatem *et al.*, 2014). The combination of automated video software with manual observer scoring of behaviours such as rearing offers a cost-effective way of maximising data collected, as well as providing experimenters with a permanent record of the raw data, which offers the benefit of allowing independent analysis, and re-analysis using different techniques.

Thigmotaxis overtook defecation as a measure of emotionality during the 1980s, due to increased use of automated detection, evidence of poor predictive value of emotionality (Wilcock & Broadhurst, 1967; Whimbey & Denenberg, 1967a; Blizard, 1971), and the suggestion that 'emotionality' is not necessarily a characteristic which is dramatically influenced by a single exposure (Candland & Nagy, 1969). Thigmotactic behaviour is characterised by movement in relation to a surface, and can take both positive (preference for proximity to a surface, e.g. "the tendency to run in contact with objects") and negative (avoidance) forms. It was first noted in protozoa such as paramecium (Kline, 1899), and positive thigmotactic behaviour (henceforth simply 'thigmotactic/thigmotaxis') was subsequently described during aversive social situations in rats during the 1920s (Stone, 1926; Sturman-Hulbe & Stone, 1929). In the 1970s thigmotactic behaviour was observed in rats following electric shocks (Grossen & Kelley, 1972), and subsequently suggested as representative of anxiety in the open field paradigm (Walsh & Cummins, 1976). In initial open field studies, low inner zone activity (high thigmotaxis) was noted in high emotionality subjects (Hall, 1934), and included as a measure of 'timidity' (Morrison & Thatcher, 1969; King, 1970) which

showed no significant sex differences (Valle, 1970). However, it was also described as having poor reliability, despite showing a negative correlation with ambulation (Ivinskis, 1968), which may be related to imprecision in the methods then used to quantify behaviour. Later studies emphasised the link between thigmotaxis and anxiety via the effects of social isolation in combination with 'agoraphobia' suggested by the aversive nature of unfamiliar spaces (Prut & Belzung, 2003), although this contradicts earlier studies indicating ambulation increases proportional to open field size (Blizard, 1971). It has also been shown that thigmotactic behaviour, at least in mice, is dependent on environmental awareness, as evidenced by mice without vibrissae showing very low thigmotaxis and high inner zone activity (Prut & Belzung, 2003). Furthermore, thigmotaxis has been recently noted as corresponding with general phobic avoidance and decreased working memory function in humans (Kallai *et al.*, 2007). In rats, it shows an inverse relationship with exploration of novel areas and is hypothesised as related to risk assessment and predator avoidance, with the presence of spontaneous or on-going pain decreasing potentially risky behaviours, such as exploration. The open field paradigm is capable of detecting these thigmotactic differences in experimental models of neuropathic pain with varying aetiologies including antiretroviral therapy-induced neuropathy (Wallace *et al.*, 2007b; Wallace *et al.*, 2008; Huang *et al.*, 2013), chemotherapy-induced neuropathy (Barzegar-Fallah *et al.*, 2014), spinal nerve transection (SNT; Blackbeard *et al.*, 2012), spinal nerve ligation (Kontinen *et al.*, 1999; Suzuki *et al.*, 2007; Ewan & Martin, 2014), chronic constriction injury (CCI; Grégoire *et al.*, 2012), and post-traumatic peripheral nerve trauma (Medico *et al.*, 2004).

Studies have shown decreases in open field activity in rats associated with pre-clinical models of myocardial infarction (Banozic *et al.*, 2014), following indole-3-acetic–induced gastritis in female but not male rats (Luo *et al.*, 2013), with a mixed picture regarding IBS, with both decreases in activity (Zhang *et al.*, 2014a), and no effect noted (Traub *et al.*, 2008). In mice, similar decreases in locomotor activity have been seen following administration of mustard oil (Maia *et al.*, 2006), AA ((Hu *et al.*, 2009b), and TNBS (Cattaruzza *et al.*, 2013). None of these studies explicitly investigated thigmotaxis, with inner zone activity only noted in a few studies (Zhong *et al.*, 2012; Zhang *et al.*, 2014a), most using immobility or ambulation/distance travelled as primary outcome measures.

Burrowing

Many fossorial mammals, birds, reptiles, and insects engage in burrowing behaviour (Kinlaw, 1999). They can be categorised as follows:

1) Primary excavators
    a) dig their own tunnels from scratch
2) Secondary modifiers
    a) inhabit and modify burrow systems created by another
3) Tertiary occupants
    a) inhabit without contributing to or modifying the existing burrow structure

Rats are an example of primary excavators, and in the wild, live underground in large colonies (Barnett, 2001). In the wild, burrowing is a necessary survival skill – a rat with poor burrowing skills will rapidly be exposed to increased risk of predation. Laboratory rats, despite their divergent appearance from their originator strain, readily burrow when given the opportunity (Stryjek *et al.*, 2012). Since the late 70s, researchers have studied the burrowing habits of rats and other rodents (Boice, 1977; Deacon, 2009; Stryjek *et al.*, 2012). Early studies tended to focus on qualitative observation of burrowing activities, highlighting associations with morphine withdrawal (Livingston *et al.*, 1988; Jones & Barr, 1995), orexin treatment (Ida *et al.*, 1999) and maternal behaviour (Jirik-Babb *et al.*, 1984; Kosten & Kehoe, 2010). Following on from this, the burrowing detour study was developed, featuring obstruction of a familiar route that could only be bypassed by burrowing (Holson, 1986; Ferguson *et al.*, 1998; Tran *et al.*, 2002). During the early 2000s', a quantitative burrowing assay was developed, based on displacement of the burrowing substrate, and found sensitive to behavioural changes associated with prion diseases (Guenther *et al.*, 2001; Deacon, 2009), hippocampal lesions (Deacon *et al.*, 2002), and numerous aetiologies of experimental pain, including traumatic (Andrews *et al.*, 2012; Lau *et al.*, 2013), drug-induced (Huang *et al.*, 2013), and inflammatory models (Rutten *et al.*, 2014b). Pharmacological validation has shown that the burrowing deficits observed in experimental models of pain are associated with nociception and the associated affective and cognitive alteration, as stimulants such as amphetamine, anxiogenics, and anxiolytics have no effect (Rutten *et al.*, 2014a), whilst drugs such as pregabalin, carbamazepine, naproxen, and morphine effectively reverse observed deficits (Andrews *et al.*, 2012; Lau *et al.*, 2013).

Other Behavioural Paradigms

Other non-conditioned, exploration-based paradigms are frequently used to detect pain-related behavioural alterations, such as the elevated plus maze (Parent *et al.*, 2012), the light-dark box (Zhang *et al.*, 2014a), and social interaction (Li *et al.*, 2014b), although most emphasise these

outcomes as related to anxiety rather than general behavioural changes related to nociceptive stimulation.

As brain areas such as the amygdala are implicated in both nociceptive responses and generation of conditioned responses, studies investigating differences in conditioned responses are instructive in elucidating how pain and nociceptive stimuli change behaviour. Conditioned place preference (CPP), traditionally used to investigate addictive properties of drugs of abuse (Jian *et al.*, 2014), can be used to detect analgesic efficiency by pairing a context with analgesic treatment and observing whether animals subjected to neuropathy display a preference for that environment (as would be expected if pain is being relieved). CPP for clonidine has been demonstrated in models of spinal nerve ligation (King *et al.*, 2009), sciatic axotomy (Qu *et al.*, 2011), spinal cord lesion models of central pain (Davoody *et al.*, 2011), spared nerve injury (SNI; Wei *et al.* 2013), and for gabapentin in a model of cisplatin-induced peripheral neuropathy (Park *et al.*, 2013). Morphine is also used to test analgesia place preference, but shows hedonic effects in naïve animals reducing the ability to properly assess analgesic effect, although the shape of the dose-response curve in CCI differs from that seen in naïve, emphasising the neural plasticity seen in neuropathic pain (Cahill *et al.*, 2013).

Place preference/avoidance paradigms have also been designed to detect avoidance of nociceptive stimuli indicative of hypersensitivity, with thermal (Yezierski *et al.*, 2010) and mechanical (LaBuda & Fuchs, 2000) stimuli studied thus far, although operant paradigm such as these require training of the animals, which could bias results though subtle differences in handling between groups, in addition to entailing increased labour and equipment costs which may not be feasible for all research groups.

A recent study suggests the use of facial grimace analysis as a means to detect responses to nociceptive and neuropathic stimuli in both mice (Langford *et al.*, 2010a; Matsumiya *et al.*, 2012) and rats (Sotocinal *et al.*, 2011). However, this measure only detects responses within the first 48 hours of an insult, and has yet to be cross-validated.

However, analysis of complex behaviour has become over-reliant on automated systems. Whilst this saves time and reduces the sources of human error inherent in manual scoring, it is at the expense of giving the experimenter a qualitative 'feel' for what the data mean - only by observing can you be aware of the different types of movement exhibited, for example the various different types of approach to the central zone (e.g. cautious, exhibiting stretch-attend postures versus walking in a straight line at a constant speed). By being aware of the variation present, the experimenter is then better equipped to interpret behavioural data collected, although it is important to minimise the

potential observer effects by maximising distance between the experimenter and the testing apparatus, and using remote observation or other means of hiding the experimenter from the test animal.

1.5 Modelling 'Pain' in Animals

When working with nociceptive models, it is vital to be familiar with legal requirements concerning the treatment of animals (Zimmermann, 1983). In the UK, the Home Office guidelines on animal welfare state that "pain, suffering, distress or lasting harm" must be minimized as far as possible, and that any procedure causing such effects must be regulated and only performed by a trained and licenced individual, working under both project and institutional licences which specify which procedures can be performed and what the ethical endpoints of each procedure are. Recent amendments now require the reporting of actual harm incurred during an experiment, in addition to pre-specification of expected severity. This will enable regulators greater awareness of actual experimental conditions, allowing identification of refinements that could improve experimental validity. Piloerection and weight loss are frequently mentioned as signs of distress, but as previously noted, the prey-animal status of most rodents means the presence of such signs is indicative of severe pain, and it is unethical to permit such suffering, therefore are not recommended as experimental endpoints. Autotomy (chewing of digits) is another outcome measure, initially noted in models of axotomy (Blumenkopf & Lipman, 1991; Kachramanoglou *et al.*, 2011), although it is now generally recognised that autotomy is associated with denervation and not necessarily representative of a pain state. Due to the obvious distress associated with autotomy, its use as an outcome measure in pain research is now discouraged.

When designing models of experimental pain, the first approach is to recreate a condition known to produce pain clinically; nerve trauma, inflammation, viral infection such as HIV and herpes zoster, and certain drugs including many anti-retroviral and chemotherapeutic agents, have all been used to varying degrees of success.

Traumatic nerve injury models, such as spinal nerve ligation (SNL; Kim & Chung, 1992; and the related model, spinal nerve transection (SNT)), and chronic constriction injury (CCI, (Bennett & Xie, 1988) use unilateral nerve damage to produce hypersensitivity of the hind paw ipsilateral to the site of injury, analogous with clinical neuropathic pain conditions associated with nerve injury. The exact presentation varies between models, but in general involves both thermal and mechanical hypersensitivities, as well as behavioural responses such as attending to the affected area. Figure 1-5 illustrates the involvement of the saphenous, tibial, sural, and peroneal nerves in the most common models of traumatic neuropathy. These all target the lumbar spinal region which innervates the

hindpaw, chosen for its well defined innervation, ease of testing, and commonality with the majority of clinical peripheral neuropathies, which target the longest nerve in the body i.e. those innervating the feet. Occasionally, cases of SNT are seen clinically, and are characterised by mechanical and cold hypersensitivity in the affected dermatome (C7; Ali *et al.*, 2002), as well as numbness and tingling (Chuang *et al.*, 1998), showing some overlap with the pre-clinical model, but also highlighting differences such as a lack of thermal abnormalities.

Chemically-induced pain can be investigated via systemic models such as alcohol-associated peripheral neuropathy (Ferrari *et al.*, 2013b), and chemotherapy (Barzegar-Fallah *et al.*, 2014) and anti-retroviral induced neuropathy (Blackbeard *et al.*, 2012), or localised models e.g. turpentine-induced bladder inflammation (Jaggar *et al.*, 1999; Farquhar-Smith & Rice, 2001), and CFA-induced peripheral inflammation (Fehrenbacher *et al.*, 2012). Application of most known irritant compounds will provoke a nociceptive response. The CFA, carrageenan (Iannitti *et al.*, 2012), and inter-peritoneal AA (Kurihara *et al.*, 2003) models of acute inflammation illustrate this well. These models result observable changes in behaviour (e.g. paw lifting, licking, writhing) or in evoked measures such as mechanical and thermal hypersensitivity.

Anti-Retroviral-Induced Neuropathy

HIV affects 35 million people worldwide, with 39% currently on anti-retroviral treatment regimens (UNAIDS, 2014). This represents a mixed population of patients taking first generation NRTIs (nucleoside and nucleotide reverse transcriptase inhibitors; e.g. Zidovudine, Didanosine, Stavudine, and Tenofovir), second generation NNTRIs (non-nucleotide reverse transcriptase inhibitors; e.g. Nevirapine, Efavirenz, and Rilpivirine), and protease inhibitors (e.g. Indinavir, Fortovase, and Ritonavir).

Current treatment regimens recommended by the WHO start with a combination of two NTRIs (Tenofovir, with either Lamivudine or Emtricitabane), and a protease inhibitor (Efavirenz), but older drugs, such a Stavudine (d4T) are still widely used in the developing world due to their low costs (Hill *et al.*, 2007; Murphy *et al.*, 2007).

d4T is a NRTI-class drug, which was approved by the FDA in 1994 (Moyle & Gazzard, 1997). In 2009 the WHO recommended it be phased out due to adverse effects (WHO, 2009), including lipodystrophy (Kampira *et al.*, 2013), and peripheral neuropathy characterised by pain and numbness in the extremities (Maritz *et al.*, 2010; Mullin *et al.*, 2011). However, it is still included on the WHO List of Essential Medicines (WHO, 2013), a fact that recognises it is still a cheap and effective

treatment widely used in the developing world, areas which show the highest infection rates (UNAIDS, 2014).

The neuropathic symptoms seen in some patients following Stavudine treatment are associated with increased levels of fatigue (Wantland *et al.*, 2011) and decreased quality of life (Mrus *et al.*, 2005), further increasing socioeconomic burden.

d4T a nucleoside analogue thymidine, and acts as both a chain terminator, directly inhibiting HIV reverse transcriptase activity (Huang *et al.*, 1992; Hurst & Noble, 1999; Martin *et al.*, 2010). *In vitro*, it has been shown to reduce neurite growth (Cherry *et al.*, 2010), inhibit stimulated autophagy in hepatocytes (Stankov *et al.*, 2012), and interfere with mitochondrial activity (Kakuda, 2000; Moyle, 2000; Dalakas, 2001; Gardner *et al.*, 2013), all effects which contribute to neuropathy.

As HIV infection itself is capable of inducing sensory neuropathy, it is difficult to isolate the effects of antiretroviral therapy in a clinical population (Dalakas & Cupler, 1996; Manji, 2000). Numerous preclinical studies have been conducted, primarily investigating side effect profile (Guimarães *et al.*, 2010), but increasingly looking at potential mechanisms of anti-retroviral-induced peripheral neuropathy. It has been shown that treatment with d4T is associated with evoked hypersensitivity (Weber *et al.*, 2007; Boateng *et al.*, 2014), and behavioural alterations in paradigms such as open field and burrowing in male rats (Huang *et al.*, 2013). Other studies looking at non-sensory consequences of neuropathy, such as free-wheel running (Weber *et al.*, 2007) and acoustic startle (Morse *et al.*, 1997; Morse, 1997) have been inconclusive.

Viral Models of Experimental Neuropathy

Pain syndromes are also associated with viral infection, such as HIV (Manji, 2000; Maritz *et al.*, 2010), post-herpetic neuralgia (varicella zoster virus, VZV; Daniel *et al.*, 2008; Attal *et al.*, 2010; Johnson & Rice, 2014), and Hansen's' disease (leprosy; (Legendre *et al.*, 2012; Haroun *et al.*, 2012) are all associated with distal peripheral neuropathy syndromes. Experimental models have been developed based on HIV, particularly using the envelope glycoprotein GP120 (Wallace *et al.*, 2007a; Kamerman *et al.*, 2012) and VZV (Fleetwood-Walker *et al.*, 1999; Garry *et al.*, 2005; Hasnie *et al.*, 2007; Boateng *et al.*, 2014), with demonstrable phenotypes of peripheral hypersensitivity with affective behavioural alterations. Although leprosy is still associated with a large clinical burden in low resource areas, attempts to develop a pre-clinical model have been thwarted by difficulties culturing *Mycobacterium leprae,* the pathogen responsible (Rees, 1988), with current models showing narrow application (Gupta, 1995).

Other options open to *in vivo* pain research involve using endogenous conditions, such as feline interstitial cystitis (FIC) and simian immunodeficiency virus (SIV) infection in primates. FIC is a naturally occurring model of chronic idiopathic cystitis which parallels the human pathology well (Buffington, 2004; Rubio-Diaz *et al.*, 2009; Keay *et al.*, 2014), whereas SIV models an HIV-like neuropathy in primates (Clements *et al.*, 2008; Burdo *et al.*, 2012; Mangus *et al.*, 2014).
Both provide useful insight into commonality between human clinical evidence and an analogous non-human condition, leading to increased translation in other non-human models.

Experimental Models of Urinary Bladder Pain

Most models of visceral pain involving the bladder tend to involve application of an irritant (e.g. intra-peritoneal acetic acid, or instillation of turpentine into the bladder), or make use of stressors such as early maternal separation and chronic psychosocial stress to induce visceral hypersensitivity. Other models use viral vectors such as pseudorabies to induce neurogenic cystitis (Rudick *et al.*, 2012), or make use of innate models, such as FIC (Westropp & Buffington, 2002). As mentioned, FIC is an endogenous model of cystitis, associated with increased ATP release (Birder *et al.*, 2003), and it is thought that the inflammatory stimuli present are capable of inducing chemosensory activity in previously unresponsive afferents (Häbler *et al.*, 1988). Interestingly, there is no evidence of overactive bladder (i.e. increased contraction frequency) in FIC cats, only a decrease in void volume (Wu *et al.*, 2011).

Acetic acid (AA) is commonly used to test acute responses to visceral pain, and when given intraperitoneally to rodents, it produces a characteristic writhing movement accompanied by abdominal contractions that can be measured using electromyography. It is responsive to pharmaceutical interventions such as milnacipran (Depoortère *et al.*, 2011), and has also been used as a conditioning stimulus producing suppression of somatic pain associated with formalin injection (Kurihara *et al.*, 2003). In addition to intraperitoneal administration, acetic acid can be instilled directly into the bladder to produce a cystitis-like syndrome with decreased bladder pressure, and urine void volume, and increased inter-contraction interval (ICI; Su *et al.*, 2013), responsive to sacral acupuncture (Hino *et al.*, 2010), which shows subtle sex differences in mice, with the male urethra being more sensitive to irritation, whereas in females, bladder tissue showed the greatest response (Yoshiyama *et al.*, 2008). Its potential as a model of testicular pain has also been investigated, with intra-testicular injection of 2-3% solutions resulting in bladder hypersensitivity and behavioural signs of pain (Yoshioka *et al.*, 2010).

Cyclophosphamide (CYP) is a chemotherapeutic agent associated with a poor side-effect profile, including haemorrhagic cystitis (Ahmed & Hombal, 1984). It is taken orally and metabolised to

acrolein, which is damaging to the mucosal layer of the bladder (Brade *et al.*, 1986). Direct instillation of acrolein is sometimes used and is associated with both abdominal hyperalgesia, and referred hypersensitivity in the hind-paw (Guerios *et al.*, 2008), dependent on TRPV1 activation (Wang *et al.*, 2008), and increased levels of FAAH (Merriam *et al.*, 2010; Wang *et al.*, 2014). It is however responsive to lidocaine pre-treatment (Guerios *et al.*, 2009), as seen clinically (Theoharides *et al.*, 2008), and importantly, heart rate, body temperature, and general activity are not documented as being altered by CYP treatment (Boudes *et al.*, 2011).

Systemic treatment with CYP, using either acute or chronic dosing, has been cited as an experimental model of bladder dysfunction akin to both OAB and IC/BPS. As with acrolein, it decreases bladder pressure and volume, while increasing ICI (Pan *et al.*, 2012; Dornelles *et al.*, 2014), and is also associated with increased bladder weight, and increased levels of inflammatory mediators, which can be normalised by NSAID treatment (Tsukimi *et al.*, 2004), whereas morphine ameliorates only hypersensitivity (Takagi-Matsumoto *et al.*, 2004; Augé *et al.*, 2013).

CYP-induced experimental cystitis is associated with macrophage activation, with inhibition of macrophage migration inhibitory factor (MIF) ameliorating the concurrent increase in inflammatory mediators (Vera *et al.*, 2010), and the cytokine CCL-2 implicated in the associated abdominal and hind-paw hypersensitivity (Arms *et al.*, 2013). TRP1A antagonists (Meotti *et al.*, 2013), and prostaglandin antagonism (Wada *et al.*, 2013) also inhibit CYP-induced contractions.

Various neurotrophic agents such as NGF (Jaggar *et al.*, 1999; Farquhar-Smith *et al.*, 2002; Guerios *et al.*, 2006), and BDNF (Frias *et al.*, 2013) have been implicated in the mechanical hypersensitivity produced by CYP, with minimal contribution from NFκB (Velasco *et al.*, 2001).

There appear to be differences in the effect produced by acute and repeated treatment, with more severe mucosal abrasion seen following acute CYP (Juszczak *et al.*, 2010), which in itself is associated with higher over-activity (e.g. decreased ICI) whereas individuals with minimal mucosal abrasion despite CYP treatment show contractility indices comparable with the control group (Andersson *et al.*, 2008).

Intravesicular instillation of agents such as acetone (Kato *et al.*, 1990; Shimizu *et al.*, 1999), mustard oil (Häbler *et al.*, 1988), croton oil (McMahon & Abel, 1987; McMahon, 1988), hydrochloric acid (Hayashi *et al.*, 2009), and turpentine (Ham & Hurley, 1965; McMahon & Abel, 1987)have been used to model painful bladder conditions. Turpentine in particular has been show to involve pathological levels of NOS (Rice, 1995), and is associated with involvement of endocannabinoids (Farquhar-Smith

& Rice, 2001), NGF (Jaggar *et al.*, 1999), bradykinin (Jaggar *et al.*, 1998a), TRPV1 (Jaggar *et al.*, 2001), and NMDA receptors (Rice & McMahon, 1994).

Neonatal bladder treatment with intravesical zymosan is associated with increased bladder permeability, and hypersensitivity to visceral but not somatic stimuli (Randich *et al.*, 2006), including increased sensitivity to bladder instillation of ice-water as an adult (Randich *et al.*, 2009), although it is important to note that clinically, the bladder cooling reflex (i.e. expulsion of liquid between 0-4°C) is seen in OAB but not IC/BPS, although IC/BPS patients do report higher levels of pain during this test (Mukerji *et al.*, 2006), therefore it is possible that cold water responses seen in this model reflect a dominant IC/BPS phenotype.

Wistar Kyoto rats (WKY) were originally bred as a control strain for the spontaneously hypertensive rat, but it was quickly noted that they possess a high pre-disposition towards affective alterations (Langen & Dost, 2011). Unlike Sprague Dawley rats, chronic water avoidance stress is capable of increasing visceral hypersensitivity in WKY rats, with associated increases in referred hyperalgesia (Robbins *et al.*, 2007).

Other models which have been suggested as useful in the study of bladder-associated pain disorders include a model of fibromyalgia (bilateral gluteal intramuscular hydrochloric acid) which shows lidocaine-reversible bladder hypersensitivity (Roppolo *et al.*, 2005), and numerous models based around both direct bacterial inflammation of the bladder (Bjorling *et al.*, 2008), or use lipopolysaccharide (LPS) isolated from gram positive bacteria to evoke an immune response (Dupont *et al.*, 2001). Bacterial infection, unlike the majority of models mentioned above, is associated with decreased thermal thresholds (Bjorling *et al.*, 2008), whereas LPS alone decreases bladder capacity and compliance, as well as affecting cytometry measures (Takezawa *et al.*, 2014).

There is considerable evidence of pelvic cross-sensitisation, with CYP increasing levels of colorectal chemosensory afferents (Brumovsky *et al.*, 2009), and colonic hypersensitivity (Bielefeldt *et al.*, 2006), and intracolonic TNBS (Trinitrobenzenesulfonic acid) increasing bladder afferent firing and sensitivity to bradykinin, capsaicin and substance P (Ustinova *et al.*, 2006).

General Comments

It is also important to bear in mind physiological and pharmacokinetic differences between rodents and humans when translating findings from basic into clinical research – for example, opioid efficacy is higher in humans than rodents (Morgan & Christie, 2011), with higher binding observed for kappa opioid agonists in clinical studies (Schattauer *et al.*, 2012), therefore it is likely that kappa agonists

tested in rodents may present as less effective than their actual clinical potential in the human population.

Additionally, there are subtle differences in behaviour, pharmacokinetics, and physiology between both different strains of experimental animals such as rats and mice, but also between different suppliers (Rex *et al.*, 1996; Swerdlow *et al.*, 2000; Palm *et al.*, 2011a; Palm *et al.*, 2011b; Langer *et al.*, 2011; O'Bryant *et al.*, 2011). Looking at rats in particular, differences have been documented in the response to stimuli such as ultrasonic sound, with the commonly used Wistar rats freezing, and the less commonly used Hooded Lister rats focussing more on escape (Neophytou *et al.*, 2000). Pharmacokinetic differences are also evident, with WKY rats showing reduced sensitivity to serotonergic drugs in the forced swim test (López-Rubalcava & Lucki, 2000), enhanced codeine metabolism seen in Sprague Dawley rats (Williams *et al.*, 2004), and variation in apomorphine sensitivity in numerous common strains of laboratory rat (Kinney *et al.*, 1999; Swerdlow *et al.*, 2000). Physiological differences between strains are highlighted by a study which showed Wistar rats have a greater L4 involvement following sciatic nerve injury (Rigaud *et al.*, 2008), whereas Lewis rats have increased levels of reactive C-fibres and a greater noradrenalin response to inflammation (Banik *et al.*, 2001). Of particular relevance to the pain field is the presence of differences in formalin test response (Lariviere *et al.*, 2006), hotplate latency (Schaap *et al.*, 2013), mechanical hypersensitivity (DeLeo & Rutkowski, 2000; Zhang *et al.*, 2003), stress-potentiation of hyperalgesia (Woolfolk & Holtzman, 1995), and NO anti-nociceptive response (Fender *et al.*, 2000). Furthermore, studies have demonstrated blunted sexual responses to a receptive female in albino rats (Sachs, 1996), and also differences in manual dexterity involved in forelimb reaching tasks, with naive Sprague Dawley rats employing a visually more similar to injured Long Evans rats (Whishaw *et al.*, 2003). An interesting study in 2012 compared swimming and digging behaviour in small numbers of laboratory bred rats with wild rats, and found that although laboratory strains were less likely to engage in exploratory behaviour and swimming, there was considerable inter-individual variation (Stryjek *et al.*, 2012). These strain and breeder-associated differences are important to be aware of, as they can have a significant influence on experimental outcome, therefore inclusion of such information in published studies is crucial to maximise the potential contribution of research.

Another factor when developing models for the study of experimental pain in animals is the inclusion of a suitable control group. In most cases, this necessitates both a naïve (i.e. un-treated, normal health), and an appropriate control that will allow identification of effects specific to manipulation of the variable of interest. For example, sham surgery groups used in models of traumatic neuropathy, involving exposure of the nerve but no manipulation, or a vehicle group that receives intra-plantar saline rather than CFA. This is particularly important as sham effects have been documented in

traumatic models such as SNI, as manifest by decreased open field behaviour (Hu *et al.*, 2009a; Wang *et al.*, 2011), and increased activation of the right amygdala (with bilateral activation seen in SNI animals; (Bourbia *et al.*, 2010).

In 2010, the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines were published in collaboration with the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs, 2014), designed to encourage transparency in the reporting of animal research (Kilkenny *et al.*, 2010). They were developed following a survey on 271 published articles on animal research which found severe shortcomings in reporting, with only 59% of studies giving a hypothesis and characteristics such as group size, sex, supplier, and housing of animals, and over 86% did not use bias reduction methods such as blinding and randomisation (Kilkenny *et al.*, 2009). The ARRIVE guidelines take the form of a 20-point check-list outlining information which should be included when reporting animal research in order to maximise clarity and enable readers to assess the quality of the research objectively. The inclusion of information on group sizes, animal characteristics (e.g. age, sex, strain, supplier), and environmental factors, and provision of error estimation will also facilitate systematic review in the pre-clinical field.

Systematic reviews have been conducted in clinical research, as a means of synthesising an answer to a hypothesis by surveying the literature in an objective and systematic fashion, since the 1980s, prompted by Archie Cochrane, who wrote extensively on the value of randomized control trials (RCT) to evidence-based medicine (Chalmers, 1993). In 1996, the CONSORT (Consolidated Standards of Reporting Trials) statement was published, providing clinicians and researchers involved in RCTs with 21-point checklist on which the ARRIVE guidelines were based (Altman, 1996). CONSORT has greatly improved the quality of the reporting of clinical trials, and allowed numerous high quality systematic reviews to be conducted, particularly associated with the Cochrane Collaboration, where the first systematic review looking at pain was registered in 1998 (Kleijnen & Mackerras, 2000). The Cochrane Collaboration is a particularly important resource as it requires pre-registration of systematic review protocols, as has been the case for RCTs, reducing the opportunity to bias findings by changing the protocol once data has been collected, and encourages regular updating of systematic reviews within its archive to allow inclusion of articles published after the initial completion of the review.

It is unlikely a 'Cochrane' style collaboration would be easy to establish in the animal research field (Sena *et al.*, 2014a; Sena *et al.*, 2014b) – there is resistance to the requirement for pre-registration of basic research, particularly as many are pilot studies, in which low group sizes are used and the object is to determine whether a hypothesis is valid before full exploration involving higher use of resources. Additionally, it is suggested that such pre-registration would crush the serendipitous

discoveries often made during exploratory studies. One way of circumventing this issue would be to distinguish pre-clinical from basic research, encouraging some degree of pre-registration, implemented by either journals or funding bodies, perhaps kept private until publication of the research to negate the opportunity for 'scooping', for studies prepared to adhere fully to the ARRIVE guidelines. Thus defined, preclinical research would move closer to the standards seen in clinical RCTs, and facilitate high quality systematic reviews of animal literature, whilst allowing for basic research, akin to case studies in clinical research, where the focus is on descriptive and exploratory studies which benefit from the freedom of being able to adapt protocols to maximise observations. The ability to distinguish these two types of animal research would greatly benefit the progression of research by allowing critical assessment of a study, appropriate to its design. This can only be facilitated by inclusion of all relevant factors and transparency regarding protocol, particularly exclusions.

1.6 Sex Differences

The majority of *in vivo* research is conducted in male animals, purportedly because the oestrous cycle of female animals adds a further level complexity which must be controlled, or at least accounted for. In terms of drug development, this makes little sense as women show differences in drug metabolism compared to men, including higher prevalence of side-effects (Campesi *et al.*, 2012; Franconi & Campesi, 2014), and would benefit from drugs which are effective irrespective of menstrual phase, with the exception being menstruation and pain conditions such as endometriosis (Hassan *et al.*, 2014), when maximum efficacy under particular hormonal conditions would be advantageous.

In rats, the female oestrous cycle lasts 4-5 days, and involves an initial small peak in follicle stimulating hormone (FSH) during day 1 (metestrous), which stimulates a larger peak in progesterone (day 2; diestrous). During day 3, or pro-oestrous, there is a large spike in estradiol levels, followed by a similarly large peak in luteinizing hormone (LH), progesterone, and FSH. The cycle ends with a small peak in estradiol (oestrous) around day 4 or 5. This cycle repeats for the entirety of the reproductive lifetime of the animal, typically 200 days in rats (~40 cycles) (Marcondes *et al.*, 2002).

Oestrous stage can be determined by taking vaginal smears and examining the cell populations present, as indicated in Table 1-3.

Table 1-3: Cell characteristics used in the staging of rat oestrus cycle
From Marcondes *et al.*, 2002

Cell Type	Characteristics	Proestrus	Oestrous	Metestrous	Diestrous
Epithelia	Round, nucleated	✓	-	✓	-
Cornified	Irregular, enucleated	-	✓	✓	-
Leukocytes	Round, small	-	-	✓	✓

The measurement of oestrous stage requires consistency, and at least 5 days consecutive testing to establish exact timings (Hubscher *et al.*, 2005). This is not always practical, as all animals should be at the same confirmed stage during testing, or group sizes determined for each stage of oestrous, increasing the numbers of animals required. Housing conditions also influence oestrous cycle length, with group housing increasing irregularity of cycles (Pallares & Gonzalez-Bulnes, 2009). Furthermore, the taking of daily vaginal swabs is a stressor, which could possibly alter behavioural responses, with some studies utilising i.p. injection as a comparable stressor when comparing with males (Nicotra *et al.*, 2014).

Sex differences have been documented in sensitivity to noxious stimuli and risk of developing chronic pain syndromes. Therefore it is important to establish experimental results in both male and female subjects, so as to be aware of any potential differences in response. In May 2014, the NIH announced an initiative to encourage studies which use female animals, recognising that awareness of sex-related differences is important in driving translational progress (National Institutes for Health, 2014).

It is clear there are sex-related differences in the reporting of and sensitivity to pain. The reasons for these are numerous but can broadly be divided into physiological differences, including hormonal differences, and psychosocial factors related to upbringing and gender identity (Bartley & Fillingim, 2013).

Sex hormones, such as estradiol and testosterone are reported to have differing effects on pain perception. The effects of reproductive hormones such as oestradiol and testosterone on pain perception are well documented, due to a substantial body of research into the effects of the menstrual cycle (Hassan *et al.*, 2014). Most studies show increased sensitivity during the luteal phase (Fillingim *et al.*, 1997; Powell-Boone *et al.*, 2005), but the effect is small in magnitude.

Testosterone is largely anti-nociceptive (Aloisi *et al.*, 2010), although has been shown to sharply decrease in both men and women in response to morphine (Aloisi *et al.*, 2009), with long-acting

opioids implicated in hypogonadism in men with chronic pain (Rubinstein *et al.*, 2013). Similar findings have also been noted in the rat (Cicero *et al.*, 1975).

Studies have shown mu-opioid receptor density is sensitive to oestradiol levels, with increased expression corresponding with decreased pain sensitivity during high-oestradiol phases of the menstrual cycle (Smith *et al.*, 2006). Clinical data also suggests sex differences in opioid use, with some studies suggesting lower post-operative use among women (Aubrun *et al.*, 2005; Periasamy *et al.*, 2014), and other showing no differences (Bartley & Fillingim, 2013). There is also possible evidence of a gender bias in opioid-based pain treatment, with physicians more likely to prescribe opioids to patients of the same sex (Weisse *et al.*, 2001).

Genetic factors that predispose to pain also show sexual dimorphism. In women, the MC1R gene, which also confers red hair and fair skin, is associated with higher levels of kappa opioid associated analgesia (Bartley & Fillingim, 2013), whereas a single nucleotide polymorphism in the mu-opioid receptor gene predisposes men, but not women, to increased pressure pain sensitivity (Fillingim *et al.*, 2005).

In terms of psychosocial factors, the picture becomes more complex, with gender stereotypes of 'stoic men' and 'emotional women' leading to suppression and enhancement of symptoms respectively (Alabas *et al.*, 2012). Non-verbal communication (e.g. grimacing, utterances, and body position) is an important part of a clinical pain diagnosis, and it has been suggested that women more likely to display and recognise non-verbal expressions of pain expression, and more likely to seek assistance from 'non-kin' networks e.g. medical practitioners (Keogh, 2014).

Overall, despite conflicting evidence regarding mechanisms, there is a case for increased sensitivity, and particularly increased prevalence of a range of chronic pain conditions in women (Hassan *et al.*, 2014), and that these have serious economic and social implications (Beckett *et al.*, 2014).

1.7 Neural Circuitry of Pain

Pain acts on multiple levels within the nervous system: sensory signals from the periphery are received centrally and interpreted in relation to previous experience and current circumstances to produce a behavioural response.

In the periphery, nociceptive stimuli such as damaging heat or cold, mechanical trauma, or chemical irritants are detected by sensory afferents, specifically unmyelinated C-fibres and myelinated Aδ fibres. These fibres possess an array of nociceptors that each respond to a defined set of stimuli, such as the thermal receptors detailed in Table 1-1. The cell bodies of these nociceptive fibres reside

in the DRG. A recent study which used novel magnetic cell sorting techniques to study the cell populations in the DRG found <10% of all cells are neurons, of which approximately 38% are CGRP positive, suggesting dominant peptidergic transmission, and used magnetic sorting to generate a 95% pure neuronal preparation featuring mainly Nav1.8 positive nociceptive neurons (Thakur *et al.*, 2014). This was used to generate a transcriptome of genes expressed exclusively in this population, providing a new technique useful in the study of how neuronal populations in the DRG are altered in pathological pain conditions.

Peripheral afferents transmit sensory information to the spinal cord via the DRG. From the dorsal horn of the spinal cord, innervation crosses the midline of the spinal cord and travels up the dorsal column medial-leminiscus (touch and proprioception) and anterolateral (pain) tracts to the ventral posterior nucleus of the thalamus, and on to the corresponding areas of the somatosensory cortex. Signals from the anterolateral tract also terminate in the parabrachial nucleus (PB) where higher connections to the amygdala, insula, and cingulate cortex are associated with modulation of the affective components of pain (Basbaum *et al.*, 2009), as illustrated in figure 1-6.

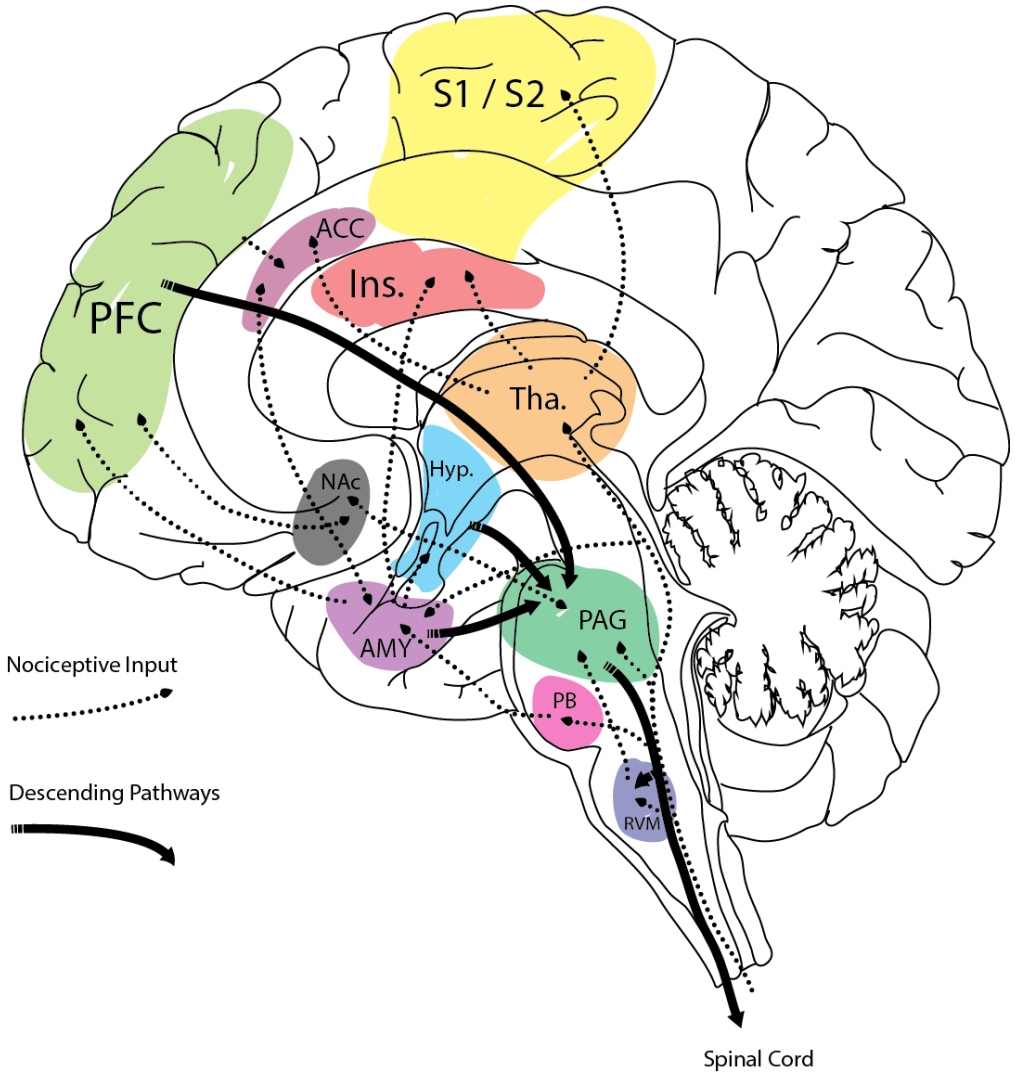


Figure 1-5: Central pathways implicated in pain processing showing nociceptive input and the resultant descending pathways
PFC - pre-frontal cortex, S1/S2 - primary and secondary somatosensory cortices, ACC - anterior cingulate cortex, Ins. - insula, Tha. - thalamus, NAc - nucleus accumbens, Hyp. - hypothalamus, AMY – amygdala, PAG – periaqueductal grey, PB – parabrachial nucleus, RVM – rostroventral medulla

Differential processing of somatic and visceral stimuli is indicated by fMRI studies in healthy volunteers which showed activation of the RVM, PAG, VTA, PB, and nucleus cuniformis (NCF) following both abdominal somatic nociceptive and rectal distension, with higher activation seen in the PAG and NCF, and PAG activation correlating with reports of anxiety (Dunckley *et al.*, 2005).

Lesion studies have assisted functional identification of brain areas involved with certain aspects of the pain experience, and have largely focussed on the amygdala. Lesions to the central amygdala (Ce) are capable of abolishing morphine's anti-nociceptive effects in rats (Manning & Mayer, 1995), which can be restored by transplantation of Ce tissue (Jain *et al.*, 2000), whereas ablation of the whole amygdala, or the basolateral (BL) nuclei alone, but not Ce prior to SNI abolished hypersensitivity (Li *et al.*, 2013). Lesions of noradrenergic neurons in the locus coeruleus in the rat

reduce hot plate latency, but have no effect on tail flick latency, indicating a role in pain modulation at the supra-spinal but not spinal level (Kudo *et al.*, 2010).

In the early 1900s, Déjerine and Roussy described "Le syndrome thalamique", or thalamic pain syndrome, one of the first studies on central post-stroke pain (CPSP), and which demonstrated the importance of the thalamus in the generation of pain (Pearce, 1988). It is now known that CPSP can be caused by lesions at any level of the central somatosensory pathway, including the medulla (Klit *et al.*, 2009). Rat models of thalamic CPSP have demonstrated bilateral cold allodynia, which can be ameliorated by high dose ketamine (Castel *et al.*, 2013), although this is not reflected clinically, with ketamine having poor efficacy in CPSP (Vranken *et al.*, 2005).

In addition to central integration of the afferent signals of nociception signals from the periphery, descending pathways originating in the RVM and PAG control endogenous analgesia by inhibiting ascending pathways in the substantia gelatinosa (Basbaum *et al.*, 2009). This role of the PAG in endogenous analgesia is supported by high levels of mu opioid receptors (Bodnar, 2013), in addition to decreases in fMRI signal and PET detection of **μ** opioid receptors associated with placebo analgesia in the PAG and several other brain areas including the thalamus, insula, and RVM (Qiu *et al.*, 2009).

The Amygdala

The amygdala or amygdaloid complex is a highly heterogeneous group of nuclei with wide-ranging and overlapping functional connections with the rest of the brain located near the temporal pole. It is primarily implicated in regulation of emotional processes, and in this complex field, the amygdala is not a single functional or structural unit. Therefore, in this discussion, areas will be distinguished based on developmental origin, namely pallial and subpallial, as shown in Figure 1-7.

History

Karl Burdach named the amygdala for its almond-like shape in the late 19th century (McDonald, 2003), and although his initial discovery only covered what is now known to be the basolateral (BL) nucleus, early anatomical study soon highlighted the heterogeneous nature of the amygdala by describing extensions of the amygdala into surrounding claustral, lenticular, striatal, and olfactory areas (Swanson & Petrovich, 1998). A seminal study by J.B. Johnston in the early 20th century laid most of the groundwork now used in definition of amygdalar nuclei, and enabled a focus on delineation based on function rather than spatial proximity (Johnston, 1923). The complexities encountered by early anatomical researchers explain the apparent misnomers associated with regions now known to be cortical yet described as nuclear (e.g. anterior cortical nuclei).

Pallial versus Sub-Pallial Amygdala

The pallial amygdala is largely involved with socio-sexual and olfactory associated function, and has strong connections with the hypothalamus and both accessory and primary olfactory bulbs. It shows allocortical composition, and can further be divided into two subgroups based on whether structures show cortical (superficial) or nuclear (deep) characteristics. The cortical pallial amygdala comprises the nucleus of the lateral olfactory tract (LOT), the bed nucleus of the accessory olfactory tract (BAOT), the cortex-amygdala transition zone (CxA), the amygdalopiriform transition area (APir), the anterior cortical amygdaloid nucleus (ACo), the posteriolateral cortical amygdaloid nucleus (PLCo), and the posteriomedial cortical amygdaloid nucleus (PMCo). The deep pallial nuclei include the BL, basomedial (BM), and lateral (La) amygdalae (originally identified as the BL amygdala), and the amygdalohippocampal transition area (AHi) (Olucha-bordonau *et al.*, 2015).

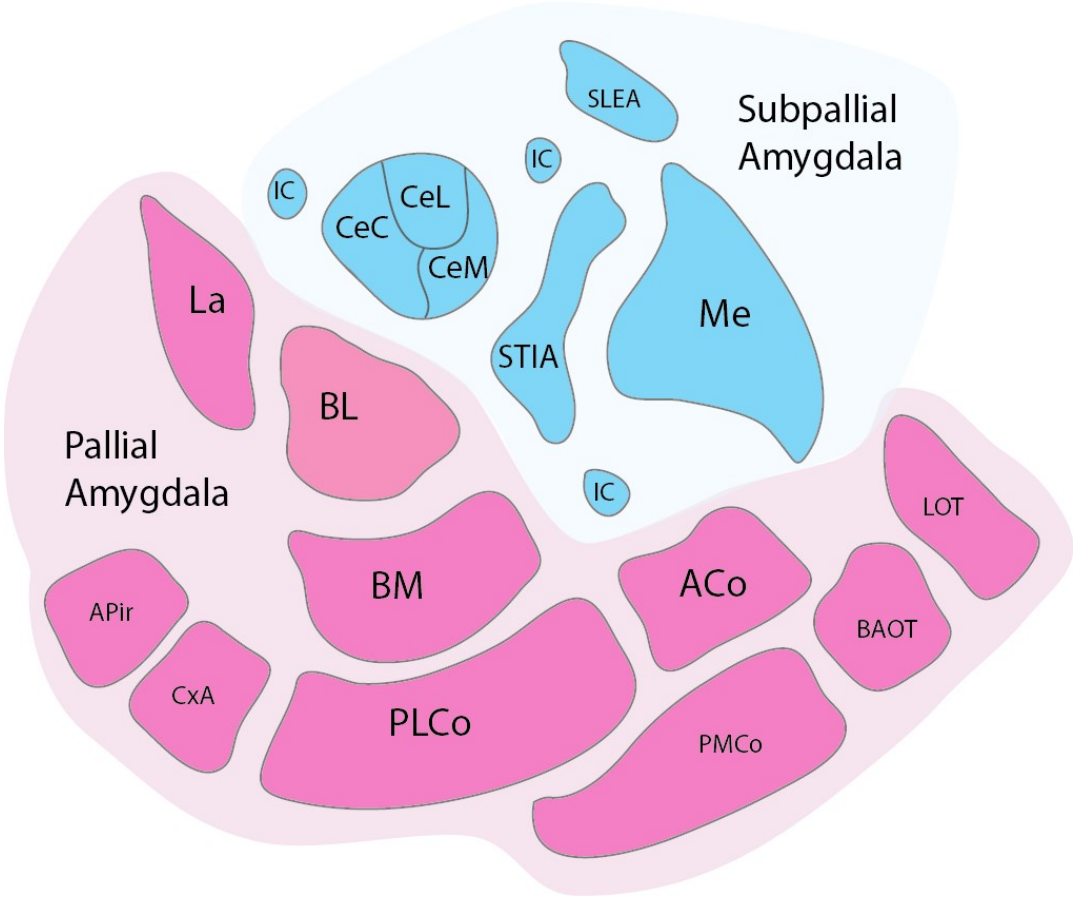


Figure 1-6: The subnuclei of the amygdala
Subpallial: Me - medial, CeC - capsular central, CeL - lateral central, CeM - medial central, ICN - intercalated cell nuclei, SLEA –sublenticular extended amygdala, STIA – intraamygdalar bed nucleus of the stria terminalis. Pallial: La – lateral, BL – basolateral, BM – basomedial, APir - amygdalopiriform transition area, CxA – cortex-amygdala transition zone, PLCo – posteriolateral cortical amygdala, ACo – anterior cortical amygdala, PMCo – posteriomedial cortical amygdala, BAOT bed nucleus of the accessory olfactory bulb, LOT – lateral olfactory tract. Note: not all nuclei are present at all levels, this is for illustrative purposes only

One characteristic of pallial nuclei compared to subpallial is the predominance of glutamatergic innervation, due to developmental origins in the pallial neuroepithelium (Guirado *et al.*, 2008). The

subpallial amygdala is also sometimes known as the extended amygdala (EA), and includes the Ce and Me amygdaloid nuclei, in addition to further EA areas including the intercalated nuclei (ICN), bed nucleus of the stria terminalis (ST), the bed nucleus of the anterior commissure (BAS), the sublenticular extended amygdala (SLEA), and anterior amygdaloid area (AAA).

Subpallial areas such as the Ce and Me possess high levels of GABAergic cells (Swanson & Petrovich, 1998), which correlates with their common origin in the ganglion eminences, from where they migrated to the cortex (Avila *et al.*, 2011). This staining pattern emphasises the link with the caudate putamen, which is also an area of high GABAergic activity (McGinty, 2007). Subpallial areas also contain the predominance of peptidergic amygdalar innervation, further emphasising the striatal origins (Real *et al.*, 2008).

Connectivity

Differences in connectivity reinforce the separation between the pallial and subpallial regions of the amygdala.

As could be predicted by their proximity, olfactory and accessory olfactory bulbs largely project to pallial structures such as the CxA, ACo, and LOT. Projections to the subpallial Me convey olfactory information from the accessory olfactory bulb (Scalia & Winans, 1975; de Olmos *et al.*, 1978). This pathway to the Me, in conjunction with activity in the BM and AHi is associated with modulation of sociosexual behaviour (Ikebuchi *et al.*, 2009; Bergan *et al.*, 2014; Hari Dass & Vyas, 2014).

Sensory information reaches the amygdala via the thalamus, with auditory, visual, and somatosensory data conveyed to the La and Ce from the thalamic posterior intralaminar complex (Turner & Herkenham, 1991). These areas also receive input from the parvicellular ventral posterior thalamic nucleus with gustatory and oral somatosensory information, and visceroreceptive thalamic nuclei innervation to the BL and BM, and La, Ce. Further viscero-receptive input is provided from primary and sensory insular cortex neurons to the same amygdaloid nuclei as the parvicellular ventral posterior thalamus (McDonald, 1998).

As the amygdala is implicated in emotional memory, numerous hippocampal inputs terminate in the pallial nuclei, BL, La, AHi, and APir (Pitkänen *et al.*, 2000), and provide contextual information used to make behavioural decisions (Ergorul & Eichenbaum, 2004). Orbitofrontal projections to the La, BL, and Ce have been shown to convey information on outcome expectancy (Schoenbaum *et al.*, 2009) and so may contribute to behaviour in the open field by modifying emotional responses in accordance with how external factors impact upon expectation.

Cholinergic basal forebrain projections to the Ce are involved in enhanced attentional processing seen in conditioning paradigms when an objects predictive value is altered (Chiba & Wada, 1995;

Han *et al.*, 1999). Dopaminergic inputs to similar areas show involvement in retrieval of fear memories (Nader & LeDoux, 1999).

The amygdala is involved in many behaviours with a hormonal element, and many neurons possess receptors for steroid hormones such as oestrogen (Rasia-Filho *et al.*, 2012), testosterone (Pfaff *et al.*, 2011; Gabor *et al.*, 2012), and corticosterone (Joëls *et al.*, 2013; Kovács, 2013; Myers *et al.*, 2014a). These cells are mainly found in the medial EA, but are also present in cortical pallial nuclei (ACo, PLCo, and PMCo). The high levels in the Me suggest steroid hormone modulation of behavioural responses, and even the sparse corticosterone innervation in the BL has been associated with stress enhanced memory consolidation (Roozendaal & McGaugh, 1997).

In addition to extrinsic inputs, there are numerous intra-amygdala connections. Chemosensory circuits in the cortical amygdala show high levels of exclusive interconnection (Martínez-Marcos *et al.*, 1999; Krusemark *et al.*, 2013), whereas networks involved in emotional behaviours connect both pallial and subpallial nuclei. The majority of pallial-subpallial interconnectivity involves the BL, BM, and La projecting to the Ce and Me, and is variously associated with odour-associated emotional memory (Ferry, 2014; Takahashi, 2014), the interplay between behaviour, emotion and motivation (Fudge & Tucker, 2009), and gated control of fear associated behaviours. Further investigations suggest projections to the Me are largely concerned with socio-sexual elements of behaviour (Dixon, 2004; Brennan & Zufall, 2006), whereas those involving the Ce mediate fear behaviours (Koo *et al.*, 2004; Ciocchi *et al.*, 2010).

The majority of amygdala projection neurons originate in the pallial regions. Projections from the La, BL, BM, and LOT to the perirhinal cortex are implicated in emotional gating of sensory input to the hippocampus (Sacchetti *et al.*, 1999).

The BL, and BM and La to a lesser extent, are reciprocally connected with the insula, and involved in conditioned taste aversion (Clark & Bernstein, 2009). An imaging study looking at activational differences between sommeliers and untrained controls gives an interesting take on the amygdala's role in interpretation of taste and found untrained individuals showed greater amygdalar activation, compared to the more analytical, non-amygdala activation seen in trained sommeliers, demonstrating the division between analytical, trained processes and emotional responses to stimuli (Pazart *et al.*, 2014).

The BL is also the origin of numerous projections to the prefrontal cortex, and it has been shown that this relationship controls acquisition and extinction of conditioned fear behaviours (Schafe *et al.*,

2001). BL connections with the hippocampal regions are also involved in fear extinction and acquisition by providing context (Zelikowsky *et al.*, 2014).

Projections from this same basolateral subgrouping target thalamic nuclei and are thought to be involved in regulation of visceral emotional associations (Van der Werf *et al.*, 2002). Again, the Ce also provides input as part of this circuit, with projections originating from CRF-positive cells in the CeL (Otake & Nakamura, 1995).

The majority of amygdaloid descending projections originate in the subpallial areas, and are involved in expression of fear and anxiety (LeDoux *et al.*, 1988). Generally, output from the medial EA to descending pathways also involves return projections through the central EA (Rizvi *et al.*, 1991). Projections from the Me proper and extended Me (primarily the STL) show different functional outcomes, with Me involved in short-term fear reactions, and STL activity associated with sustained fear or anxiety-like behaviours (Walker *et al.*, 2009).

The Ce is also the origin of projections to the hypothalamus, but unlike those from the Me, these are associated with control of fear-associated stress responses. Ablation is associated with decreased circulating corticosterone (Feldman & Conforti, 1985), and the inverse is seen during Ce stimulation (Redgate & Fahringer, 1973).

The PAG is implicated in pain pathways, and receives amygdala input via the Ce, specifically CRF, somatostatin, and substance P immunoreactive cells in the CeM and CeL (Chen *et al.*, 2009; Zhao *et al.*, 2009; Penzo *et al.*, 2014) are involved in controlling the expression of endogenous analgesia (Nakamura *et al.*, 2013), fear (Canteras *et al.*, 2012), and foraging behaviour (Mota-Ortiz *et al.*, 2009).

Sex differences have been noted in strength of pain-associated connectivity between the Ce and PAG, with men showing increased connectivity, despite similar increases in activation and VAS ratings in response to heat stimuli seen in both men and women (Linnman *et al.*, 2012).

The PB receives input from the central CeL, and sends projections back via the CeC (Bray, 2000) involving gustatory circuits implicated in the development of conditioned taste aversion (Yamamoto, 2007), in addition to homeostatic control of water and sodium intake (Johnson *et al.*, 1999).

The CeL/CeM and central EA areas such as the STL also project to the dorsal vagal complex, modulating stress-induced gastrointestinal activity (Johnson *et al.*, 2012).

Table 1-4 summarises the immunohistological characterisation of the Ce subdivisions in the rat. Other studies investigating immunohistology in the amygdala often fail to distinguish the subdivisions (Katona *et al.*, 2001; Badowska-Szalewska *et al.*, 2006), or use simplified schema e.g. medial and lateral areas of the Ce (Równiak *et al.*, 2008). It is also worth bearing in mind that amygdala outputs are less extensive in the rat compared to primates, suggesting caution when translating what is seen in the rat to the clinical population as neurochemical differences may also be present (Amaral & Price, 1984), and additionally, some transmitters such as relaxin3 show extensive receptor staining without detectable relaxin3 innervation (Ma *et al.*, 2007).

Functionality

As this study is looking at amygdala activity associated with nociception and how this influences behavioural responses to a contextual evaluation of the environment, the majority of amygdala functionality discussed will relate to this.

The amygdala is a key centre for control and generation of emotional behavioural responses in both mammals (LeDoux, 2000; Price, 2006) and other organisms (Moreno & González, 2007).

Table 1-4: Immunohistochemical properties of the nuclei of the central amygdala
Highlighting similarities between lateral and capsular areas. ++ indicates dense staining, + positive staining, - negative staining. Data from Cassell *et al.*, 1986; Gray & Magnuson, 1987; Ma *et al.*, 2007*;Spicer 2013 (unpublished data))*

	Medial	Lateral	Capsular
General description	Small clustered cells	Small dense cells	Loosely packed cells
NAPDHd	++	-	-
AChE	+	-	-
Substance P	++	++	+
Somatostatin	++	+	+
Galanin	++		
Calbindin		+	
CRF		+	
Neurotensin		+	
CCK		++	+
CGRP		+ (perineuronal nests)	+
Relaxin3		++*	++
Enkephalin			+

Early studies in primates identified a syndrome named Kluver Bucy associated with functional loss of amygdala activity due to temporal lobe damage characterised by placidity, hypersexuality, and hyperorality (Terzian & Ore, 1955; Bucy & Kluver, 1955), although to date, little is known about the pain phenotype associated with this condition (Muller *et al.*, 2005). Other studies into amygdala function have investigated individuals with bilateral amygdala damage and noted an apparent lack of fear (Adolphs *et al.*, 2005), however this only seems to apply to externally initiated fearful stimuli as studies looking at responses to low CO_2 were able to evoke fear in patients with bilateral amygdala defects (Feinstein *et al.*, 2013), suggesting some other brain areas must also be capable of evoking feelings of fear to internally-generated physiologically-induced stimuli, separate from the contribution from the amygdala.

The lateral amygdala (La) is strongly implicated in development of fear conditioned responses, with lesions abolishing development of such responses (Amano *et al.*, 2011), and studies demonstrating synaptic potentiation in the La enables fear-conditioned responses (Lazzaro *et al.*, 2010; Hong *et al.*, 2012). The La also projects to the Ce (direct and via the BM), mediating fear-associated behaviours such as freezing (Duvarci *et al.*, 2011). The BL is also involved in some elements of fear conditioned behaviour, particularly learning-related aspects (Roche *et al.*, 2010), whereas the Me is implicated in unconditioned neuroendocrine responses to stress (Masini *et al.*, 2009). Some studies have also shown involvement of the BM in responses to live predators, emphasising the refinement of the amygdaloid system, with differential responses to present and unseen dangers (Takahashi, 2014). Cortical nuclei are not exempt from fear associated behavioural modulation, and are suggested to play a role in processing aversive chemosensory stimuli (Dhungel *et al.*, 2011). Furthermore, a recent study demonstrated the ability of toxoplasma gondii to supress predator-odour evoked fear behaviours in rats via epigenetic modulation of the medial amygdala (Hari Dass & Vyas, 2014), emphasising how the amygdala's role in behavioural modulation can be hijacked.

Few studies have considered the direct effect of open field exposure on amygdala activity, and how this contributes to behaviours observed. However, studies looking at response to predators implicate the Me and BL/BM in generating evasive behavioural responses, with little involvement of the Ce – the Ce is, however activated in response to a compound extracted from fox faeces, trimethylthiazoline (TMT), although ablation studies have shown this activation is not required for a behavioural response (Day *et al.*, 2004; Fendt & Endres, 2008).

Numerous functional imaging studies have also shown increased activation in the amygdala associated with pain states (Bornhovd *et al.*, 2002; Hadjikhani *et al.*, 2013), however, variation in the areas defined as "the amygdala", a lack of spatial precision (Chen *et al.*, 2003; Soher *et al.*, 2007), and statistical complications associated with analysing large interdependent data sets (Bennett *et al.*,

2011) suggest caution is required when interpreting such data. It was recently shown that the accuracy of diffusion-weighted MRI in determining connectivity is very low, and dependent on analysis algorithms used when compared to traditional tract tracing (Thomas *et al.*, 2014).

The evidence for lateralisation in amygdala activation is inconclusive, with studies showing left (Baas *et al.*, 2004; Beraha *et al.*, 2012), right (Carrasquillo & Gereau, 2008), or variable dominance (Dyck *et al.*, 2010). With unilateral peripheral stimulation, activation is often observed on the contralateral side (Brügger *et al.*, 2011), but there is evidence of functional division between left and right amygdalae (Chen *et al.*, 2014).

From Table 1-5, it is clear there is low overall consistency in lateralisation of amygdala activation, with both left and right showing dominance in different studies. The problem with comparing such information is that the amygdala is such a heterogeneous brain area that studies reporting simply activation are actually giving very little useful information. In studies related to pain and associated behavioural responses, activity in the Ce appears to show a left bias, although bilateral activation dependent on time after SNT injury has been described (Gonçalves & Dickenson, 2012), with implantation of corticosterone pellets into either Ce resulting in hypersensitivity and decreased open arm activity in the elevated plus maze, but bilateral implantation was required for expression of visceral hypersensitivity (Tran & Greenwood-Van Meerveld, 2012).

Connections between the PFC and amygdala are implicated in top-down control of behavioural responses, with an indirect pathway involving excitation of the BL by the medial PFC, which in turn leads to inhibition of the Me and associated brainstem connections (Likhtik *et al.*, 2005).

The different subdivisions of the amygdala receive sensory input from areas such as the thalamus and spinal cord (capsulo-lateral portion of the Ce, via the La and BL), and provides output via the medial nucleus of the central amygdala to the PFC and hypothalamus, suggesting involvement in the assessment and generation of emotional associations that form an integral part of the pain experience (Neugebauer *et al.*, 2009). Studies have shown pain-associated increases in amygdala activity both clinically and pre-clinically: Pancreatitis (Frøkjær *et al.*, 2011), cluster headache (Seifert *et al.*, 2011), back pain and arthritis (Baliki *et al.*, 2008), fibromyalgia (Harris *et al.*, 2009), menstrual pain (Tu *et al.*, 2010).

c-Fos

c-fos is a proto-oncogene from the Fos family of transcription factors that encodes a 62kDa nuclear protein (c-Fos). It forms a heterodimer with c-Jun (also a transcription factor), known as activator

protein 1 (AP-1), which binds DNA and enables the conversion of extracellular signals into changes in gene expression (Bullitt, 1990).

Within 30-40 minutes of neurotransmitter stimulation, c-Fos mRNA is rapidly induced in neurons, with protein levels peaking 90-120min after the initial stimulation. It is involved in early cell responses to growth factors (Biały & Kaczmarek, 1996), and has been shown to be up regulated in cancer (Durchdewald *et al.*, 2009; Healy *et al.*, 2013). Additionally, it is also commonly used as a marker of neuronal activation, with increased levels associated with stimuli including drug of abuse (Armario, 2010), stress (Nishi *et al.*, 2013; Reul, 2014), and pain (Hunt *et al.*, 1987; Harris, 1998; Coggeshall, 2005).

In studies investigating nociceptive stimuli, up-regulation of c-fos RNA or c-Fos protein has been shown in the spinal cord following both visceral inflammation (Dinis *et al.*, 2004) and CCI. In the brain, the type of noxious insult influences the areas activated: visceral models of acute CYP-induced haemorrhagic cystitis (Bon *et al.*, 1998), colorectal distension (Traub *et al.*, 1996) and intraperitoneal acetic acid increase c-Fos immunoreactivity in the Ce, whereas intradermal formalin is associated with c-Fos activity in the BL but not the Ce of the amygdala (Nakagawa *et al.*, 2003). Formalin has also been shown to increase activation in the hippocampus and RVM (Roche *et al.*, 2010), whereas studies looking at central activation following SNT have shown both increases in the Ce on one hand (Morland *et al.*, Manuscript in Preparation) and no change in the amygdala or hypothalamus (Narita *et al.*, 2003). This 2003 study also found higher levels of c-Fos in the frontal cortex, thalamus, and PAG, with decreases in the nucleus accumbens (NAc) and VTA following SNT in male rats. Non-nociceptive stressors are also capable of inducing up regulation of c-Fos. Restraint stress increases c-Fos immunoreactivity in the paraventricular nucleus of the hypothalamus (PVN) (Umriukhin *et al.*, 2012), and also in the prefrontal cortex, and amygdala (Shoji & Mizoguchi, 2013). There is some evidence that open field exposure increases c-Fos immunoreactivity in a number of brain areas, including the Ce, enhanced by chlordiazepoxide treatment, but the authors failed to include information on activation period allowed for c-Fos, so it is possible these increases were due to other factors (Shaw *et al.*, 2011).

Levels of amygdala c-Fos activation have been shown both sensitive to behavioural phenotype, with dominant animals showing higher levels following open field exposure (Wrona *et al.*, 2013), and dominant males, but not females exhibiting sensitivity to early life 'Denial of Expectation Reward' experiences resulting in increased adult Ce c-Fos response to fear conditioning (Diamantopoulou *et al.*, 2013).

Increases in Ce c-Fos have been observed 4 hours after initiation of CYP treatment, but not at later stages, indicating an acute amygdala response to bladder insult (Bon *et al.*, 1997; Bon *et al.*, 1998).

It is important to be aware of the limitations associated with using c-Fos as a marker for neuronal activation. Firstly, c-Fos alone will only detect recent cell activation, giving no indication whether the signal transmitted was excitatory or inhibitory, a limitation also seen in imaging techniques such as MRI. Additionally, not all cells express c-Fos (Dragunow & Faull, 1989), such as those in the dorsal root ganglia (Hunt *et al.*, 1987). Studies have also found unexpectedly low levels of c-Fos in areas previously identified as involved in pain mechanisms such as the ventral postero-lateral thalamus (Bullitt, 1990). Together, this evidence suggests caution in the interpretation of negative results, as the absence of evidence of neuronal activation following nociceptive stimulation cannot be taken as evidence that the brain area in question is not being activated.

Table 1-5: Summary of evidence for lateralisation of amygdala activation, showing the variation present in studies to date

ABBREVIATIONS – AMY: amygdala, PET: positron emission tomography, fMRI: functional magnetic resonance imaging, LORETA: Low Resolution Electromagnetic Tomography, Ce: central amygdala, CeL: lateral central amygdala, PHN: post-herpetic neuralgia, CRF: corticotrophin-releasing factor, IBS: irritable bowel syndrome, OCD: obsessive compulsive disorder, ERK: extracellular signal-regulated kinase, mGluR5: metabotropic glutamate receptor 5, EPhys: electrophysiology, CORT: corticosterone, SNT: spinal nerve transection, SR: systematic review, IHC: immunohistochemistry

Study Details	Type	Condition	Method	Area	Details	Left	Right	Both
(Sutherland *et al.*, 2010)	Basic	Stress	Gene Expr.	AMY	CRF	✓		
(Naliboff *et al.*, 2003)	Clinical	Visceral	PET	AMY	IBS; Enhanced in women	✓		
(Hindi Attar *et al.*, 2010)	Clinical	Psychiatric	fMRI	AMY	Facial processing	✓		
(Beraha *et al.*, 2012)	Clinical	Negative Affect	fMRI	AMY		✓		
(Liu *et al.*, 2013)	Clinical	Neuropathic Pain	fMRI	AMY	PHN	✓		
(Baas *et al.*, 2004)	SR	Emotional processing - 54 studies	fMRI/PET	AMY		✓		
(Akitsuki & Decety, 2009)	Clinical	Psychiatric	fMRI	AMY	Social context	✓		
(Kwon *et al.*, 2003)	Clinical	Psychiatric	MRI	AMY	OCD compared to schizophrenia	✓size		
(Carrasquillo & Gereau, 2008)	Basic	Inflammation	IHC	Ce	ERK		✓	
(Ji & Neugebauer, 2009)	Basic	Inflammation	EPhys	CeL	Monoarthritis		✓	
(Kolber *et al.*, 2010)	Basic	Inflammation	IHC/behaviour	Ce	mGluR5		✓	
(Veltman *et al.*, 2004)	Clinical	Psychiatric	PET	AMY	Phobia		✓	
(Strigo *et al.*, 2008)	Clinical	Depression	fMRI	AMY	Painful Stimuli		✓	
(Proverbio *et al.*, 2009)	Clinical	Empathy	LORETA	AMY	Effect seen only in women		✓	
(Farahbod *et al.*, 2010)	Clinical	Depression	LORETA	AMY	Facial processing		✓	
(Gaffrey *et al.*, 2011)	Clinical	Depression	fMRI	AMY	Juveniles - facial processing		✓	
(Kim *et al.*, 2014)	Clinical	Alcohol Dependence	fMRI	AMY			✓ ↓	
(van der Plas *et al.*, 2010)	Clinical	Fear	MRI	AMY	Juveniles - enhanced in women w/history of depression		✓size	
(Tran & Greenwood-Van Meerveld, 2012)	Basic	CORT-implantation	Behaviour	Ce	Affective, behaviour & somatic/visceral hypersensitivity			✓
(Gonçalves & Dickenson, 2012)	Basic	Neuropathic Pain	EPhys	Ce	SNT - differential effect of time			✓
(Dyck *et al.*, 2010)	Clinical	Psychiatric	fMRI	AMY	Dependent on stimuli			✓
(Chen *et al.*, 2014)	Clinical	Depression	fMRI	AMY	6 right - 12 left			✓
(Rosenberger *et al.*, 2009)	Clinical	Stress	fMRI	AMY	Rectal distension and stress			✓

1.8 Inflammation in Pain

Inflammation describes the reaction of the body to tissue damage. It can be divided into innate and adaptive responses: innate immune responses are the first line of defence and are most active within the first 12 hours of an insult. Innate immunity involves natural barrier systems such as the epithelium and endothelium, phagocytic cells such as monocytes and macrophages, granulocytic cells, including neutrophils, eosinophils, and basophils, and other cells types including mast cells, dendritic cells, complement protein systems, and natural killer (NK) cells. In response to damage to epithelial barriers, these cells release a range of cytokines and inflammatory mediators that act to isolate and limit damage caused by insults including bacterial infection, and chemical or physical trauma.

Adaptive immunity, a more specialised form of immune response, becomes involved a day or so after the original insult. It involves highly specialised cells known as T and B lymphocytes, which generate antibodies towards foreign agents such as bacteria, and possess 'memory' whereby previously encountered antigens elicit a stronger response. In terms of inflammation and pain, the contribution of adaptive immunity is minimal.

Released during innate immune reactions, cytokines are small protein molecules involved in a broad range of activities associated with cell signalling. There are various specific subtypes of cytokines, including chemokines and interleukins. Chemokines are cytokines specifically involved in cell chemotaxis, acting to guide and modulate cell migration, whereas interleukins were initially identified in leukocytes and act prominently in the immune response. There are also a number of other mediators involved in the inflammatory response, including growth factors and enzymes such as inducible nitric oxide synthase (iNOS), and prostaglandin-endoperoxide synthase 2 (PTGES2/COX2), which catalyse reactions producing the potent vasodilators nitric oxide and prostaglandin respectively.

Tumour necrosis factor (TNF), interleukin-1β (IL-1β), and IL-6 are endogenous pyrogens and are involved in the increase in body temperature that occurs during inflammatory processes. In general, inflammatory mediators can be pro-inflammatory (e.g. IL-1β), anti-inflammatory (e.g. IL-10), or show activity in both directions dependent on pathways activated (e.g. IL-6) (Arango Duque & Descoteaux, 2014).

As many painful situations involve tissue damage without foreign bodies present, innate immunity is more prominent compared to adaptive, and as there is no 'memory' involved in an innate response,

the magnitude of response is modulated by factors including severity and spread of insult, not familiarity.

Neurogenic inflammation, where release of neuropeptides such as substance P and CGRP from peripheral sensory afferents acts on local vasculature and soft tissue to increase vasodilation and inflammation levels, is also implicated in chronic pain conditions such as CRPS (Krämer, 2012).

The TRPA1 receptor is thought to be involved in the neurogenic process via peripheral axon and central dorsal root reflexes (Koivisto *et al.*, 2014), and has been suggested as a drug target in neurogenic and central pain conditions (Pertovaara & Koivisto, 2011).

The triple response of Lewis is a classic example of an inflammatory response whereby a non-penetrative scratch on the skin initially turns red due to capillary dilation, followed by a wider area of reddening (flare) caused by neurogenic reflex activity resulting in arteriole dilation, with the final component of the response being oedema/wheal following the shape of the initial scratch (Kong, 1960).

Inflammation has been shown to play an important role in many pain conditions including arthritis (Lee *et al.*, 2014), CRPS (Borchers & Gershwin, 2014), and migraine (Bruno *et al.*, 2007). Systemic *e.coli*-induced inflammation has been shown to decrease pain thresholds to pressure and electrical stimulation, whilst also increasing the pain experience during the cold pressor test (de Goeij *et al.*, 2013). Numerous studies have investigated the cytokine profile associated with pain conditions including chronic pelvic pain syndrome (Murphy *et al.*, 2014), IC/BPS (Schrepf *et al.*, 2014), and neuropathic pain (Ellis & Bennett, 2013). In particular, Dawes and co-workers demonstrated commonality between cytokines up-regulated following UVB-irradiation in clinical and pre-clinical models, showing the pro-inflammatory cytokine CXCL5 is up-regulated and capable of producing hypersensitivity in otherwise naïve rats (Dawes *et al.*, 2011). Further, it has been documented that following surgery, innate immunity suffers stress-associated suppression (Cardinale *et al.*, 2011; Cata *et al.*, 2013), emphasising the complexity of the inflammatory contribution to pain.

Mast cells are myeloid stem cell derived immune cells actively involved in the inflammatory response (Sant *et al.*, 2007). They produce a range of mediators including nitric oxide (NO), COX-2, NGF, serotonin, and histamine, in response to inflammatory mediators, including NGF itself via TrkA receptors (Rice *et al.*, 2002), and also in response to bradykinin (Cochrane *et al.*, 1982). Bradykinin is associated with increased pain sensitivity (Paterson *et al.*, 2013), and acts to balance the

inflammatory response in tandem with other mast-cell secretions, such as interleukin-10 (IL-10) possessing anti-inflammatory properties (Gautam *et al.*, 2011).

Other mediators such as NO are released from other cells, including neurons, during tissue damage and are involved in vasodilatation (Cirino, 1998). NO is highly expressed in healthy bladder tissue (Austin *et al.*, 2003), and has been implicated in bladder cancer (Ryk *et al.*, 2010; Ryk *et al.*, 2011), and ESSIC type 3 IC/BPS (IC featuring Hunner's ulcers) (Logadottir *et al.*, 2012). The high expression in neurons, is implicated in modulation of motor behaviour in mice, with systemic, intra-cerebroventricular and intra-striatal administration of a NOS inhibitor associated with anxiolytic effects in the elevated plus maze, as well as reduced locomotion and rearing in the open field (Del Bel *et al.*, 2005). It also has similar effects in rats, with studies identifying the PAG as a site with high NO activity, wherein application of NOS inhibitors induced anxiolytic effects, with NO donors having the opposite effect (Guimarães *et al.*, 2005).

Interleukin-1β (IL-1β) is also highly implicated in behavioural changes associated with inflammation. It stimulates the release of CXCL8, CCL2, and CSF2, and is supressed by prostaglandin E2, another inflammatory mediator (Mosher *et al.*, 2012). Behaviourally, systemic IL-1β reduces ambulation and rearing activity in both male and female rats, with female rats in oestrous showing greater sensitivity compared to non-oestrous and male rats (Avitsur *et al.*, 1995). Furthermore, socially isolated mice show increased pre-frontal expression of IL-1β following spared nerve injury (SNI), a situation replicated by application of an oxytocin-inhibitor (Norman *et al.*, 2010). IL-1β is also up regulated following Stavudine treatment (Lagathu *et al.*, 2007) and implicated in numerous autoinflammatory pain conditions (Chen & Meckfessel, 2013).

It is clear that inflammatory mediators are involved to different degrees in numerous pain conditions, and offer attractive targets for drug development; therefore identification of characteristic inflammatory profiles is important in the study of pain.

2 Study Aims and Statement of Hypothesis

Aims

Affective dysfunction associated with chronic pain is a major obstacle to successful treatment. The aims of this thesis are to investigate the behavioural alterations associated with the turpentine model of visceral inflammation, and determine whether such changes are correlated with activation in the central amygdala, an area implicated in pain-related behavioural responses. The secondary aims are to examine differences between visceral inflammation and a model of anti-retroviral-associated neuropathy, and further investigate open field phenotype in naïve animals to increase translational understanding of affective aspects of the pain experience.

Hypotheses

1. Variation seen in Naïve animals is due to trait differences associated with defined behavioural phenotypes
2. Urinary bladder inflammation in the female laboratory rat is associated with pain-related changes in ethologically-relevant behaviours, which are accompanied by increases in activation in the central nucleus of the amygdala
3. d4T-induced peripheral neuropathy in female rats is associated with evoked mechanical hypersensitivity and increases in thigmotactic behaviour

3 Materials and Methods

3.1 Ethical Statement

All animal experiments conformed to the British Home Office Regulations (Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 (SI 2012/3039)) under the authority of United Kingdom Home Office Project Licence 70/7162 and the IASP guidelines for *in vivo* research (Zimmermann, 1983; and http://www.iasp-pain.org). Experiments were designed according to Good Laboratory Practice standards (Macleod *et al.*, 2009) and reported in accordance with the ARRIVE Guidelines (Kilkenny *et al.*, 2010; Rice *et al.*, 2013).

3.2 Experimental Animals

Adult female Wistar rats (Charles River, UK) were housed in groups of 2-4 in individually ventilated cages with free access to tap water and standard rat chow (RM1 (P), Special Diet Services, UK). Male Wistar rats (Charles River, UK) housed under the same conditions were used in the repeated open field study.

Animals were maintained under a 12-hour light cycle (07:00-19:00) under temperature and humidity-controlled conditions (25°C, 30% ± 5). Cages were cleaned weekly on a Tuesday (p.m.), and housed in a holding room containing mice and rats of both sexes. Rats and mice were housed at opposite ends of the holding room, and each cage was individually ventilated to avoid exposure to olfactory stressors. Animals were habituated to the holding room for a minimum 48 hours after delivery. Weight, estimated age, and sex of experimental animals used in each study are detailed in Table 3-1.

Table 3-1: Weight details of experimental animals
Estimated age taken from UK growth chart (http://www.criver.com/products-services/basic-research/find-amodel/wistar-rat)

Study	Sex	Weight at start of study (median (95% CI))	Estimated age*
1 Naïve repeated Open Field	Male	235.2g (231.6-238.8)	7 weeks
2 A Acute Bladder Inflammation + Open Field	Female	237.4g (232.6-242.2)	11 weeks
B Acute Bladder Inflammation + Burrowing	Female	248.6g (242.1-255.1)	11 weeks
C Repeated Bladder Inflammation + Open Field	Female	231.3g (228.3-234.2)	11 weeks
3 d4T anti-retroviral neuropathy + Open Field	Female	229.7g (216.0-243.4)	11 weeks

3.3 Study Design

Table 3-2 highlights the major domains of good laboratory practice, including bias reduction.

Table 3-2: Major Domains of Good Laboratory Practice (Macleod et al., 2009)

Characteristic	Description of procedures
Sample size calculation	Group size was determined by sample size estimation for each experiment using SigmaStat software, version 3.5 (ANOVA sample size, desired power = 0.8, $\alpha = 0.05$). Effect sizes for estimation were derived from previous studies in our group. Details of sample size calculation can be found in the appropriate study design sections
Inclusion and exclusion criteria	Animals that died prior to, or during model induction were excluded from further study. Animals were only excluded from open field analysis if video footage was compromised; animals were excluded from c-Fos analysis if >90min elapsed between open field and perfusion, or if hemisphere were indistinguishable.
Randomization	Animals were randomised to model group by cage using a pseudorandom ABCBCACAB labelling system.
Allocation concealment	To maintain concealment of group allocation, a second experimenter prepared the syringes with either olive oil or turpentine. Although I (model creator) was theoretically unaware of the allocation to treatment group, due to the pungent odour of turpentine, allocation concealment was difficult to maintain. Procedures were still followed, using the blinding procedure described below, as well as masking cage labels or turning around the cages before each behavioural assessment session.
Reporting of animals excluded from analysis	All animals excluded are reported in the relevant tables below.
Blinded measurement, assessment, and analysis of outcome	Codes were assigned to different treatments by an independent person and kept in a sealed envelope. The codes were not broken until the analysis had been completed. The experimenter was blinded to the experimental group to which an animal was randomized. In addition, open field videos were renamed by an independent person before analysing, and immunohistochemistry sections were identified by animal not group code.

Study 1

Naïve, male Wistar rats were used to test the effects of repeated open field exposure on behavioural outcome measures commonly used in pain research. There were no differences in how the animals were handled over the three-week testing period. Each animal was exposed to the open field on three occasions, exactly 1 week apart. 26 animals were used in this study, with no exclusions. Figure 3-1 illustrates the experimental timeline.

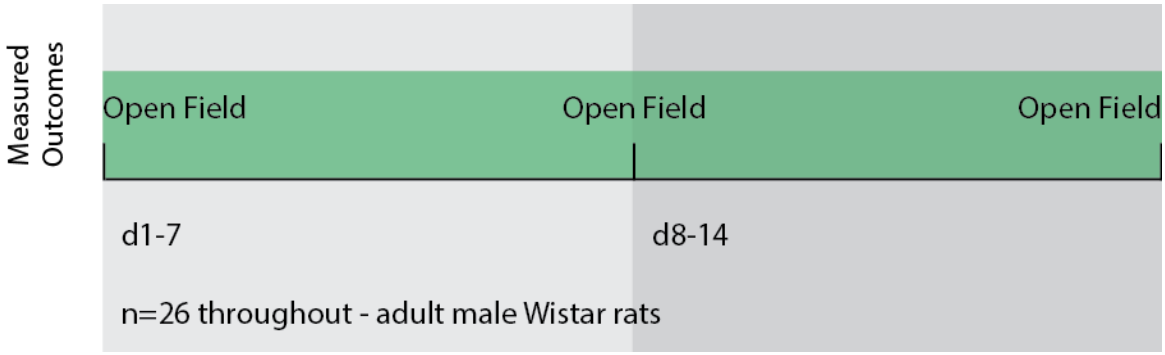


Figure 3-1: Experimental timeline for the repeated open field study using naïve male rats. No interventions were involved, with open field outcomes measured at three points during the study.

Table 3-3 details the information used when calculating sample sizes. As this was an explorative study looking at longitudinal changes over time, rather than comparing the effects of treatment, it was not possible to do a classic sample size calculation. Instead, the following equation was used:

$$n = \frac{z'\sigma}{MOE}$$

Where z is the critical value (1.96 for a 95% confidence interval), σ represents the standard deviation of duration in the inner zone for naïve male rats (5.11), and MOE representing the margin of error, (2). Using data from 12 naïve male rats, this calculation yielded a sample size of 25. 26 animals were used in the study to avoid uneven group housing. This was an approximate method of estimating sample size based on confidence interval (CI), as the sample size calculator used (OriginPro) did not account for repeated testing, and as this study utilised repeated testing, the sample size was deemed appropriate for detecting phenotypic alterations in this study.

Table 3-3: Information used when calculating sample sizes for study 1

	Duration	Frequency
Mean/Median	8.9s	13.5
Standard Deviation/IQR	5.11	8.5-19.5

Study 2

In the bladder inflammation experiments, there were three main experimental groups: Naïve (N), Instrumentation (I), and Turpentine (T). Two forms of bladder inflammation were employed – acute (tested 24 hours following induction of inflammation), and repeated (tested 24 hours following the final session of inflammation, two weeks after initial induction). In the repeated model, there was an additional Anaesthesia Only (AO) group, subjected to a two-hour period of isoflurane anaesthesia with no other intervention. This group was introduced to determine the contribution of anaesthesia to behavioural alteration. Twenty-four hours following model completion, animals were either exposed to the open field (acute and repeated) or burrowing (acute only) paradigms. Tissue for immunohistochemistry (brain) and cytokine analysis (bladder) was harvested within 90-120 minutes of open field exposure to capture peak c-Fos activation.

For full details of exclusions and group sizes, see Table 3-5 and Figure 3-2 for experimental timelines.

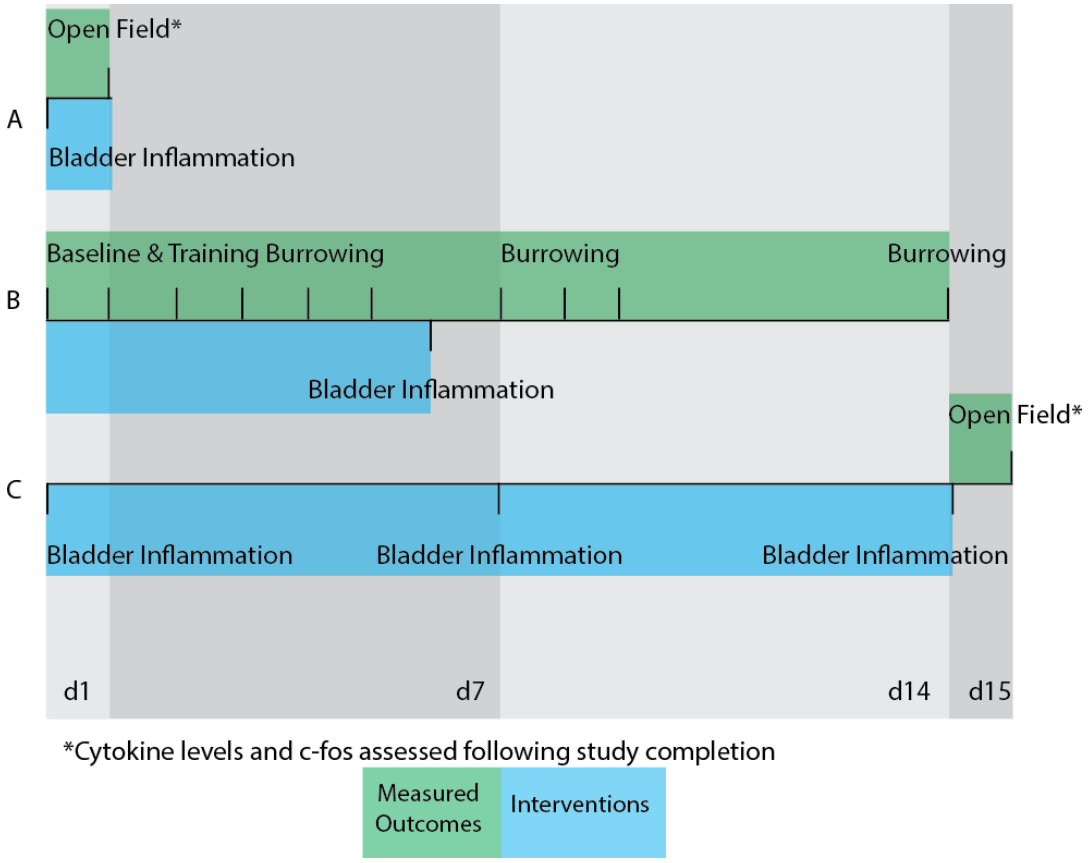


Figure 3-2 - Experimental timeline for Bladder Inflammation studies, illustrating the three substudies – A) acute bladder inflammation with a single open field exposure, B) baseline burrowing measurements followed by a single session of bladder inflammation and four testing sessions for burrowing, C) Repeated bladder inflammation (x3) followed by a single open field exposure.

Table 3-4: Group sizes and exclusions for study 2, acute and repeated bladder inflammation

	Acute Bladder Inflammation		
	Naïve	Instrumentation	Turpentine
Model Induction	14	9	17
Exclusions	*1 found dead in cage prior to study start*		*3 died during model induction*
Open Field	13	9	14
Exclusions			
c-Fos immunoreactivity	9	8	13
Exclusions	*3 due to quality of staining, 1 due to timing*	*1 due to quality of staining*	*1 due to quality of staining*
Cytokine Microarray	4		4
Exclusions	*Animals chosen based on behaviour median frequency)*		*Animals chosen based on behaviour (close to (close to median frequency)*
	Acute Bladder Inflammation and Burrowing		
Model Induction	14	14	14
Exclusions		*3 died during model induction*	*2 died during model induction*
Burrowing	14	11	12
Exclusions	*2*	*2 (all due to baseline burrowing <500g)*	*2*
	Repeated Bladder Inflammation		

	Naive	AO	Instrumentation	Turpentine
Model Induction	16 (20 inc. *)	8	10	14 (18 inc. *)
Exclusions				*2 died during model induction*
Open Field	16	8	10	12
Exclusions				
c-Fos IR	15	8	10	8
Exclusions	*1 due to quality of staining*			*4 due to quality of staining*
Cytokine Microarray	3*	NT	4*	4*
Exclusions	*1 due to PCR replication failure; as above*		*As above*	*As above*

* n=4/group extra animals in repeated model for cytokines only; NT, not tested

Table 3-5 details the information used when calculating sample sizes. Frequency of entry to the inner zone data from a previous study conducted in this laboratory looking at thigmotaxis in the open field following SNT (Morland *et al.*, Manuscript in Preparation). Using a power of 0.08 and alpha value of 0.05, a sample size of 8 was calculated using OriginPro (applicable to both 1-Way ANOVA and t-test).

Table 3-5: Information from previous work into the spinal nerve transection model (SNT) used when calculating sample sizes for study 2

	Naïve	SNT	
Mean	7.67	4.17	Difference = 4.17
Standard Deviation	3.84	1.65	Mean = 2.745

Study 3

In the study of d4T-induced neuropathy, there were three main experimental groups: Naïve (N), Vehicle (V), and d4T. Animals were tested for hindpaw sensitivity (mechanical, thermal, cold) on days 4, 7, 10, and 13, and exposed to the open field on post-injection day 14. Table 3-6 shows group sizes, Table 3-7 gives the data used to calculate sample sizes, and Figure 3-3 illustrates the experimental timeline.

Table 3-6: Group sizes used in the d4T study

Group	Initial Group Size	Open Field	Evoked Nocifensive Measures
Naïve	12	12	12
Saline	12	12	12
d4T	12	12	12

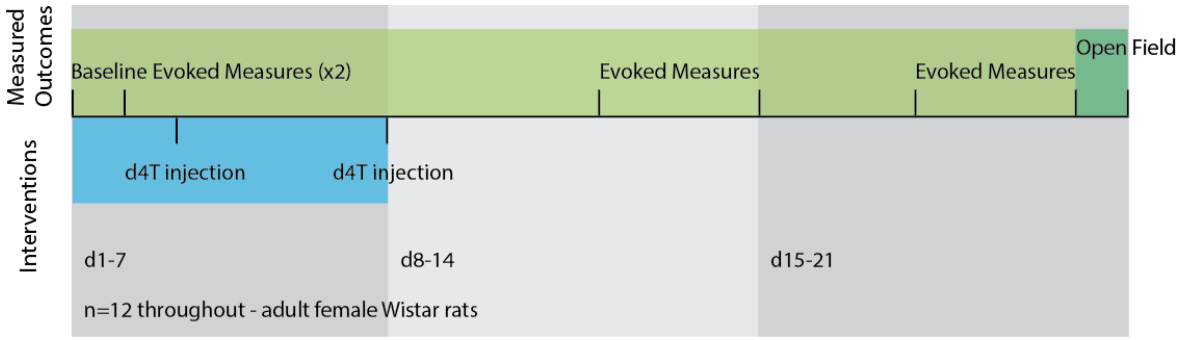


Figure 3-3: Experimental timeline for the d4T study illustrating the measurement of baseline evoked measures before two injections of d4T. Evoked measures were tested at a further 4 points, completed with a single open field exposure.

Frequency of entry to the inner zone data from a previous study conducted in this laboratory looking at thigmotaxis in the open field following d4T (Stavudine) treatment in male rats (Huang *et al.*, 2013) was used. Using a power of 0.08 and alpha value of 0.05, a sample size of 12 was calculated using SigmaStat (applicable to both 1-Way ANOVA and t-test).

Table 3-7: Information used when calculating sample sizes for study 3

	Naïve	d4T	
Mean	15.67	4.71	Difference = 10.96
Standard Deviation	2.87	1.13	Mean = 2

3.4 Test Environment

All behavioural experiments were conducted during the light phase (08:00-18:00) in a dedicated behavioural laboratory, with surgical procedures carried out in a separate but adjacent surgical room. *In vivo* studies were conducted in batches of 2-3 animals per group (n=6-9/batch) due to capacity and protocol constraints. Humidity and temperature were maintained at 25°C and 30% respectively. Light levels outside the isolation chamber during open field paradigms ranged from 70300 lux, measured using an IsoTech ILM350 lux meter (IsoTech, Industrial Partner, USA). As rodents have a greater auditory range than humans, ultrasonic sound recordings were taken using a Mini-3 Bat Detector (Ultra Sound Advice, UK) to determine the background levels of high frequency sound generated by equipment. Fluorescent lighting and computer equipment emitted signals within the 20-50 kHz range - this equipment was switched on for the duration of each experiment and animals were allowed to acclimatise to the testing room for 30 min prior to testing.

3.5 Model Induction

Study 1: Repeated Open Field

Naïve animals were used without prior intervention.

Study 2: Bladder Inflammation

Visceral inflammation was induced via turpentine inflammation of the urinary bladder. Turpentine inflammation has been previously shown to induce both cytometric changes in bladder activity (Rice & McMahon, 1994; Jaggar *et al.*, 1998b; Farquhar-Smith *et al.*, 2002), and referred thermal hyperalgesia in the hind paws (Jaggar *et al.*, 1999; Farquhar-Smith & Rice, 2001) but this is the first study looking at the effect on ethological behaviours such as thigmotaxis following turpentine inflammation of the bladder. Female rats were used for anatomical reasons associated with catheterisation.

Under isoflurane anaesthesia, (1.5-3% in 2 L/min O_2), bladder inflammation was induced as described by McMahon and Abel (1987). With the rat in a supine position, a transurethral catheter (diameter 1.02mm) was introduced into the bladder and position verified by applying gentle abdominal pressure to empty the bladder. 0.5 ml olive oil (instrumentation control; Marks &

Spencer, UK) or turpentine (50% in olive oil; Rustins Pure Turpentine, Rustins, UK) was slowly introduced to the bladder using a 0.5ml syringe attached to the catheter with a luer lock. The instillation was maintained under anaesthesia for 2 hours before removal of the catheter. The animal was allowed to recover in a separate area before returning to the home cage. To avoid hypothermia, a warming mat was used, and 1ml sterile saline (B. Braun Melsungen AG, Germany) was subcutaneously administered at hourly intervals (start, middle, and end) to prevent dehydration. Isoflurane levels were monitored and adjusted throughout the procedure to maintain an appropriate depth of anaesthesia.

Based on the above protocol, a model of persistent bladder inflammation was developed, involving three instillations, each one week apart. Animals were weighed at the start of each experimental session to monitor changes in weight.

Study 3: d4T-induced Neuropathy

d4T is an antiretroviral known to induce peripheral neuropathy. Patients are generally administered d4T orally, and previous studies have demonstrated in rats that both oral gavage and i.v. administration routes produce similar nocifensive behavioural profiles (Weber *et al.*, 2009; Huang *et al.*, 2013). We selected i.v. administration as it is a comparatively less stressful procedure than oral gavage, and is more consistent in terms of absorption rates (Kaul *et al.*, 1999). The treatment regimen (two 50mg/kg i.v. injections) was chosen on the basis of previous studies (Huang *et al.*, 2013), which showed a robust phenotype of mechanical hypersensitivity in male rats. Female rats were used to enable comparison with the repeated model of bladder inflammation, and contrast the effects with that seen following d4T-associated neuropathy, to investigate differences and similarities between two models of non-unilateral pain with differing aetiologies.

Animals were briefly anaesthetised (isoflurane, 1.5-3% in 2 L/min O_2), and their tail placed in warm water to facilitate i.v. injection. Once the tail vein was sufficiently dilated, the needle was gently inserted, bevel-up into the tail vein, and 0.5 mL of either 0.9% saline (sterile 10ml ampoules, B. Braun Melsungen AG, Germany) or d4T (a gift from Pfizer Ltd., UK; 50 mg/kg in sterile saline) was slowly injected. A second identical i.v. injection was given 4 days later.

3.6 Outcome Measures

All Studies: Thigmotaxis

Thigmotaxis is an ethological outcome measure that detects changes in the innate behavioural conflict between exploration and predator avoidance. It has been shown effective in detecting negative behavioural responses associated with traumatic peripheral nerve injury (Leite-Almeida *et*

al., 2009; Blackbeard *et al.*, 2012), HIV-induced (Wallace *et al.*, 2008), VZV-associated neuropathy (Hasnie *et al.*, 2007) and d4T-induced (Huang *et al.*, 2013) neuropathies in male rats. This is the first study to investigate whether visceral inflammation is also capable of inducing thigmotactic changes, and also the first investigating effects in female animals.

The open field paradigm was used to assess thigmotaxis. Animals were exposed to the open field at the following points in each study:

1. Naïve Study: At weekly intervals over three weeks in naïve animals
2. Bladder Inflammation: 24 hours after acute model induction and the final session of bladder inflammation (repeated)
3. d4T-associated neuropathy: 14 days after the final injection of d4T

Rats were taken from their home cage using a gloved hand and introduced into the near corner of a matt black 100 cm^2 arena, enclosed in an isolation chamber (115 x 115 x 255 cm) to minimise fluctuations in light and sound levels and minimise observer effects on behaviour (i.e. the effect of observation on that which is being observed). Light levels within the open field were 12 lux (measured in the centre of the arena). Animals were positioned in the near corner facing the centre of the arena floor, and allowed to explore for 15 minutes. The arena was cleaned with 0.02% Distel (formerly Trigene, Tristel Solutions Ltd., UK) between trials. Behaviour was captured during the 15-minute exploration session by high sensitivity camera (VCB 3372; Sanyo, Japan), and analysed using Ethovision XT 10.1 (Tracksys, UK (for Noldus, Netherlands)). The primary outcome measure was frequency of entry to a virtual central zone (40 cm^2; defined using Ethovision software). Secondary outcome measures were duration in the central zone, and rearing. Total distance travelled was used as a measure of general locomotor activity, and was compared to a range derived from previously published studies of 6000-8000 cm. The distance travelled by naïve animals was used as an internal control, allowing identification of data that may be compromised, either due to environmental conditions, or issues with the animals themselves. Rearing was defined as both forelimbs elevated, either against a wall, or freestanding, and measured under blinded conditions by a trained observer watching at 4 x playback speed. Animals were excluded if the environmental conditions were disrupted during testing (e.g. noise).

In study 1, investigating the effects of repeated open field exposure, StopWatch+ software (Georgia State University, USA; http://www.cbn-atl.org/research/stopwatch.shtml) was used to collect rearing behaviour data, allowing differentiation of free-standing and wall-supported rearing, in addition to recording duration and latency behaviour. Furthermore, mean velocity and latency to

enter the inner zone were also analysed using Ethovision, to enhance characterization of the response to repeated open field exposure.

Study 2: Burrowing

Burrowing activity was measured using a protocol developed within the IMI Europain work package 2 burrowing sub group (IMI Europain, 2012; Rutten *et al.*, 2014b), and cross-validated across 10 separate laboratories covering five countries, with both academic and pharmaceutical industry participants. The protocol involves training, baseline, and testing phases. During training, animals were allowed to acclimatize to the testing room before being transferred, in pairs, to empty, tissue paper-lined burrowing cages. After 30 minutes habituation, a burrowing tube (grey plastic, 320mm x 100mm diameter) containing 2.5kg clean, dry pea shingle gravel (2-4mm diameter) was introduced to each cage, and left in situ for 60 minutes, after which it was removed and weight displaced noted. If the pair did not burrow, one member of the pair was swapped an individual rat from a known burrowing pair during subsequent habituation sessions as required. This was based on the idea that a non-burrower may be encouraged to burrow in the presence of a burrower. Baseline and testing involved a similar protocol as habituation, but animals were singly placed into the burrowing cages. After three baseline sessions, animals were randomized into three groups using a balanced design to ensure consistent baseline burrowing activity between groups. Burrowing activity was tested on days 1, 2, 3, and 7 following acute bladder inflammation, and weight displaced recorded. Following each trial, the gravel was rinsed under clean tap water and allowed to air-dry overnight. At the end of the study, gravel was sterilized before storage.

Animals were excluded from final analysis if their mean baseline weight displaced was less than 500 g.

Study 2: Cytokine Activation

Increased production of inflammatory mediators have been shown in models of experimental pain (Cunha *et al.*, 2008; Dawes *et al.*, 2011; de Oliveira *et al.*, 2014). Collaboration with John Dawes and co-workers at Kings College, London, enabled the inclusion of a qRT-PCR Array study, looking at changes in mRNA levels of 92 different inflammatory mediators (compared to four house-keeping genes). Cytokines were categorized into cytokines, chemokines, growth factors, enzymes, and 'other' (Table 3-8).

Tissue from whole bladders, which were snap frozen during the saline phase of perfusion, was used for RNA extraction. Samples were homogenized while still frozen, and total RNA obtained using "hybrid" method of phenol extraction (Trizol, Invitrogen, USA) and column purification (RNeasy, Qiagen, USA). All samples were deoxyribonuclease (DNase, Qiagen, USA) treated to avoid genomic contamination, and purity and integrity confirmed with an RNA 6000 Nano Chip (Agilent, USA).

Complementary DNA (cDNA) was synthesized from RNA with the SuperScript II reverse transcriptase kit (Invitrogen, USA). Each cDNA sample was diluted with PCR-grade water and added in a 1:1 ratio to TaqMan® Universal master mix to produce a final concentration of 1 ng/µl cDNA. Samples were loaded into the appropriate ports (1 µl/well) according to manufacturer's guidelines. Cards were placed into a 7900HT Fast Real-Time PCR system (Applied Biosystems, USA), and samples subjected to 40 cycles of amplification. Expression of each transcript was measured with the $\Delta\Delta C_t$ (cycling time) method normalised to the geometric mean of the four housekeeping genes using the R package NormqPCR (Perkins *et al.*, 2012). Relative changes in transcript levels are presented as fold change (FC). When transcript numbers were undetermined for a given detector in <50% of samples, the average C_t value was calculated with the remaining data values. If transcript numbers were undetermined in >50% of transcripts for a given sample, a default C_t of 38 was assigned. If these conditions coincided, no FC value was calculated. Significant differences in gene expression were detected using significance analysis of microarray (SAM) technique, involving calculation of false discovery rates (FDR), represented as a *q* statistic describing the % probability of the result observed being a false positive (Xiao *et al.*, 2002; Lin *et al.*, 2008).

Table 3-8: Cytokine qRT-PCR Array Card Targets

Chemokines	Cytokines	Growth Factors	Enzymes	Complement System	Other	Housekeeping Genes
CCL1	CSF1 / MCSF	AREG	ALOX5	C3	AIF1	18S
CCL2	CSF2 / GMCSF	ARTN	ALOX15	C5	EDN1	ACTB
CCL3	CSF3 / GCSF	BDNF	LTA4H		PROK2	GAPDH
CCL4	IFNG	BTC	NOS2			HPRT1
CCL5 / RANTES	IL-1α	EREG	PTGES			
CCL6	IL-1β	FGF7	PTGS2			
CCL7	IL-2	HBEGF				
CCL9	IL-3	KITLG				
CCL11	IL-4	NGF				
CCL12	IL-5	NRG1				
CCL17	IL-6	NRG1				
CCL19	IL-7	(EXON				
CCL20	IL-9	J)				
CCL21B	IL-10					
CCL22	IL-11					
CCL24	IL-12A					
CCL25	IL-12B					
CCL26	IL-13					
CCL27	IL-14 / TXLNA					
CCL28	IL-15					
CX3CL1	IL-16					
CXCL1	IL-17 / CTLA-8					
CXCL2	IL-18					
CXCL3	IL-19					
CXCL4 / PF4	IL-20					
CXCL5 / CXCL6	IL-21					
CXCL7 / PPBP	IL-23A					
CXCL9	IL-24					
CXCL10	IL-27					
CXCL11	IL-33					
CXCL12	IL-35 / EBI3					
CXCL13	LIF					
CXCL14	MIF					
CXCL16	TNF					
CXCL17 XCL1						

Study 2: Amygdala Activation

Animals were humanely killed with 0.65ml pentobarbitone sodium given via i.p injection (20% w/v Solution for Injection, Pentoject, AnimalCare Ltd, UK) and transcardially perfused with 0.9% heparinized saline followed by 4% paraformaldehyde, both at 4°C. The brain was removed and postfixed in 4% paraformaldehyde for 6 hours and cryoprotected in 30% sucrose for at least 3 days prior to sectioning (50 μm) on a freezing microtome (model no. HM450, Thermo Scientific, USA). Staining of sections took place over several consecutive weeks due to capacity constraints. 12 sets of sections were processed during each batch. Sections were washed in phosphate buffered saline (PBS; 1($NaH_2PO_4.2H_2O$):9($Na_2HPO_4.12H_2O$):7(NaCl) in dH_2O), quenched with 0.03% H_2O_2 (Sigma-Aldrich, UK), washed again in PBS, blocked for one hour in 5% normal goat serum (NGS, Millipore, UK; PBS with 0.3% TX (Triton X-100, BDH, UK)), before incubation overnight at 4°C with 1:20,000 polyclonal rabbit IgG anti-c-Fos (sc-52, Santa Cruz, USA; 0.3% PBS-TX, 2% NGS). The following day, sections were washed in PBS and incubated for two hours at room temperature with 1:250 Biotin-SP-conjugated Affinipure goat anti-rabbit IgG (F(ab')2 fragment specific; Jackson, USA; 0.3% PBS-TX, 2% NGS). Staining was visualised using a Vectastain ABC kit (avidin-biotin-peroxidase complex, VectorLabs, UK) and DAB (3,3'-Diaminobenzidine) with nickel salt intensification (VectorLabs, UK). Sections were mounted, counterstained with toluidine blue to enhance cyto-architecture, and cover-slipped using DePex mounting media (VWR, UK) prior to image capture. The bregma position of each mounted section was determined with reference to Paxinos & Watson Rat Brain Atlas (Paxinos & Watson, 2006), and only those between -1.44mm and 3.36mm (range of the central amygdala) were captured using a Leica DM R light microscope (Leica Microsystems, Germany). Image analysis was conducted using Photoshop CS5 (Adobe, USA), and the mean number of positively stained cells per mm^2 calculated for each subdivision of the central amygdala (medial, lateral, and capsular), with lateralization and rostro-caudal axis position noted. A positively stained cell was defined as having clear round dark nuclei (blue/black) - sections without cFos positive cells in areas out with the central amygdala were excluded. Staining areas outside distinct round puncta with a strong blue-black colour were ignored. Image analysis was conducted blind to intervention. Animals were excluded if the open field-perfusion interval exceeded 120 minutes. Sections were excluded from analysis if the area containing the amygdala was bilaterally damaged, or if there were no c-Fos positive cells present in the section.

Study 3: Evoked Measures of Hypersensitivity

To detect signs of neuropathy associated with anti-retroviral treatment, evoked responses to mechanical, thermal, and cold stimuli were assessed. These measures are thought to correlate with qualitative sensory testing procedures used to assess neuropathic and chronic pain conditions in the

clinical setting. Testing was conducted in the following order: Mechanical, thermal, cold, and sites selected as shown in Figure 1-4, and Figure 1-5.

Mechanical Sensitivity

Hind-paw withdrawal threshold in response to punctuate static mechanical stimulation was measured with an electronic von Frey device of 0.5 mm^2 probe tip area (SENSEBox System, Somedic Sales AB, Sweden). Before each habituation or testing session, animals were allowed to acclimatise to the testing room for 20 minutes before being transferred individually to Perspex testing chambers (20 x 20 x 14 cm). Rats were allowed to acclimatize until exploratory behaviour ceased. During two habituation sessions, animals were left undisturbed in the chamber for 50min. During the two baseline sessions that followed, and all subsequent testing sessions, the probe tip was used to apply force to the mid-plantar hind paw, which increased gradually at a rate of 8–15 g/s until the paw was withdrawn. The application was performed 5 times with a minimum interval of 1 min between applications. The hind paw reflex thresholds were determined at days four, seven, ten, and thirteen after initial d4T injection for inclusion and exclusion purposes. We only measured hypersensitivity in the hind paws as anti-retroviral-induced neuropathy predominantly affects the feet and is less frequent in the hands due to increased vulnerability with increasing nerve length.

Thermal Sensitivity

Thermal sensitivity was measured using the Hargreaves Test (Hargreaves *et al.*, 1988). The apparatus was calibrated using a radiometer before first use to ensure consistent IR emissions. Before each habituation or testing session, animals were allowed to acclimatise to the testing room for 20 minutes before being transferred individually to Perspex testing chambers (20 x20 x14 cm) placed on the Hargreaves apparatus (Ugo Basile, Italy). Rats were allowed to acclimatize until exploratory behaviour ceased. During two habituation sessions, animals were left undisturbed in the chamber for 50min. During the two baseline sessions that followed, and all subsequent testing sessions, thermal stimuli were directed at the mid-plantar hind paw and latency to withdraw recorded. To avoid thermal damage, the heat source had an automatic cut-off of 20 seconds. The application was performed 5 times with a minimum interval of 1 min between applications. The hind paw thermal sensitivity thresholds were determined at days four, seven, ten, and thirteen after initial d4T injection for inclusion and exclusion purposes.

Cold Sensitivity

The acetone drop test was used to assess cold hypersensitivity. Before each habituation or testing session, animals were allowed to acclimatise to the testing room for 20 minutes before being transferred individually to Perspex testing chambers (20 x 20 x14 cm) placed on plastic mesh. Rats

were allowed to acclimatize until exploratory behaviour ceased. During two habituation sessions, animals were undisturbed in the chamber for 50min. During the two baseline sessions that followed, and all subsequent testing sessions, a syringe was used to apply a drop of acetone to the mid-plantar hind paw and latency to withdraw recorded. The application was performed 5 times with a minimum interval of 1 min between applications. The hind paw cold sensitivity thresholds were determined at four, seven, ten, and thirteen days after initial d4T injection for inclusion and exclusion purposes.

3.7 Statistical Analysis

Sample size calculations were performed to determine group sizes in all studies. For the bladder inflammation studies, data from previous studies involving male SNT animals (n=8, open field and c-Fos), and CFA inflammation (n=12, burrowing) was used; for the d4T study, data from previous d4T studies using male animals was used (n=12); for the naïve behavioural characterization study, pooled naïve data from previous studies was examined and sample size calculations designed to detect difference in behavioural phenotype based on frequency of entry to the inner zone were conducted using this pooled naïve data (n=26).

Normally distributed, continuous outcome measures was analysed using 1-way ANOVA, with Kruskal-Wallis ANOVA on ranks utilised for data that was not normally distributed or non-continuous (e.g. activity count in the open field). 2-way ANOVA was used to investigate the interaction between group and hemisphere, rostro-caudal axis position, and nuclear subdivisions of the central amygdala (CeA), and also to investigate the effect of behavioural phenotype. 2-way repeated measure (RM) ANOVA was additionally used to study the difference across time and between behavioural phenotypes in the naïve open field study. Where overall ANOVA detected significant differences, multiple comparison procedures were used - Holm-Sidak for normally distributed data and Dunn's multiple comparison for non-parametric tests. Correlations between behavioural and immunohistological outcomes were assessed using Pearson correlation coefficients. Inflammatory mediator analysis was conducted using Significance Analysis of Microarrays (SAM), and the false discovery method (FDR), which take into account dependence between transcripts and uses nonparametric techniques. Significance was taken as q=0%.

Exploratory secondary analysis to look for thigmotactic phenotype based on open field was also conducted during the bladder inflammation study. Animals were classified as "Low Thigmotaxis" if they entered the central zone more than the pooled median or "High Thigmotaxis" if they were below the pooled median. Pooled medians were calculated separately for acute and repeated

studies. 2-way ANOVA was used to investigate the effect of responder phenotype on other behavioural outcomes, and cFos immunoreactivity in the central amygdala.

When examining responder phenotype in the naïve repeated exposure study, a further level of differentiation was required, to take into account both behavioural phenotype, and changes over time. Therefore, the pattern of high or low phenotype across the three sessions was assessed, and animals categorised further into one of 4 categories, as shown in Table 3-9.

Table 3-9: Behavioural Phenotypes defined by thigmotactic activity, showing the four different behavioural phenotypes involved and the progression of increasing levels of thigmotaxis

Week of testing	1	2	3
Low Thigmotaxis	Low	Low	Low
Slow Thigmotaxis	Low	Low	High
Rapid Thigmotaxis	Low	High	High
High Thigmotaxis	High	High	High

Summary statistics are expressed as mean (95% CI) when data was normally distributed, or median (Interquartile Range: IQR) when data failed normality testing. Significance was taken at $p<0.05$. Biological limitations were taken into account when expressing data e.g. cell density c-Fos data given to one significant figure.

Graphical representation of data using box and dot plots involved display of both median and mean (black) lines, a box covering the IQR (25-75%), and whiskers indicating the extent of the 95% CI.

All statistical tests were performed, sample sizes calculated, and appropriate graphs generated using OriginPro v9.1 (OriginLab, USA) and SigmaPlot v10 (Systat Software Inc., USA).

4 Results - Study 1: Repeated Open Field Exposure in Naïve Rats

The aim of this study was to characterise naïve behaviour in the open field to enable better interpretation of study data, and gain understanding into how rat behaviour in the open field changes with repeated exposure. This will allow improved design of experiments utilising the open field paradigm by enabling baseline testing so changes in thigmotaxis can be related to trait behavioural phenotype. Naïve male Wistar rats were used as male rats are most commonly used in research, and were repeatedly exposed to the paradigm over three weeks. In addition to the primary (frequency of entry) and secondary (duration in inner zone, latency to enter inner zone, rearing, velocity, and distance travelled) parameters, weight and faecal count during open field were also monitored.

Exclusions

No animals were excluded from this study. The total number of animals studied was 26.

All animals gained weight over the course of the three-week experiment, from 235.2g (8.84) during week one, to 285.0g (16.71) in week 2, and 326.91g (17.28) in the final week (1-way RM ANOVA $p<0.001$). There was no difference in faecal count during the open field paradigm (1-way RM ANOVA $p=0.314$).

4.1 Open Field

Thigmotaxis

Activity in the inner zone of the open field decreased over time. Frequency of entry decreased from 4 (1-7) in week one to 1 (0-3) in week three (1-way RM ANOVA $p=0.022$). There was no difference between week two activity (1.5 (6-0)), and either week one or week three activity. There was no significant difference in duration in the inner zone ($p=0.88$), with durations of 6.48s (3.58-9.39), 4.65s (2.59-6.70), and 3.66s (1.01-6.32) recorded for weeks one, two, and three respectively (Figure 4-1).

Rearing

In contrast with studies 2 (bladder inflammation) and 3 (d4T peripheral neuropathy), rearing analysis for this study was done using StopWatch+ software, enabling free-standing and wall-supported rearing to be distinguished, in addition to recording duration and latency of rearing activity.

2-way RM ANOVA was performed to compare wall-supported with free-standing rearing across time.

During the first exposure to the open field, animals made 52.5 (39-70) wall-supported (WS) rears, and 20 (13-28) free-standing (FS) rears. This decreased significantly to 35 (21-47) WS and 8 (4-16) FS during the second exposure ($p<0.001$), and even further to 22.5 (13-38) WS and 8 (3-16) FS during

week 3 ($p<0.001$ WS). There was also a significant difference between weeks 2 and 3 in WS rear frequency ($p=0.017$), which was not seen in FS rears. There were significantly more WS rears than FS ($p<0.001$).

Duration spent engaging in wall-supported rearing activity also decreased over time. WS rears dropped from 34.28s (29.46-39.10) in week 1 to 24.81s (19.42-30.21) during week 2, and even further to 19.08 (13.93-24.22) in week 3 ($p<0.001$). FS rearing duration was significantly lower than WS ($p<0.001$) but showed no difference over time. No difference between weeks 2 and 3 was seen in either form of rearing behaviour (Figure 4-1).

Latency to perform WS rearing was not significantly changed over time ($p=0.341$), but in FS there was a significant increase from 28.75s (22.88-34.62) during week 1, to 118.56s (70.09-167.03) in week 2 ($p<0.001$), and 94.68s (39.46-149.91) in week 3 ($p=0.008$, Figure 4-2).

There was no effect of time on average rear duration, but there was a significant difference between WS and FS during week 3, with a significant decrease in duration of FS rears ($p=0.001$).

Locomotor Activity

Both total distance travelled, and mean velocity decreased over time. Distance travelled decreased significantly from 5802cm (5117.95-6487.58) during week 1, to 4410.25cm (3615.81-5204.69) in week 2, and 3188.59cm (2458.77-3918.41) in week 3 (1-way RM ANOVA $p<0.001$). Mean velocity also showed a significant difference, decreasing from 6.45cm/s (5.69-7.21) in week 1, to 4.94cm/s (4.07-5.82) in week 2, and 3.54cm/s (2.73-4.35) in week 3 (1-way RM ANOVA $p<0.001$).

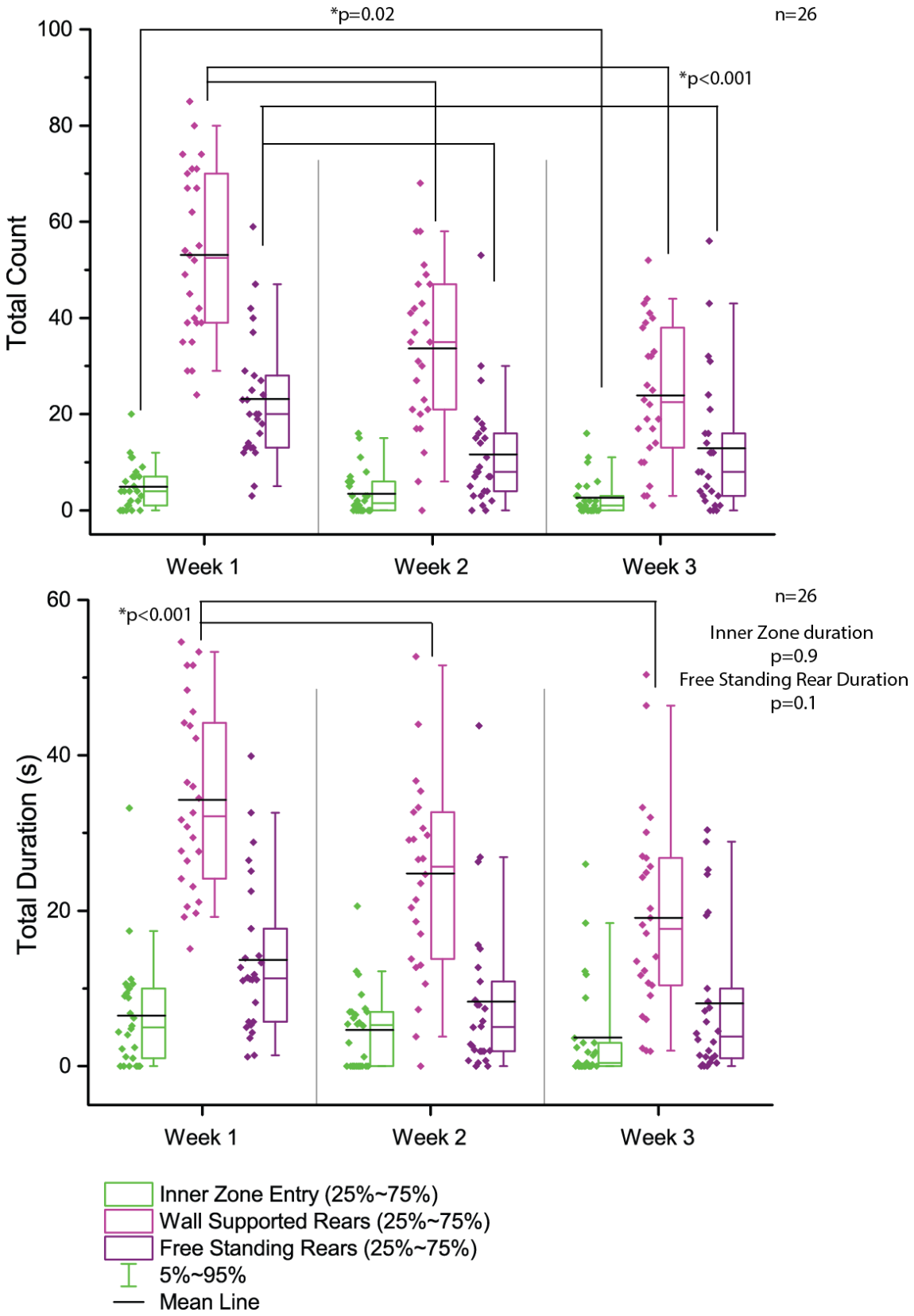


Figure 4-1: Frequency and duration of inner zone and rearing activity in the open field following repeated open field exposure Activity significantly reduced over time, with decreases noted in frequency of inner zone activity and rearing, and a decrease in time spent engaging in wall-supported rearing behaviour All outcomes tested using 1-way repeated measures ANOVA

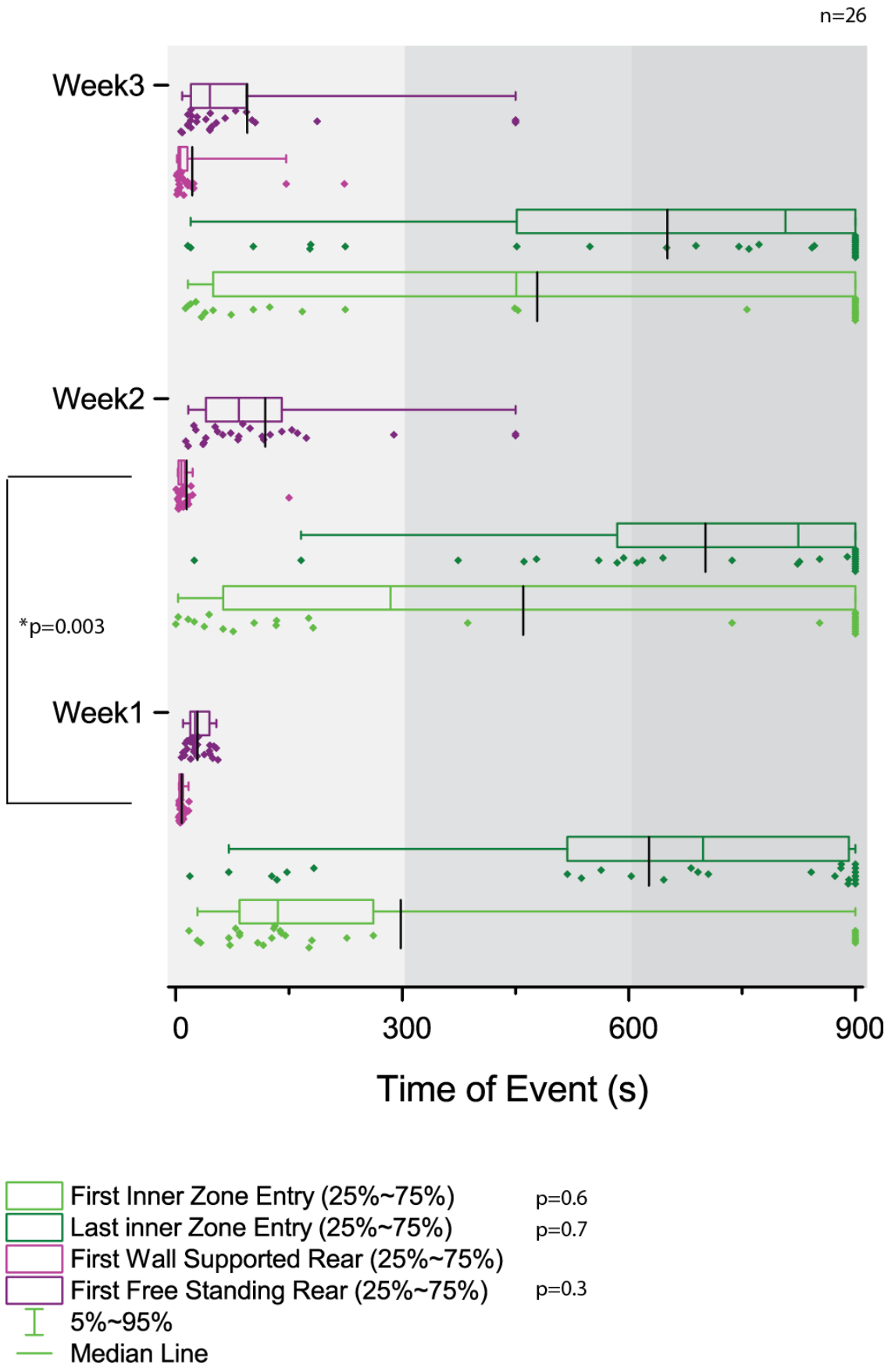


Figure 4-2: Latency to enter in the inner zone and rearing behaviour following repeated open field exposure
Latency to exhibit free-standing rearing increased between weeks 1 and 2. There were no other significant differences. All outcomes tested using 1-way repeated measures ANOVA

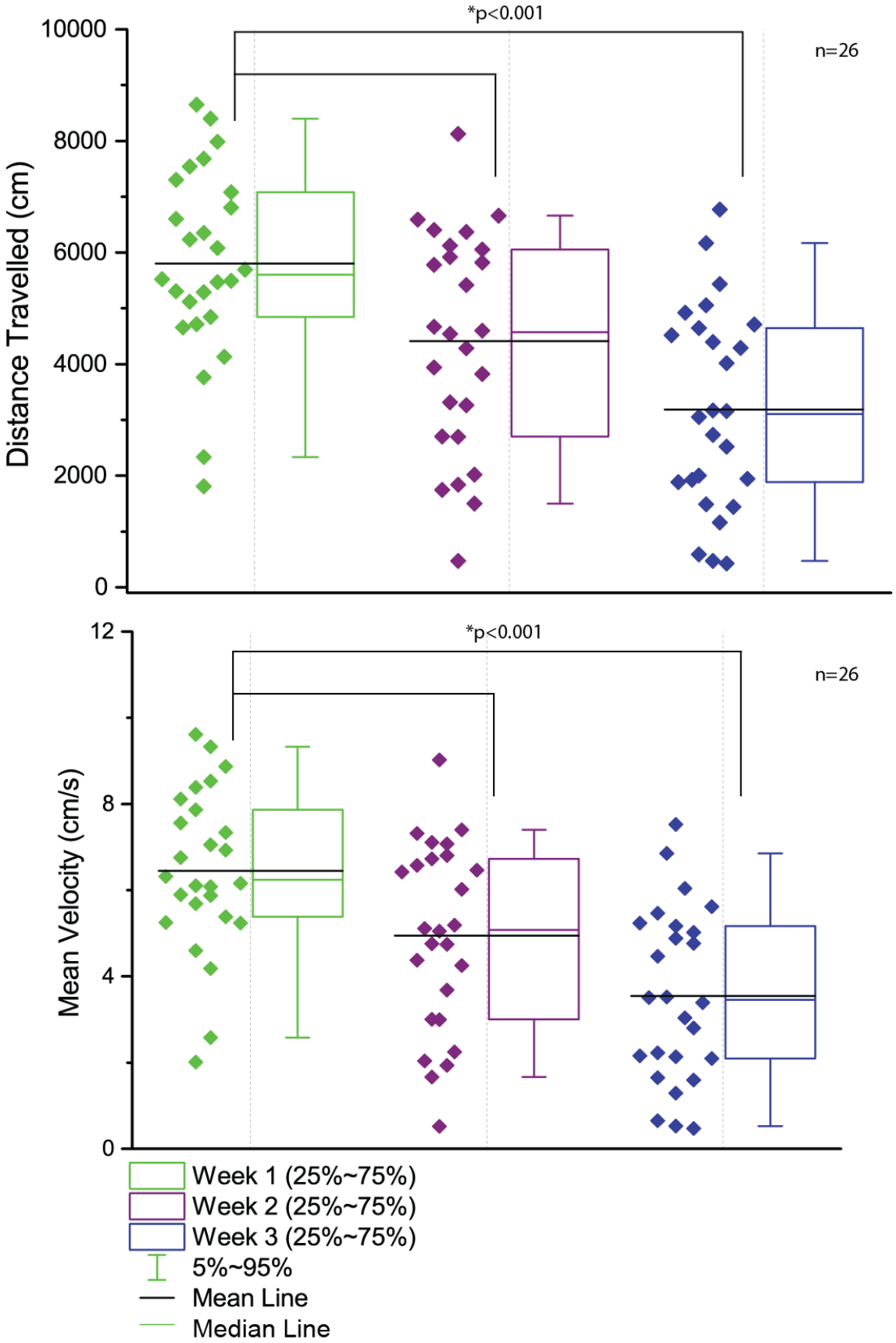


Figure 4-3: Distance travelled and velocity in the open field following repeated exposure Significant decreases were observed in both outcome measures. All outcomes tested using 1-way repeated measures ANOVA

Responder Analysis

The sample size of this study was calculated to take into account the potential for different behavioural phenotypes present in the naïve population (n=26). Based on frequency of entry to the inner zone, the animals were classified as low (>2) or high (≤2) thigmotaxis. The pattern of high or low phenotype across the three sessions was assessed, and animals categorised further into one of 4 categories, as shown in Table 3-9. 3 animals showed low thigmotaxis, 8 showed a slow thigmotactic phenotype, 10 a rapid thigmotactic phenotype, and 5 showing consistently high levels of thigmotaxis across the three weeks of testing.

Behavioural outcomes were then analysed using 2-way RM ANOVA, with week of exposure, and the thigmotactic phenotypes as factors.

Frequency of inner zone entry was significantly different between low (11 (6-16)) and high (0 (0-1)), slow (3 (1-6.5)), and rapid (1.5 (0-5)) thigmotactic groups, and between high and slow thigmotactic groups ($p<0.001$). Within groups, there was an effect of week, with low and both rapid and high thigmotactic animals showing significantly different frequency of entry each week tested ($p=0.003$). High and slow thigmotactic animals were only significantly different during week 1 ($p=0.02$), and there were differences between low and slow thigmotaxis groups during week 1 ($p=0.007$) and 3 ($p=0.001$, Figure 4-4).

Duration spent in the inner zone was significantly different between the low thigmotaxis (14.73s (7.23-22.23)), and slow (4.87s (3.12-6.61)), rapid (3.97s (2.00-5.94)), and high (1.08s (0.03-2.13)) thigmotaxis groups. No effect of week was detected.

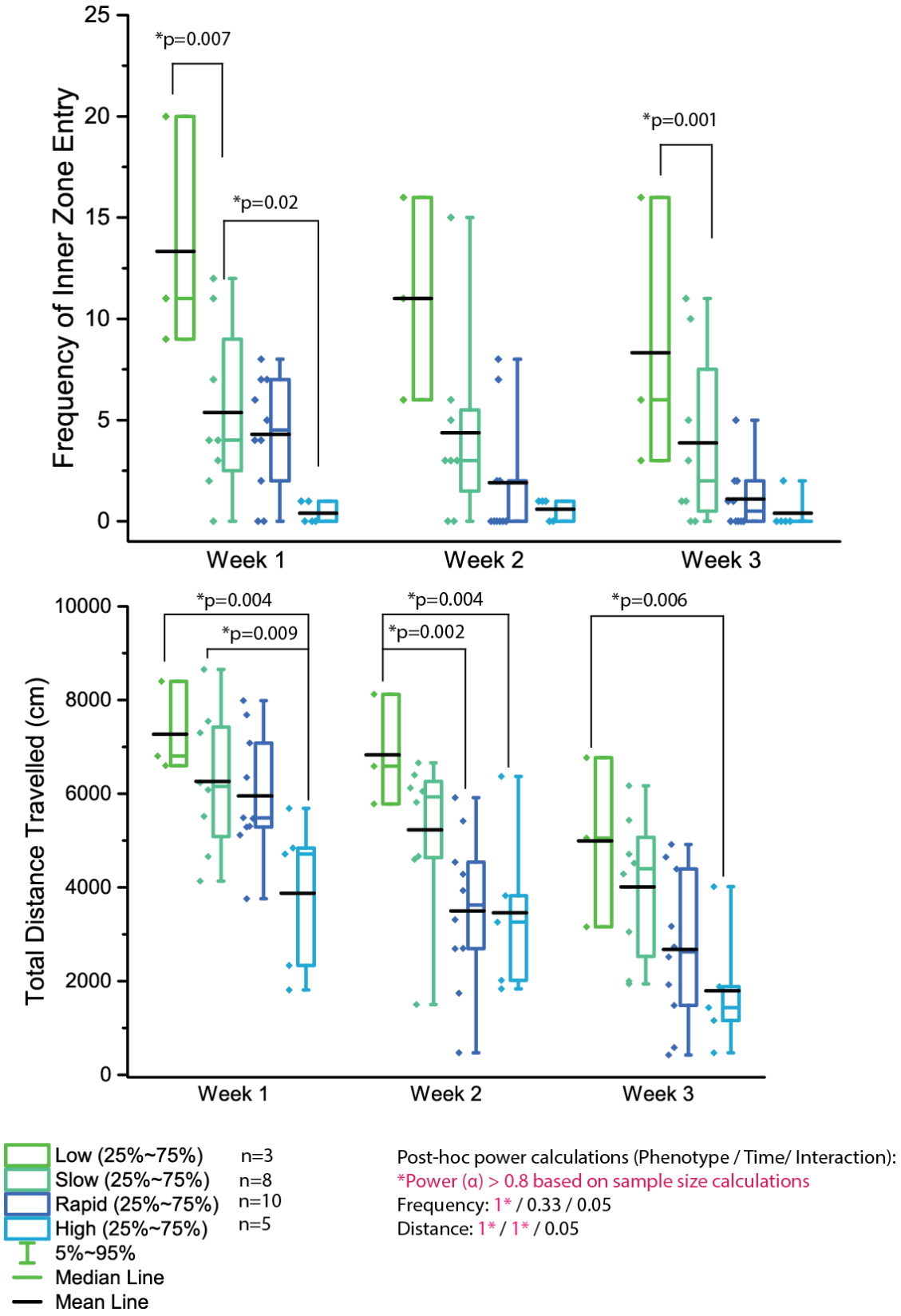


Figure 4-4: Frequency in inner zone and total distance travelled in the open field by thigmotactic phenotype There were significant differences between responder groups at all three time points. All outcomes tested using 2-way repeated measures ANOVA

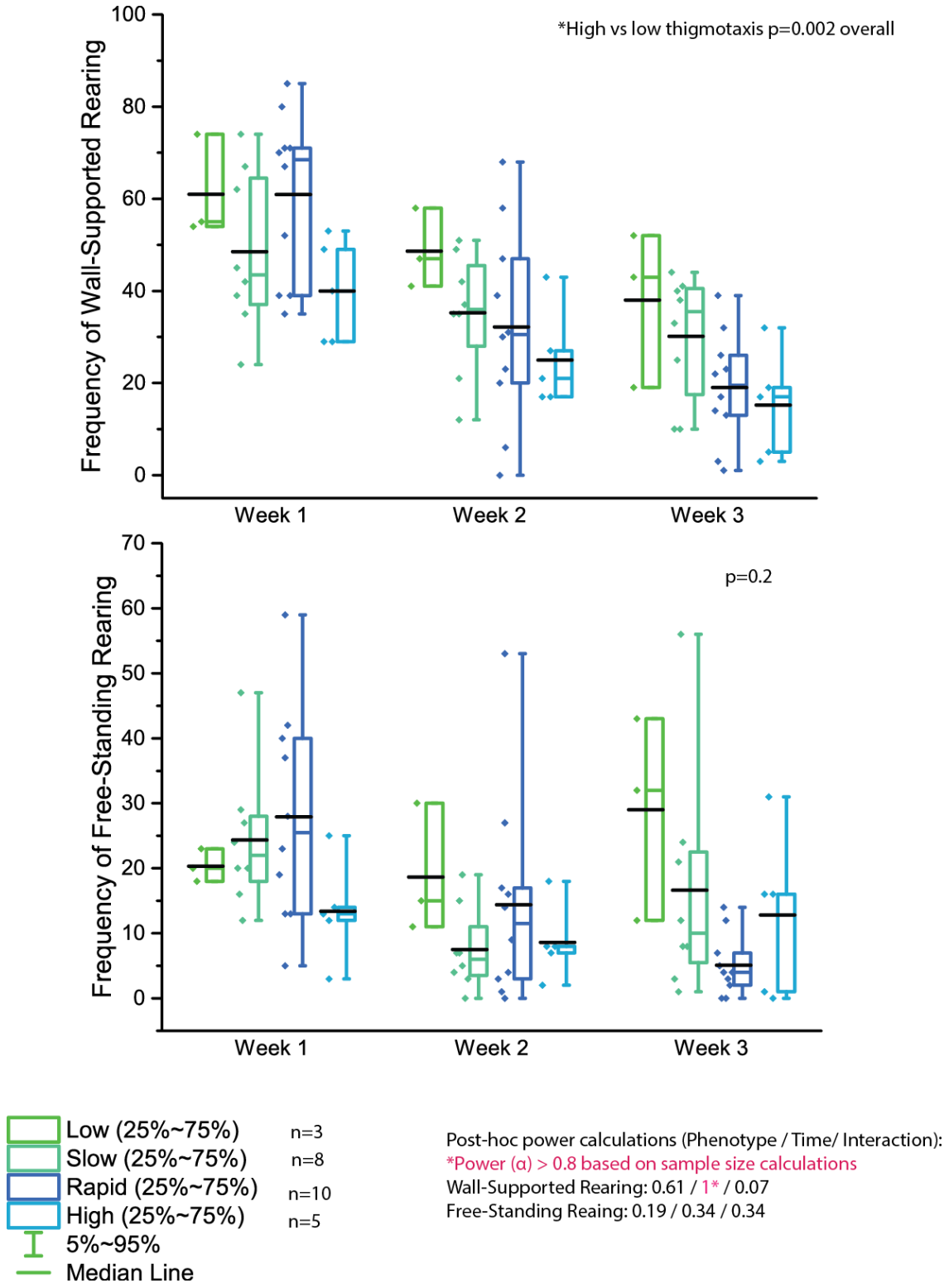


Figure 4-5: Frequency of wall-supported and free-standing rearing behaviour in the repeated open field by thigmotactic phenotype. All outcomes tested using 2-way repeated measures ANOVA

Wall-supported but not free-standing rear frequency showed significant differences

Behavioural phenotype contributed significantly to frequency of wall-supported rearing (p=0.02), with multiple comparison procedure indicating a significant difference between high and low thigmotactic groups (p=0.003). Differences between week 1 and subsequent weeks were seen in both high thigmotaxis (vs. week 3; p=0.01) and rapid thigmotaxis groups (vs. week 2 and 3; p<0.001).

A similar effect was seen in duration of wall-supported rears ($p=0.04$), again with strongest effect between high and low thigmotaxis groups ($p=0.007$), and an effect of week observable in the rapid thigmotaxis groups (vs. 2: $p=0.002$; vs. 3: $p<0.001$). Free-standing rearing only showed an effect of behavioural phenotype on latency to rear in the slow thigmotaxis group ($p=0.009$). No effect was seen on average rear duration for either free standing or wall-supported rears, or on frequency and duration of free-standing rears (Figure 4-5).

Distance travelled revealed significant effects of group between low (6355.23cm (5148.78-7581.68)) and high (3045.55 (2060.58cm (2060.58-4030.53)), rapid (4046.08cm (3280.51-4811.66)), and slow (5170.37cm (4418.00-5922.74)) thigmotaxis groups ($p<0.001$), and between slow and both rapid ($p=0.01$), and high thigmotaxis ($p<0.001$). There were significant effects seen between weeks in both slow and rapid thigmotactic groups ($p<0.01$ Figure 4-4).

Velocity in the inner zone also showed an effect of behavioural phenotype, with significant difference between high and low thigmotactic animals present overall ($p<0.001$) and at each time point tested ($p=0.004$, $p=007$, and $p=0.006$ respectively. Overall effects were also detected between high and slow thigmotaxis ($p=0.003$, week 1: $p=0.004$), and low and rapid thigmotactic groups ($p=0.0004$, week 2: $p=0.002$, Figure 4-3).

Key findings

- Activity in the open field gradually decreases with repeated exposure
- Four behavioural phenotypes were detected, which take into account both frequency of inner zone entry during each session, and the pattern of behaviour over time – the majority of animals showed high inner zone activity during the first and/or second exposures, with a significant minority exhibiting consistently high or low levels of thigmotaxis across the three trials
- Frequency and duration of inner zone entry, distance travelled, and velocity showed consistent phenotypes
- Thigmotactic outcomes (frequency and duration) showed highest re-test stability between weeks 1 and 2

4.2 Correlations

To gain further understanding of open field behaviour and the relationships between different behaviours, Pearson's correlation coefficients were calculated. As numerous significant correlations were detected, only those equal to or greater than $\rho=0.8$ will be discussed. Full details of

correlations between behavioural outcomes are shown in Table 4-1, with between-week correlation data shown in Table 4-2.

Highlighting the strongest correlations in the overall data, velocity and distance travelled were significantly correlated ($\rho=1$, $p<0.001$), and frequency of inner zone entry was significantly correlated with duration spent in the inner zone ($\rho=0.89$, $p<0.001$). Both distance and velocity were also significantly correlated with the frequency ($\rho=0.85$, $p<0.001$) and duration ($\rho=0.80$, $p<0.001$) of wall-supported rearing. Duration and frequency were significantly correlated with both wall-supported ($\rho=0.91$, $p<0.001$) and free-standing rearing ($\rho=0.87$, $p<0.001$).

Comparing correlations week by week revealed a time-dependent increase in ρ, with 4 significant correlations greater than $\rho=0.8$ observed in data from week 1, compared to 8 in week 3. However, correlations between weeks were poor, showing commonality only with the adjacent time point (i.e. week 1 with 2, and 2 with 3, but not 1 with 3).

Looking at correlations within thigmotactic phenotypes, velocity and distance were strongly correlated in all subgroups($\rho=1$, $p<0.001$). Correlations between distance travelled and frequency and duration of wall-supported rearing were strongest in the rapid ($\rho=0.90$, $p<0.01$), and high ($\rho=0.87$, $p<0.001$) thigmotaxis animals. Low thigmotaxis animals also showed a correlation with frequency of wall-supported rears ($\rho=0.95$, $p<0.001$).

Combining responder type with across week comparison to investigate the relationship between week 1 behaviour and that observed during subsequent weeks revealed differences between the thigmotactic phenotypes as would be expected by their temporal phenotype e.g. those in the rapid thigmotaxis group showed negative correlations between weeks 1 and 2, whereas in the low and slow, there were positive correlations.

Duration in the inner zone was consistently correlated over time, suggesting use as a predictor of behavioural phenotype.

Key findings

- Duration in the inner zone was the only outcome measure consistently correlated with itself over repeated testing
- Velocity and distance travelled was strongly correlated in every phenotype and time point tested
- Frequency and duration measures of the same outcome (e.g. rearing, inner zone entry) show strong correlations
- Repeated exposure to the open field strengthens correlations observed

Table 4-1: Overall Pearson's correlation coefficients between open field behavioural outcomes
*There was a strong relationship between velocity and distance moved. * denotes significant correlations present between parameters*

			Mean Velocity*	2*	3*	4*	5*	6*	7*	8*	9*	10*	11*
2	Total Distance moved*	ρ	1*										
		Sig.	0.00										
3	Frequency of Inner Zone Entry*	ρ	0.64*	0.64*									
		Sig.	0.00	0.00									
4	Duration of Inner Zone Activity*	ρ	0.56*	0.56*	0.89*								
		Sig.	0.00	0.00	0.00								
5	Latency to first enter Inner Zone	ρ	-0.22	-0.21	-0.25	-0.11							
		Sig.	0.12	0.14	0.07	0.44							
6	Latency to last enter Inner Zone*	ρ	0.47*	0.48*	0.64*	0.54*	0.30*						
		Sig.	0.00	0.00	0.00	0.00	0.03						
7	Wall-supported Rear Frequency*	ρ	0.85*	0.85*	0.57*	0.51*	-0.20	0.52*					
		Sig.	0.00	0.00	0.00	0.00	0.16	0.00					
8	Wall-supported Rear Duration*	ρ	0.80*	0.80*	0.51*	0.46*	-0.12	0.53*	0.91*				
		Sig.	0.00	0.00	0.00	0.00	0.41	0.00	0.00				
9	Wall-supported Rear Latency*	ρ	-0.38*	-0.38*	-0.18	-0.17	0.31*	-0.01	-0.38*	-0.36*			
		Sig.	0.00	0.00	0.11	0.13	0.03	0.94	0.00	0.00			
10	Free-standing Rear Frequency*	ρ	0.47*	0.46*	0.45*	0.39*	-0.31*	0.31*	0.57*	0.51*	-0.23*		
		Sig.	0.00	0.00	0.00	0.00	0.03	0.03	0.00	0.00	0.05		
11	Free-standing Rear Duration*	ρ	0.40*	0.38*	0.35*	0.37*	-0.28*	0.17	0.45*	0.43*	-0.17	0.87*	
		Sig.	0.00	0.00	0.00	0.00	0.05	0.23	0.00	0.00	0.13	0.00	
12	Free-standing Rear Latency*	ρ	-0.26*	-0.26*	-0.15	-0.10	0.19	0.10	-0.34*	-0.30*	0.45*	-0.40*	-0.34*
		Sig.	0.02	0.02	0.20	0.39	0.19	0.48	0.00	0.01	0.00	0.00	0.00

Table 4-2: Summary of Pearson's correlation coefficients calculated between weeks
*To investigate predictive value of previous behaviours. * denotes significant correlations present between parameters*

			WEEK 1						WEEK 2					
			Velocity*	Distance*	Inner Zone Freq.*	Inner Zone Duration*	WS Rear Freq.*	FS Freq.						
week 2	WS Rear Frequency*	ρ	0.42*	0.42*	0.28	0.20	0.45*	0.46*						
		Sig.	0.03	0.03	0.16	0.33	0.02	0.02						
	Inner Zone Frequency	ρ	0.34	0.34	0.40*	0.36	0.28	0.12						
		Sig.	0.09	0.09	0.04	0.07	0.17	0.55						
	Inner Zone Duration	ρ	0.39*	0.38*	0.44*	0.41*	0.33	0.11						
		Sig.	0.05	0.05	0.02	0.04	0.10	0.58						
						*			Velocity*	Distance*	Inner Zone Freq.*	Inner Zone Duration*	WS Rear Freq.	FS Rear Freq.
week 3	Inner Zone Frequency*	ρ	0.13	0.13	0.33	0.42*	-0.11	-0.17	0.41*	0.42*	0.74*	0.61*	0.27	-0.08
		Sig.	0.53	0.54	0.10	0.03	0.61	0.41	0.04	0.03	0.00	0.00	0.18	0.71
	Inner Zone Duration*	ρ	0.13	0.13	0.37	0.46*	-0.07	-0.19	0.25	0.26	0.63*	0.54*	0.13	-0.10
		Sig.	0.53	0.54	0.07	0.02	0.75	0.34	0.23	0.21	0.00	0.00	0.54	0.63
	FS Rear Frequency*	ρ	0.03	0.02	0.06	0.06	-0.23	-0.22	0.28	0.29	0.47*	0.40*	0.21	-0.13
		Sig.	0.90	0.92	0.77	0.75	0.25	0.28	0.17	0.15	0.01	0.04	0.30	0.52

5 Results - Study 2: Bladder Inflammation

Acute and repeated turpentine bladder inflammation was induced in female Wistar rats to investigate the behavioural consequences of visceral inflammation.

Bladder inflammation as a model of visceral inflammation was well tolerated; the main cause of mortality was surgical complications relating to technical issues associated with anaesthesia. In the repeated model, all deaths occurred during, or immediately following the final inflammation session. Following recovery from anaesthesia and return to the home cage, animals were alert and outwardly indistinguishable from naïve.

5.1 Inflammatory Mediator mRNA

Intensity of inflammatory response was assayed using whole bladder tissue and a qRT-PCR array featuring 92 inflammatory mediator transcripts and four house-keeping genes.

Acute Bladder Inflammation

Both bladder weight and RNA concentration were increased following turpentine bladder inflammation as shown in Table 5-1.

Table 5-1: Bladder weight and total RNA concentration following a single episode of turpentine bladder inflammation. Mean (IQR)

	Bladder Weight (g)	Total RNA (ng/nl)	Relative to Naive
Naïve	0.13 (0.01-0.25)	368.35 (195.8-540.9)	1
Turpentine	0.28 (0.18-0.35)	615.03 (250.7-979.3)	1.67

A total of 81/92 cytokine transcripts were analysed for magnitude and significance of transcript upregulation following acute bladder inflammation, as measured by fold change (FC) compared to naïve tissue. 11 markers (CCL1, CCL28, CTLA-8, CCXL17, IFNG, IL-2, IL-3, IL-4, IL-9, IL-13, IL-27) were excluded from the acute inflammation model due to high cycle time (>38), indicative of low levels or issues in detection. The top ten up-regulated transcripts were iNOS (159,110.28 FC), PROK2 (11,060.01 FC), CXCl2 (3,679.32 FC), CCL3 (2,541.99 FC), IL-1α (1,827.32 FC), IL-1β (403.78 FC), CSF3 (350.52 FC), CXCL3 (254.33 FC), IL-10 (222.75 FC), and IL-6 (153.21 FC) (see Table 5-2). Figure 5-1 shows the rank FC for all markers analysed. SAM was used to identify 25 mRNAs significantly different in the turpentine group compared to naive (FC >1.5, Δ = 1.61, FDR=0%). Of these, 13 were classified as chemokines (CCL2, CCL3, CCL4, CCL6, CCL7, CCL12, CCL20, CXCL1, CXCL2, CXCL3, CXCL5/6, XCL1), 9 as cytokines (CSF2, CSF3, IL-1α, IL-1β, IL-6, IL-10, IL-24, IL-35/EBI1, TNF), 2 as enzymes (iNOS, COX-2), 2 as growth factors (ARTN, EREG), and 1 as 'other' (PROK2), as seen in Table 5-4.

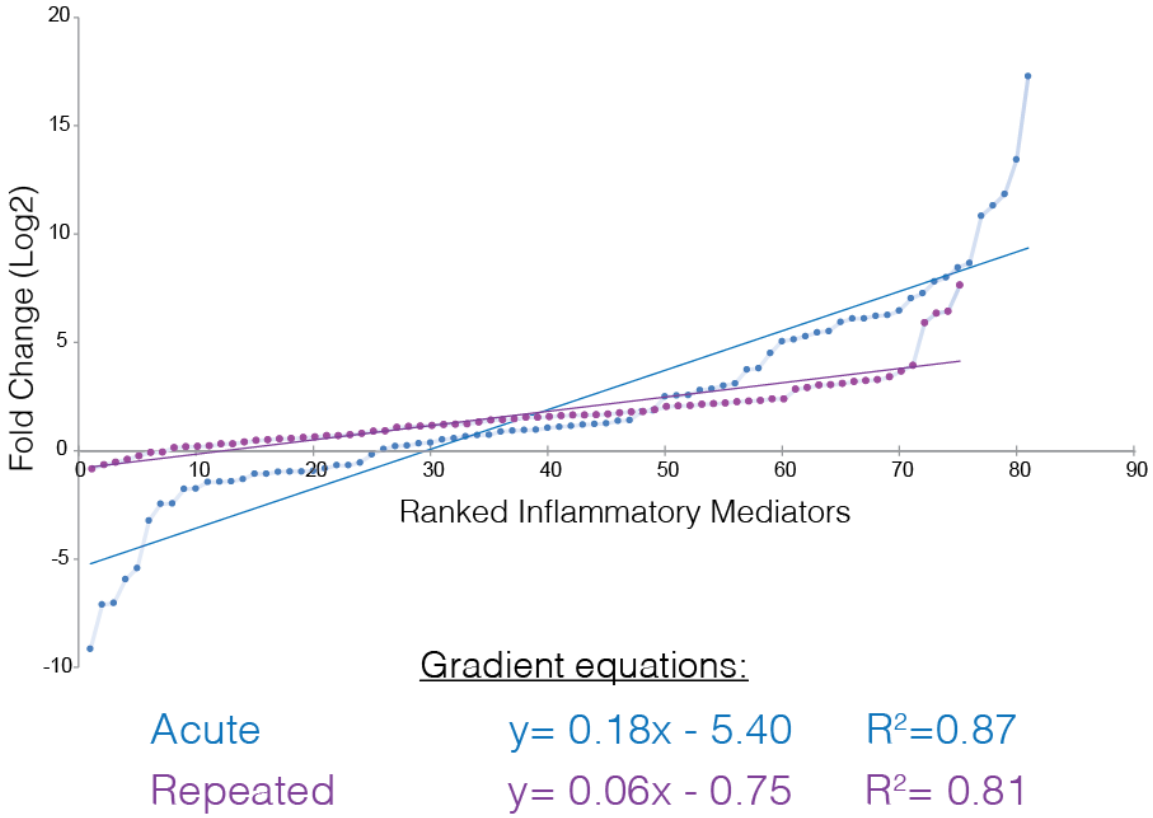


Figure 5-1: Fold change of inflammatory mediators following acute and repeated turpentine bladder inflammation. Markers were ranked by fold change.

Table 5-2: Top 10 up-regulated transcripts in bladder tissue from turpentine group
Ranked by mRNA FC, and normalised to naive values. Data presented as mean FC (95% CI), n=3-4, * Significant difference (p<0.05)

	Acute Bladder Inflammation			Repeated Bladder Inflammation		
Rank	**Gene**	**FC**	**95% CI**	**Gene**	**FC**	**95% CI**
1	NOS2	159110.28	-127366.6-445587.2	CCL12	169.65	67.05-272.25
2	PROK2	11060.01	-12741.5-34861.5	CTLA-8	75.098	-56.21-206.4
3	CXCL2	3679.32	-4618.0-11976.7	CXCL17	71.2	-22.64-165.03
4	CCL3	2541.99	-1417.4-6501.3	CXCL11	53.27	-71.05-177.6
5	IL-1α	1827.32	-2717.6-6372.2	NOS2	14.2	-9.46-37.87
6	IL-1β	403.78	-328.2-1135.7	BDNF	11.9	-2.06-25.86
7	CSF3	350.52	-269.9-971.0	CXCL2	9.94	-9.78-29.65
8	CXCL3	254.33	-131.8-640.5	PROK2	9.12	-10.24-28.49
9	IL-10	222.75	-399.6-845.1	IL-21	8.88	-17.20-34.96
10	IL-6	153.21	-42.8-349.3	IL-1β	8.63*	4.62-12.65

Repeated Bladder Inflammation

Both bladder weight and RNA concentration were increased in the turpentine and instrumentation groups as shown in Table 5-3.

Table 5-3: Bladder weight and RNA concentration in the repeated bladder inflammation model showing mean (IQR)

	Bladder Weight (g)	**[RNA] (ng/nl)**	**RNA relative to Naïve**
Naïve	0.15 (0.07-0.22)	2082.75 (-1164.7-5330.2)	1
Instrumentation	0.30 (0.16-0.44)	3548.5 (-172-7269)	1.70
Turpentine	0.30 (0.16-0.43)	2600.25 (165.1-5035.4)	1.25

A total of 75/92 cytokine transcripts were analysed for magnitude and significance of transcript up-regulation following repeated bladder inflammation. 16 markers (CCL1, CCL3, CCL25, CCL26, CXCL12, IL-2, IL-3, IL-4, IL-4, IL-9, IL-13, IL-19, IL-20, IL-27, C5, and IFNG) were excluded from the repeated inflammation model due to high cycle time (>38). One housekeeping gene (18s) was excluded as it was significantly up-regulated in the turpentine group. FC was therefore calculated normalized to the remaining housekeeping genes (HPRT, ACTB, and GAPDH).

Figure 5-1 shows FC rank for all cytokines analysed in the turpentine groups. The top 10 mRNA transcripts up-regulated following repeated bladder inflammation with turpentine (normalised to naïve data) were CCL12 (169.65 FC), CTLA-8 (75.10 FC), CXCL17 (71.20 FC), CXCL11 (53.27), iNOS (14.2 FC), BDNF (11.9 FC), CXCL2 (9.94 FC), PROK2 (9.12 FC), IL-21 (8.88 FC), and IL-1β (8.63 FC) (see Table 5-2). Using SAM, we identified 4 mRNAs that were significantly different in turpentine compared to naive (FC >1.5, Δ = 1.61, FDR=0%). Of these, 3 were classified as chemokines (CCL7, CCL12, CXCL17), and 1 as cytokine (IL-1β), as seen in Table 5-4.

Analysis of bladders from the repeated instrumentation group also revealed significant up-regulation of CCL12 (108.55 FC), and of PPBP (FC 34.42) compared to naïve animals (FC >1.5, Δ = 1.67, FDR=0%).

Table 5-4: Significance analysis of qRT-PCR array (SAM) results for acute and repeated bladder inflammation
Significant fold difference in acute model[a], repeated model[b], or both[c]; Significance level, FDR q=0%

		Acute			Repeated		
		FC	**Score (d)**	**q-value (%)**	**FC**	**Score (d)**	**q-value (%)**
Chemokines	CCL2[a]	**43.47**	4.63	0	3.36	1.43	8.44
	CCL3[a]	**1656.98**	8.89	0	*transcript excluded*		
	CCL4[a]	**95.66**	2.55	0	6.74	1.13	8.44
	CCL6[a]	14.34	2.52	0	3.98	1.9	5.52
	CCL7[c]	**17.96**	2.37	0	**5.44**	3.47	0
	CCL12[c]	**149.46**	3.7	0	**120.42**	8.28	0
	CCL20[a]	**66.21**	3.69	0	9.71	1.4	8.44
	CXCL1[a]	**218.46**	2.71	0	3.42	1.21	8.44
	CXCL2[a]	**9412.93**	5.23	0	5.63	1.22	8.44
	CXCL3[a]	**75.99**	2.61	0	*transcript excluded*		
	CXCL6[a]	**106.35**	4.74	0	5.24	1.17	8.44
	CXCL17[b]	*transcript excluded*			**34.53**	4.15	0
	XCL1[a]	**0.01**	-4.93 0.00	0	2.38	0.45	15.95
Cytokines	CSF2[a]	**91.78**	5.87	0	2.2	0.57	15.95
	CSF3[a]	1355.83	4.75	0	5.23	0.87	10.67
	IL-1α[a]	**2857.26**	4.63	0	5.46	1.6	5.52
	IL-1β[c]	**272.74**	5.88	0	**7.15**	2.68	0
	IL-6[a]	**522.18**	4.78	0	4.57	1.11	8.44
	IL-10[a]	**334.63**	3.67	0	2.83	0.48	15.95
	IL-24[a]	**233.58**	4.22	0	0.11	-0.98	21.98
	IL-35 / Ebi3[a]	**45.77**	2.46	0	3.15	1.1	8.44
	TNF[a]	**20.44**	3.2	0	2.42	1.13	8.44
Enzymes	NOS2[a]	**81737.07**	11.43	0	13.87	1.98	5.52
	COX-2 / PTGS2[a]	**8.42**	2.66	0	0.96	-0.07	21.98
Growth Factors	ARTN[a]	**0.37**	-2.46	2.26	0.39	-1.61	20.25
	EREG[a]	**140.2**	3.15	0	24.38	1.91	5.52
Other	PROK2[a]	**4443.04**	7.25	0	8.52	1.34	8.44

Comparison of Inflammatory mediator up-regulation in Acute and Repeated Bladder Inflammation

From a possible list of 92 transcripts, 73 were detected in both models. Table 5-5 shows the mean rank, standard deviation, and super rank value (SRV) for the top ten transcripts. Of these IL-1β was significantly up-regulated in both (FDR q=0%). Two other chemokines, CCL7 (acute: 272.74 FC; repeated: 7.15 FC), and CCL12 (acute: 17.96 FC; repeated: 7.15 FC), were also significantly upregulated in both models (FDR q=0%).

Table 5-5: Super rank values (SRV) for cytokines in bladder tissue up-regulated following acute and repeated bladder inflammation

SRV	Gene	Mean Rank	SD
1	NOS2	3	1.41
2	CXCL2	5	0.71
3	PROK2	5	7.07
4	IL-1β	8	2.83
5	CCL12	9.5	4.24
6	IL-1α	13.5	7.07
7	BDNF	15	8.49
8	C3	18.5	4.95
9	IL-35 / EBI3	19	4.95
10	EREG	19.5	10.6

Comparison of Repeated Instrumentation and Turpentine

CCL12 was significantly up-regulated in both turpentine and instrumentation groups following repeated bladder inflammation. PPBP was up-regulated in instrumentation but not turpentine groups.

Key findings

- Both acute and repeated visceral inflammation increase levels of 4 inflammatory mediators
- Number of inflammatory mediators up-regulated by repeated inflammation is lower than that seen in acute inflammation, suggesting habituation to the stimuli
- CCL12 is also up-regulated in the instrumentation group, suggesting involvement in a general inflammatory response, rather than irritant-specific (i.e. turpentine)

5.2 Open Field Behaviour

Thigmotaxis in the open field has been observed in traumatic and HIV/ART-associated models of neuropathic pain (Hasnie *et al.*, 2007; Wallace *et al.*, 2007a; Huang *et al.*, 2013), but as yet not in models of visceral inflammation. Therefore, open field behaviour was assessed 24 hours after either a single (acute), or the third (repeated) session of bladder inflammation. Behaviour was recorded for 15 minutes.

Acute Bladder Inflammation

Animals in both the instrumentation (7, 0-12, p=0.004), and the turpentine (11, 1-16, p=0.03) groups entered the inner zone significantly less frequently than naïve (10, 2-27) (Kruskal-Wallis overall 1way ANOVA, p=0.045, Figure 5-2).

Duration in the inner zone was not significantly different between groups (Kruskal-Wallis 1-way ANOVA, p=0.216). The time spent in the inner zone was 11.52 s (6.88-26.88), 6.8 s (2.88-12.96), and 8.64 s (2.72-13.92) for naïve, instrumentation, and turpentine groups respectively (Figure 5-2).

Rearing behaviour was not significantly different between groups either (Kruskal-Wallis 1-way ANOVA, p=0.179), with median rear counts of 58 (40-61), 45 (32-55), and 44 (26-50) for naïve, instrumentation, and turpentine groups respectively (Figure 5-3).

In the turpentine group, distance travelled significantly reduced from 8964.82 cm (8153.48-9776.16) in naïve animals to 6204.49cm (4513.71-7895.26) in turpentine (p=0.0018). No difference was observed between instrumentation (7038.95 cm (6011.39-8066.50) and naïve (p=0.1029) or turpentine (overall 1-way ANOVA p=0.0045, Figure 5-3).

Repeated Bladder Inflammation

No significant differences in inner zone activity (frequency or duration) were seen between any groups, with naïve animals entering the inner zone 12 (8-14) times for 13.76s (9.12-24), instrumentation entering 7.5 (5-14) times with a duration of 14.48 (6.56-27.2), and turpentine animals entering the inner zone 8.5 (1.5-10) times with a median duration of 6.96s (1.76-16.72) (Kruskal-Wallis 1-way ANOVA p=0.39, and p=0.348; Figure 5-4). No difference was seen in rearing activity (p=0.241), with median rear counts of 58 (49-71), 48 (44-63.5), and 53.5 (45-56) for naïve, instrumentation, and turpentine groups respectively (Figure 5-4).

A decrease in distance travelled was seen in both instrumentation (p=0.016, 6797.29 cm (5626.187968.39)) and turpentine (p=0.00091, 5974.04 cm (4678.10-726.99)) groups compared to naïve (8432.59 cm (7904.75-8960.42); overall 1-way ANOVA p=0.002, Figure 5-5).

To control for the effects of repeated anaesthesia, an additional control group was introduced for the repeated model. These “anaesthesia only” (AO) animals were not significantly different compared to control, with an average distance travelled of 8316.25cm (95% CI: 7852.49-8780.01), 13 inner zone entries (IQR: 8-18), and a median duration in the inner zone of 14.24s (IQR: 11.04-24.32).

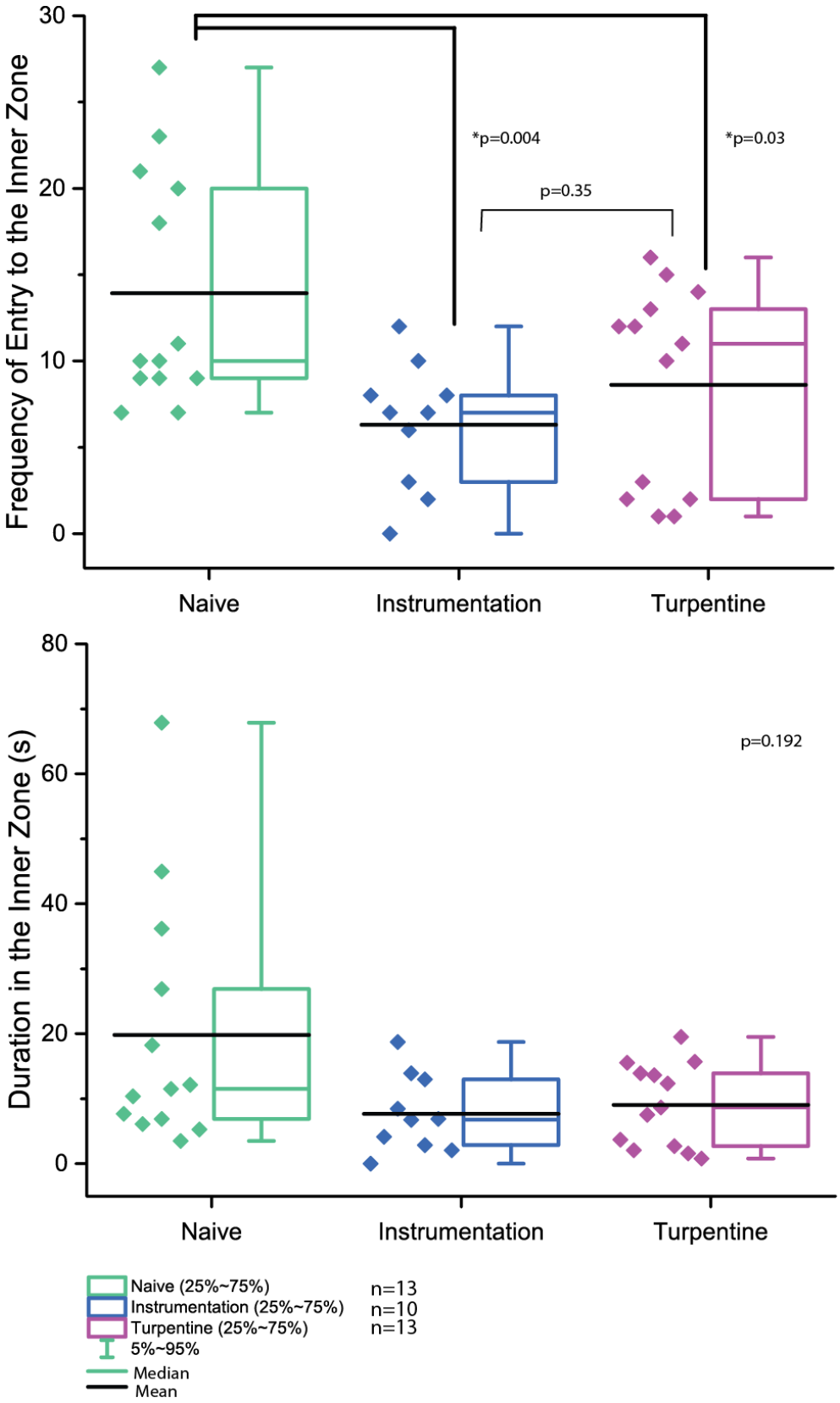
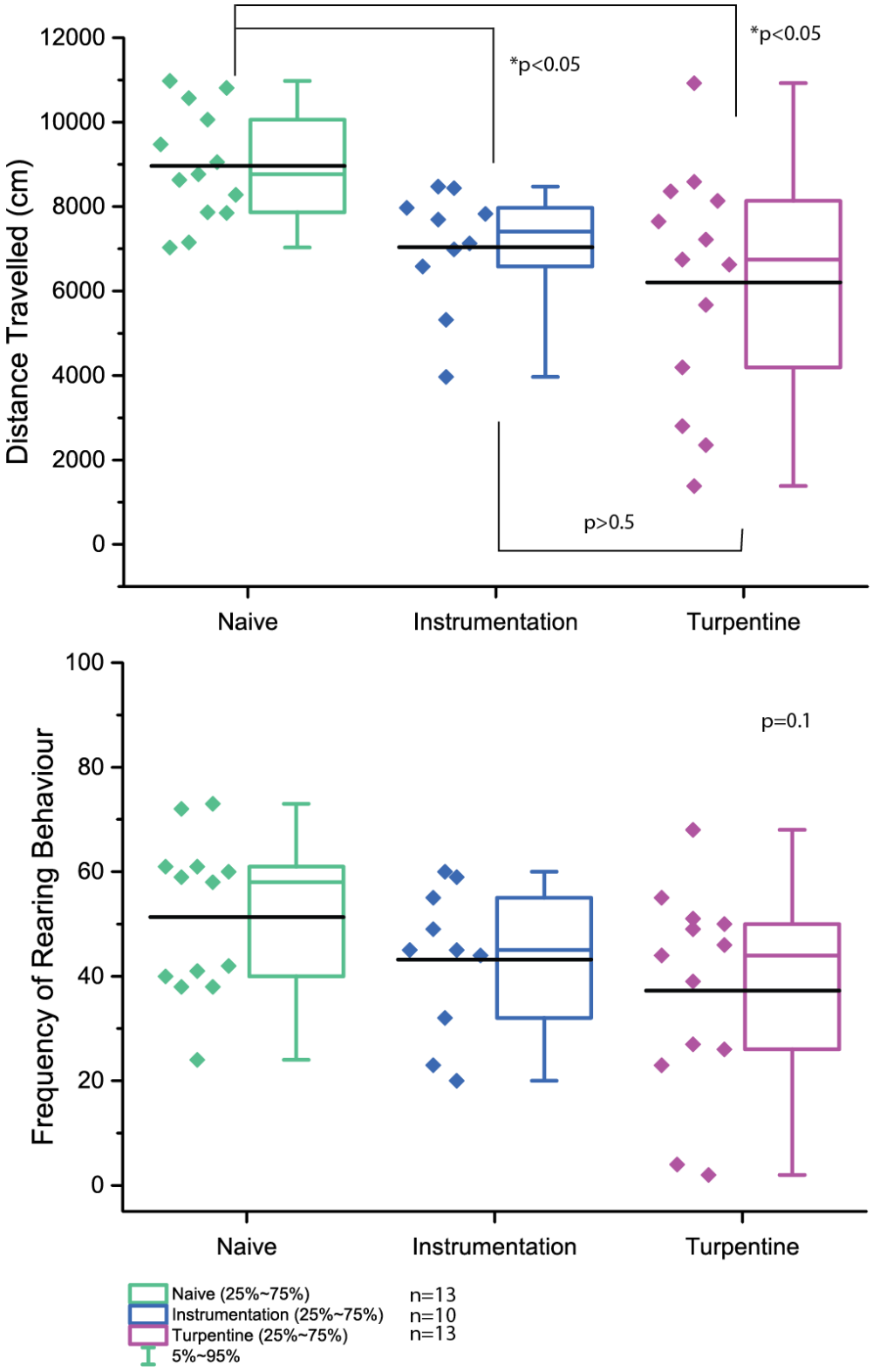


Figure 5-2: Inner zone activity (frequency and duration) following acute bladder inflammation
There was a significant decrease in inner zone entry in both turpentine and instrumentation groups.
Frequency analysed using Kruskal-Wallis ANOVA on ranks, Duration by 1-way ANOVA



5-3: Total distance travelled (cm), and rearing activity in the open field following acute bladder inflammation Significant decreases in total distance travelled were seen in both turpentine and instrumentation groups. Distance by 1-way ANOVA , Rearing analysed using Kruskal-Wallis ANOVA on ranks.

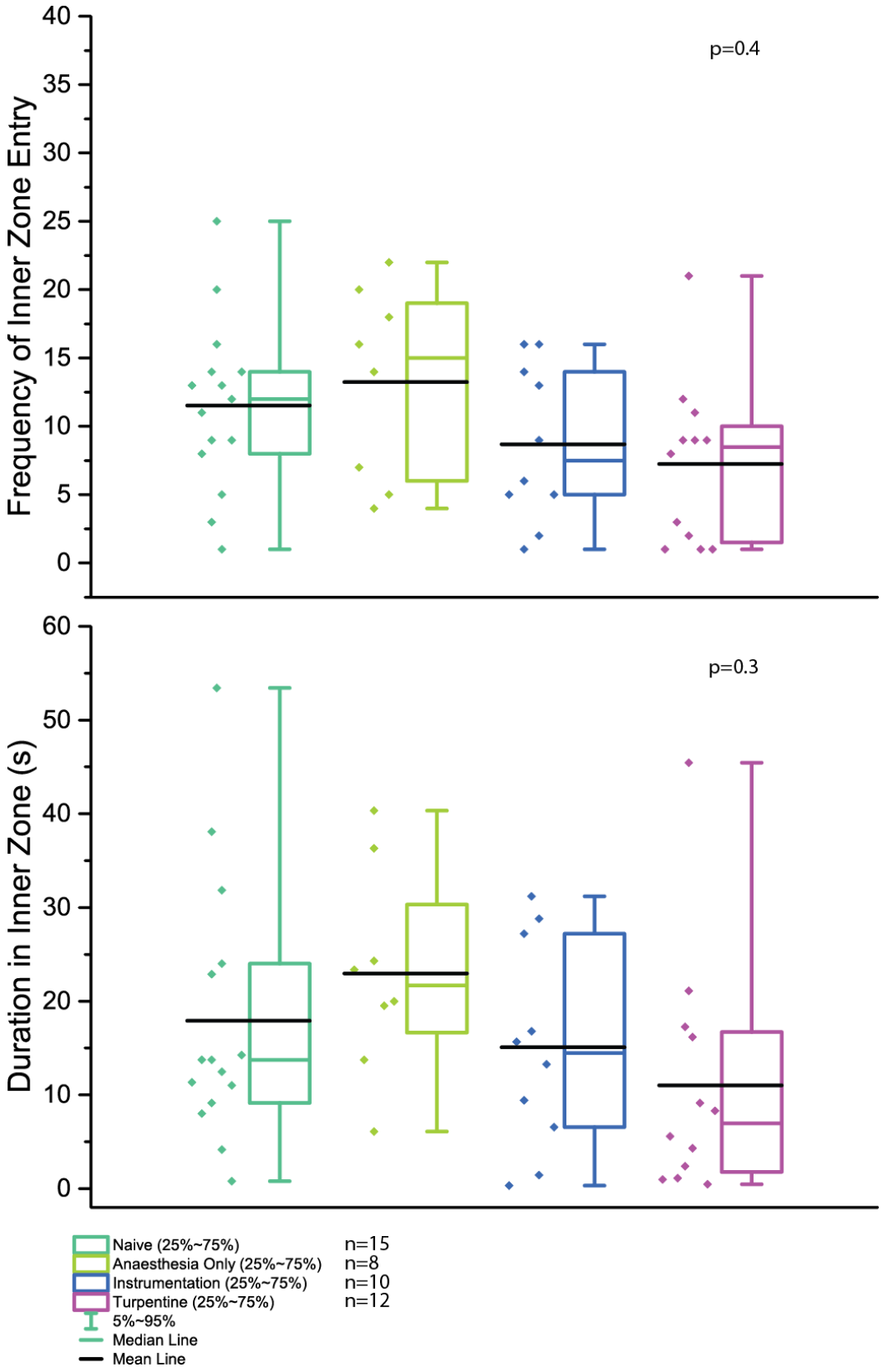


Figure 5-4: Inner zone activity following repeated bladder inflammation No significant differences in inner zone activity were recorded. Frequency analysed using Kruskal-Wallis ANOVA on ranks, Duration by 1-way ANOVA

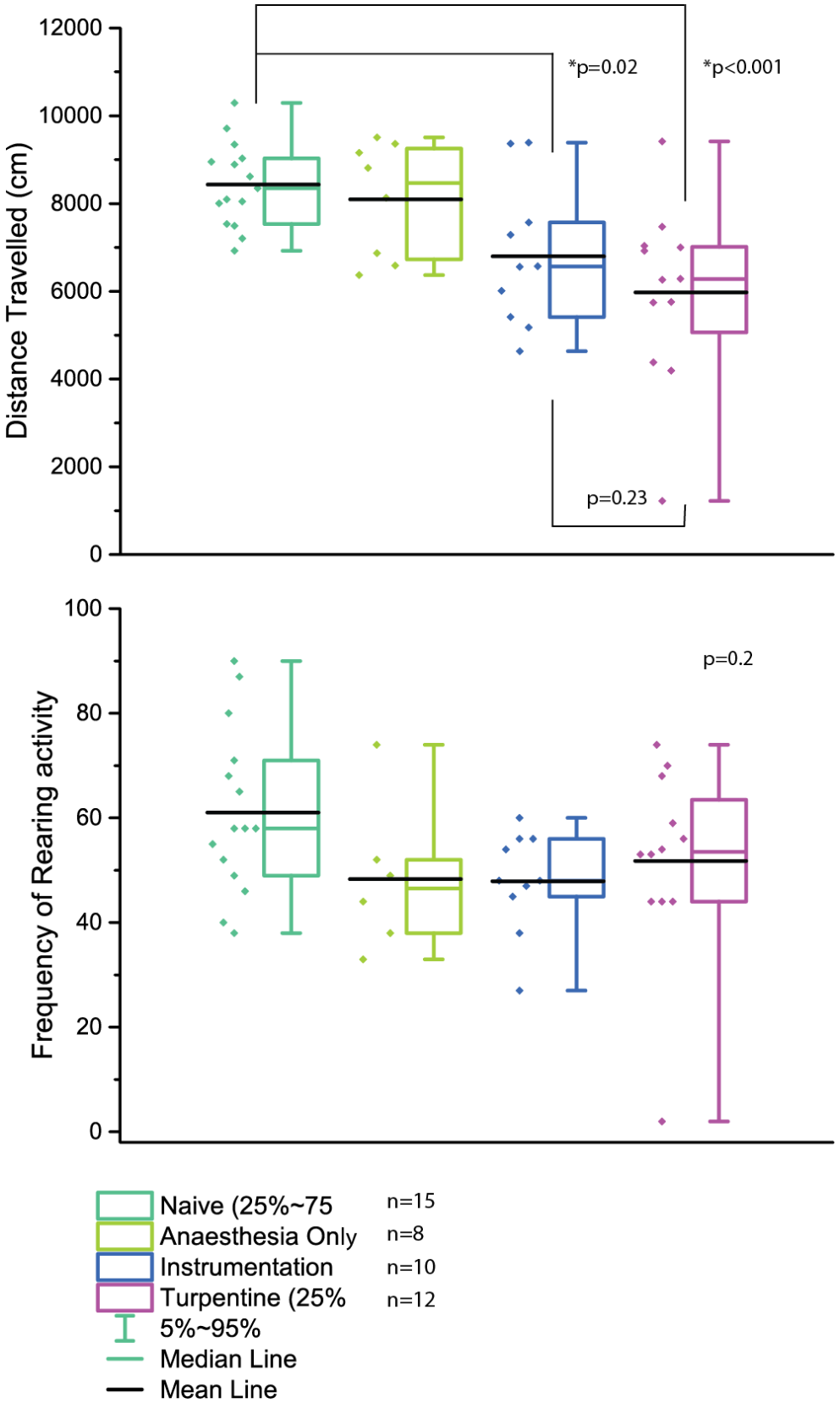


Figure 5-5: Distance travelled (cm) and rearing activity in the open field following repeated bladder inflammation Significant decreases in total distance travelled were seen in both turpentine and instrumentation groups when compared to naïve. Distance by 1-way ANOVA , Rearing analysed using Kruskal-Wallis ANOVA on ranks.

Comparison of Acute and Repeated Bladder Inflammation

2-way ANOVA was conducted to investigate how these models compare in terms of behavioural outcomes. No significant difference between acute and repeated bladder inflammation were seen for distance travelled ($p=0.4$), frequency of inner zone entry ($p=0.8$), or duration in the inner zone ($p=0.4$). Rearing was significantly higher in the repeated model (54, 46-60) compared to acute ($p=0.01$; 45, 35-58.5). Turpentine inflammation had a significant effect on behaviour, independent of the number of instillations, with significant decreases seen in distance ($p<0.001$), frequency ($p=0.004$), duration ($p=0.01$), and rearing ($p=0.01$), with higher rear frequencies in repeated turpentine when compared to acute turpentine ($p=0.03$). Instrumentation had a less pronounced effect, significantly decreasing distance travelled ($p=0.001$), and frequency of entry to the inner zone ($p=0.004$) only. Figure 5-6 and Figure 5-7 illustrate the overall behavioural effects of acute and repeated bladder inflammation.

Key findings

- Acute but not repeated bladder inflammation significantly increases thigmotaxis
- Both acute and repeated models showed a decrease in locomotor activity
- There was a strong effect of instrumentation, suggesting catheterisation alone is sufficient to induce behavioural alterations such as thigmotaxis
- 2-way ANOVA found no differences between acute and repeated inflammation on distance travelled, frequency of entry to the inner zone, and duration in the inner zone
- Rearing activity was higher in the repeated model than in the acute

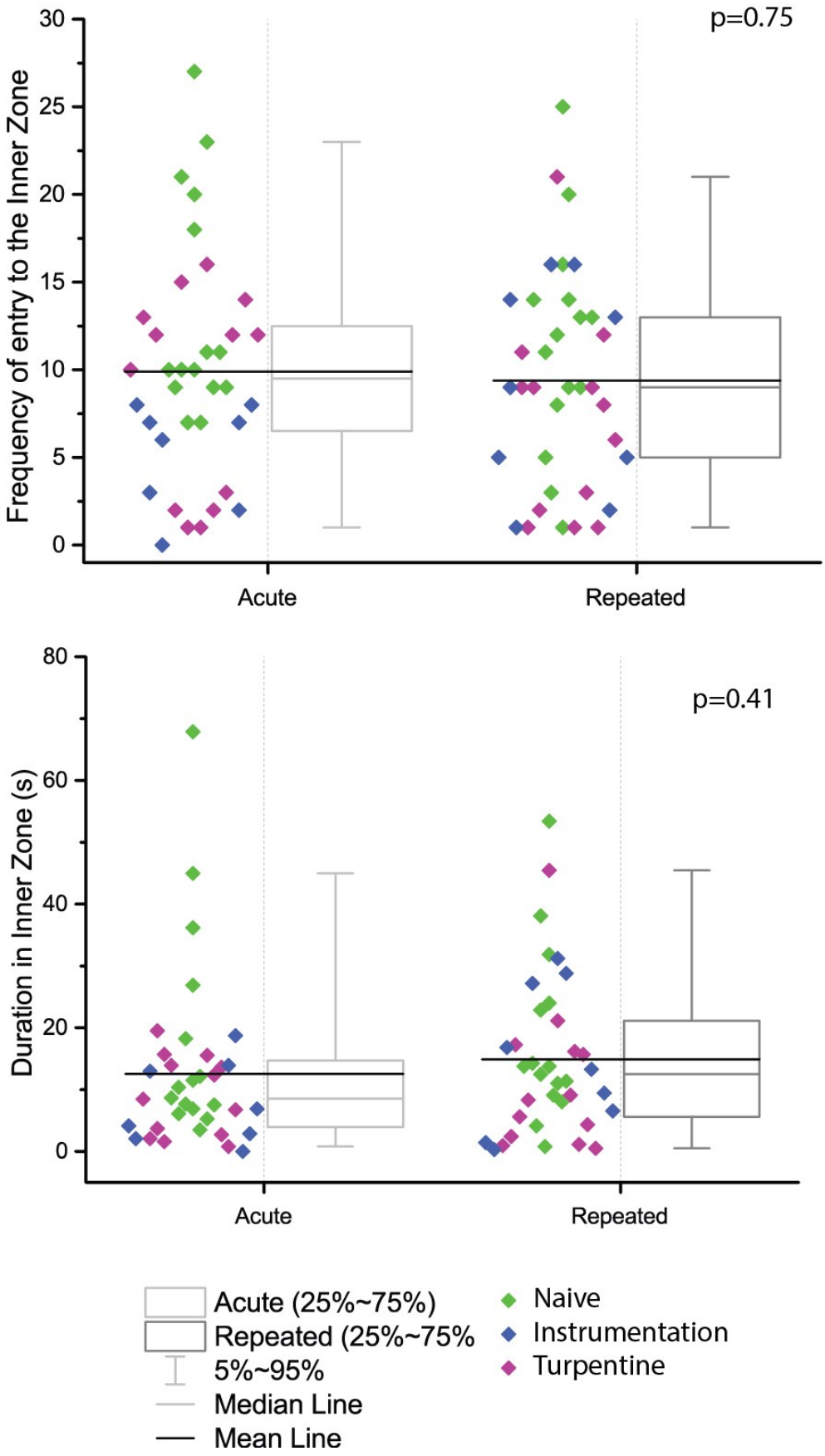


Figure 5-6: Comparison of thigmotactic outcomes (frequency and duration in the open field) following acute and repeated bladder inflammation p-values shown for 2-Way ANOVA comparison between acute and repeated inflammation

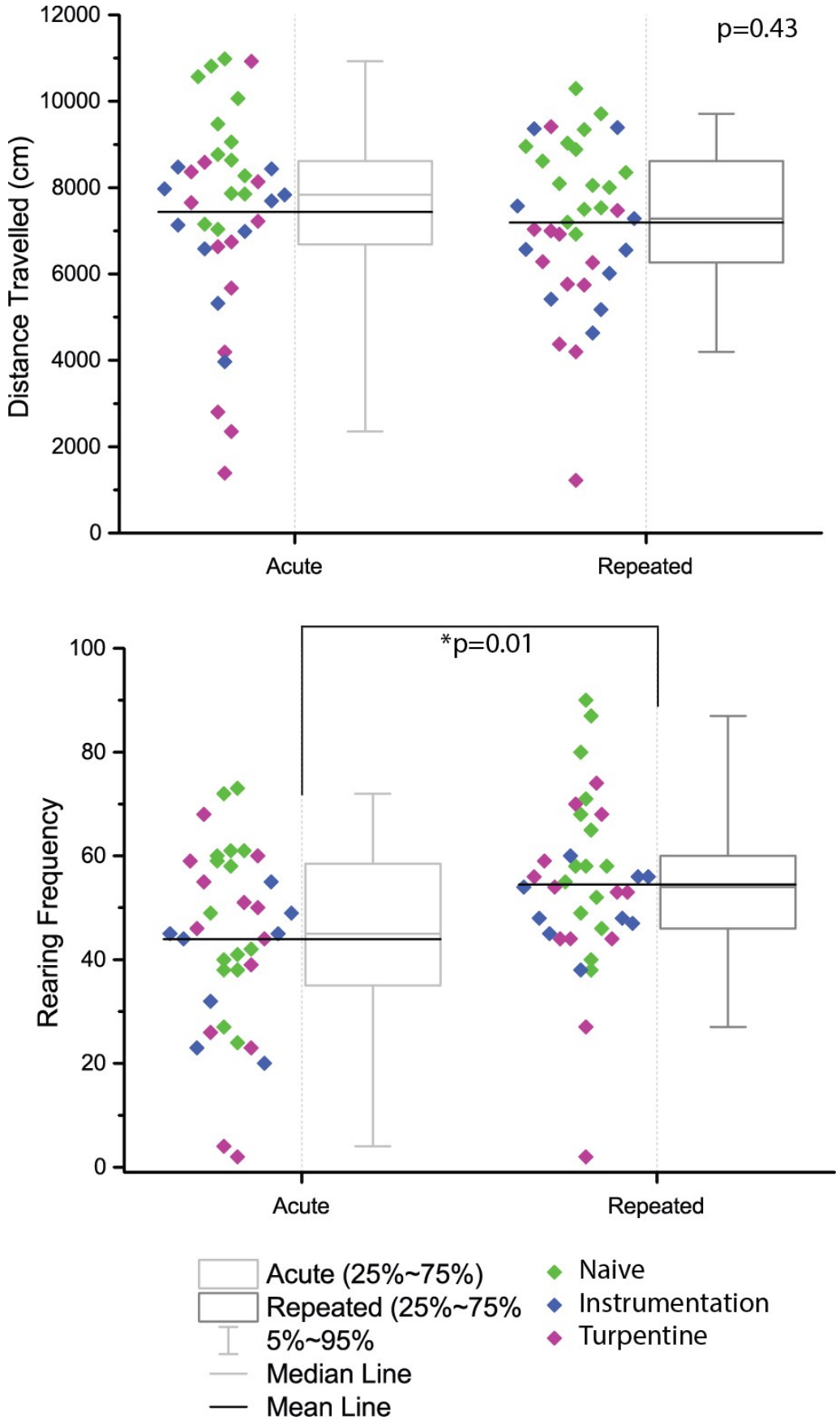


Figure 5-7: Comparison of distance travelled and rearing activity following acute and repeated bladder inflammation. p-values shown for 2-Way ANOVA comparison between acute and repeated inflammation

5.3 Burrowing Paradigm

Burrowing is a relatively new ethological assay, which has detected deficits associated with experimental models of pain such as CFA inflammation (Rutten *et al.*, 2014a), and spinal nerve transection (Andrews *et al.*, 2012). It models burrow maintenance behaviour, and deficits in burrowing have been associated with decreases in quality of life measures such as impaired ability to maintain activities around the home and workplace seen in many chronic clinical conditions (Breivik *et al.*, 2006).

A separate group of female rats had burrowing activity assessed following acute turpentine bladder inflammation. Briefly, three paired training, and three individual baseline sessions of burrowing were conducted prior to model induction. 2500g gravel was used, and the weight displaced during each hour-long session recorded. Bladder induction was induced as previously described, with naïve, instrumentation, and turpentine groups. 24 hours later, animals were re-introduced to the burrowing paradigm and weight displaced recorded.

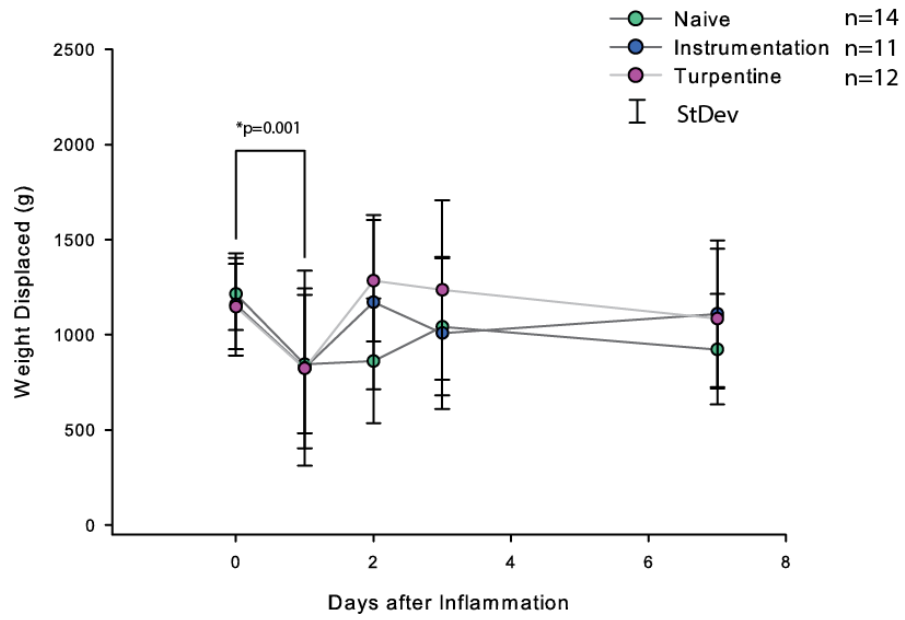
This was repeated at 2, 3, and 7 days after bladder inflammation. Data was analysed using 2-way Repeated Measure ANOVA and showed equal variance but was not distributed normally. Data below presented as median (IQR).

Exclusions:

Animals were assigned to experimental group based on mean baseline burrowing activity, and those with baseline values below 500g were equally distributed between groups but excluded following completion of the study (n=2/group). 5 animals in total were lost during model induction due to surgical complications, yielding final group sizes of 14 (naïve), 12, (instrumentation), and 11 (turpentine).

Bladder inflammation did not significantly influence weight displaced during the burrowing paradigm ($p=0.46$) as shown in Figure 5-8. There was a significant effect of time, with animals in all groups significantly decreasing gravel displacement between baseline (Naïve: 1214.6g (1006.0-1274.4g), Instrumentation: 1169.9g (998.5-1415.9g), and Turpentine: 1155.0g (901.8-1363.7g)) and 24 hours post-inflammation (Naïve: 849.6g (499.3-1110.3g), Instrumentation: 834.6g (178.84-1781.0g), and Turpentine: 812.2g (231.0-1331.5g)) ($p=0.001$). No other differences were observed.

A



B

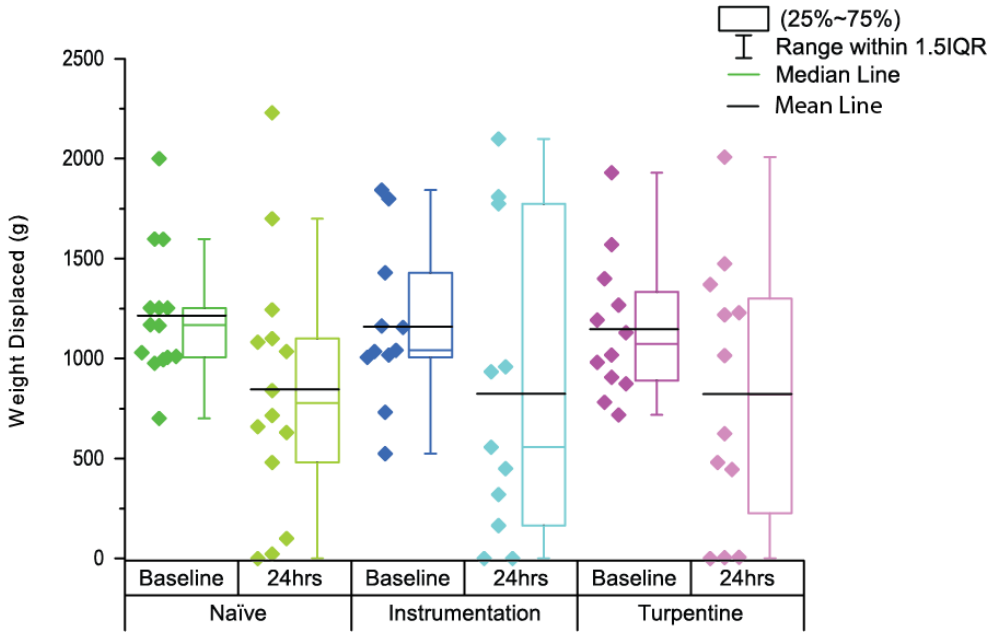


Figure 5-8: Weight displaced during 1hr burrowing session 24hrs after acute bladder inflammation

A) data from baseline (average of 3 measurements), to 1, 2, 3, and 7 days. All groups burrowed significantly less during the first test session (1 day)

B) Box and Dot representation of the significant difference seen between baseline activity and that 24hrs following acute bladder inflammation

Data analysed using 1-way Repeated Measures ANOVA

Key findings

- Acute bladder inflammation has no effect on burrowing activity
- Burrowing activity is highly variable

5.4 c-Fos Immunoreactivity in the Central Amygdala Following Open Field

c-Fos is an immediate-early oncogene specific to neurons. It shows up-regulation associated with neuronal activation (Gao & Ji, 2009), with a window of expression 90-120 minutes after a stimulus (Hunt *et al.*, 1987). Numerous studies have demonstrated up-regulation of c-Fos in the central amygdala in response to nociceptive stimuli such as intra-peritoneal acetic acid (Tanimoto *et al.*, 2003), intra-plantar formalin (Nakagawa *et al.*, 2003), adjuvant-induced arthritis (Jawed *et al.*, 2014), and dental pain (Yamashiro *et al.*, 1998). In addition, studies have showed social stress influences cFos expression in the central amygdala (Daskalakis *et al.*, 2014), and that c-Fos expression in the central amygdala correlates with novelty-seeking behaviour (Majkutewicz *et al.*, 2012). Therefore, cFos immunoreactivity in the central amygdala was investigated to determine whether alterations in activation mirror the effect of visceral noxious inflammatory stimuli on open field behaviour.

Acute Bladder Inflammation

Levels of c-Fos immunoreactivity were higher in the left hemisphere, with observed densities (cells/mm^2) of 19.7 (2.8-36.6), 23.1 (11.5-34.7), and 27.6 (5.1-50.1) compared to 12.2 (2.4-22), 7.4 (1.4-13.3), and 12.5 (2.8-22.2) in naïve, instrumentation, and turpentine groups respectively ($p=0.024$). Overall, c-Fos immunoreactivity was present at densities of 23.9 (14.1-33.6) cells/mm^2 in the left hemisphere, compared to 10.9 (6.2-15.5) in the right, as shown in Figure 5-9.

No other differences in c-Fos immunoreactivity were observed in the central amygdala at the overall level (Figure 5-10).

Figure 5-11 show examples of c-Fos staining and amygdala delineation in sections from rostral, intermediate, and caudal areas.

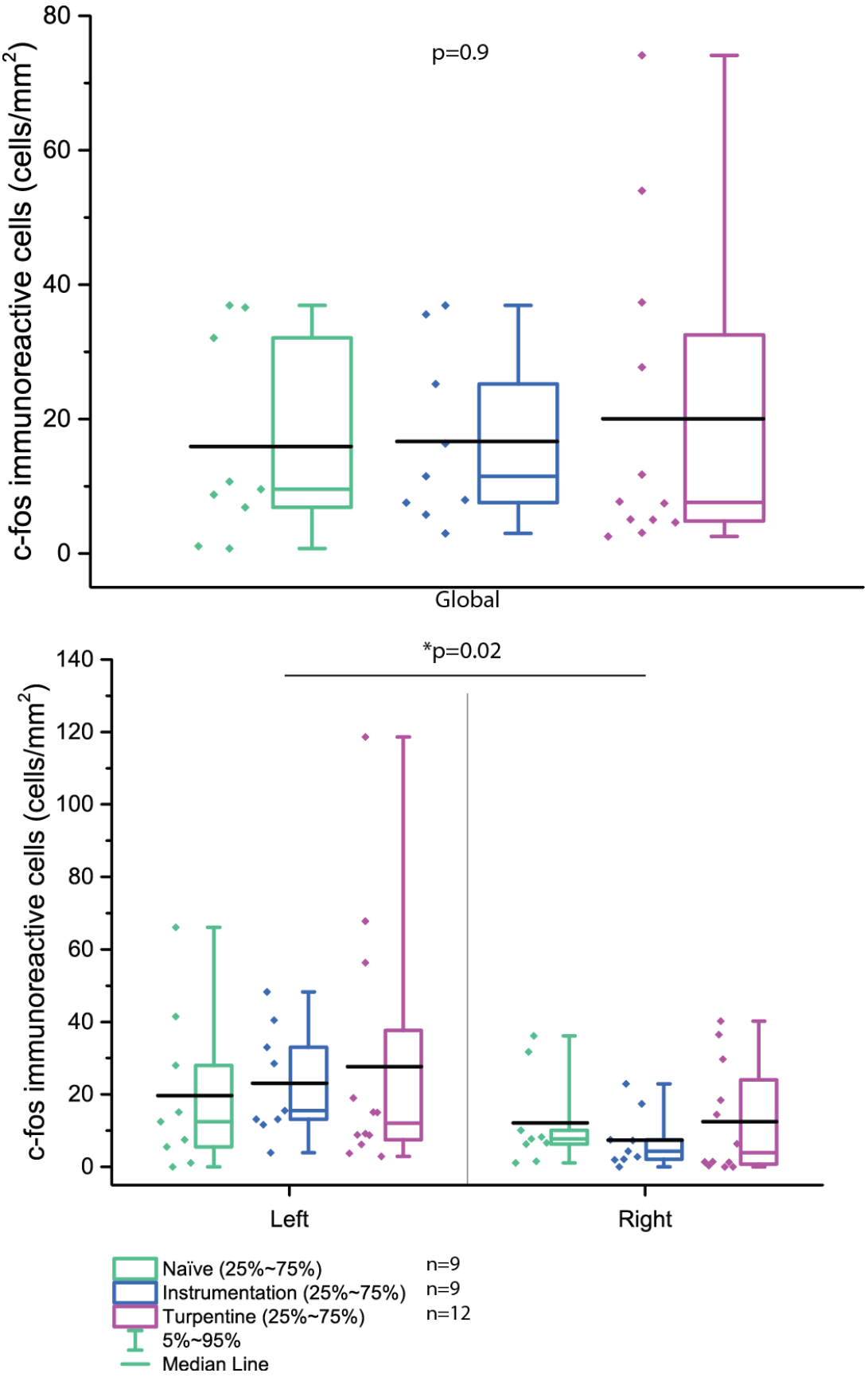


Figure 5-9: c-Fos immunoreactivity in the central amygdala globally and in left/right hemispheres following open field exposure (Acute bladder inflammation). Analyses conducted using 1-way and 2-way ANOVA for global and hemispheric respectively.

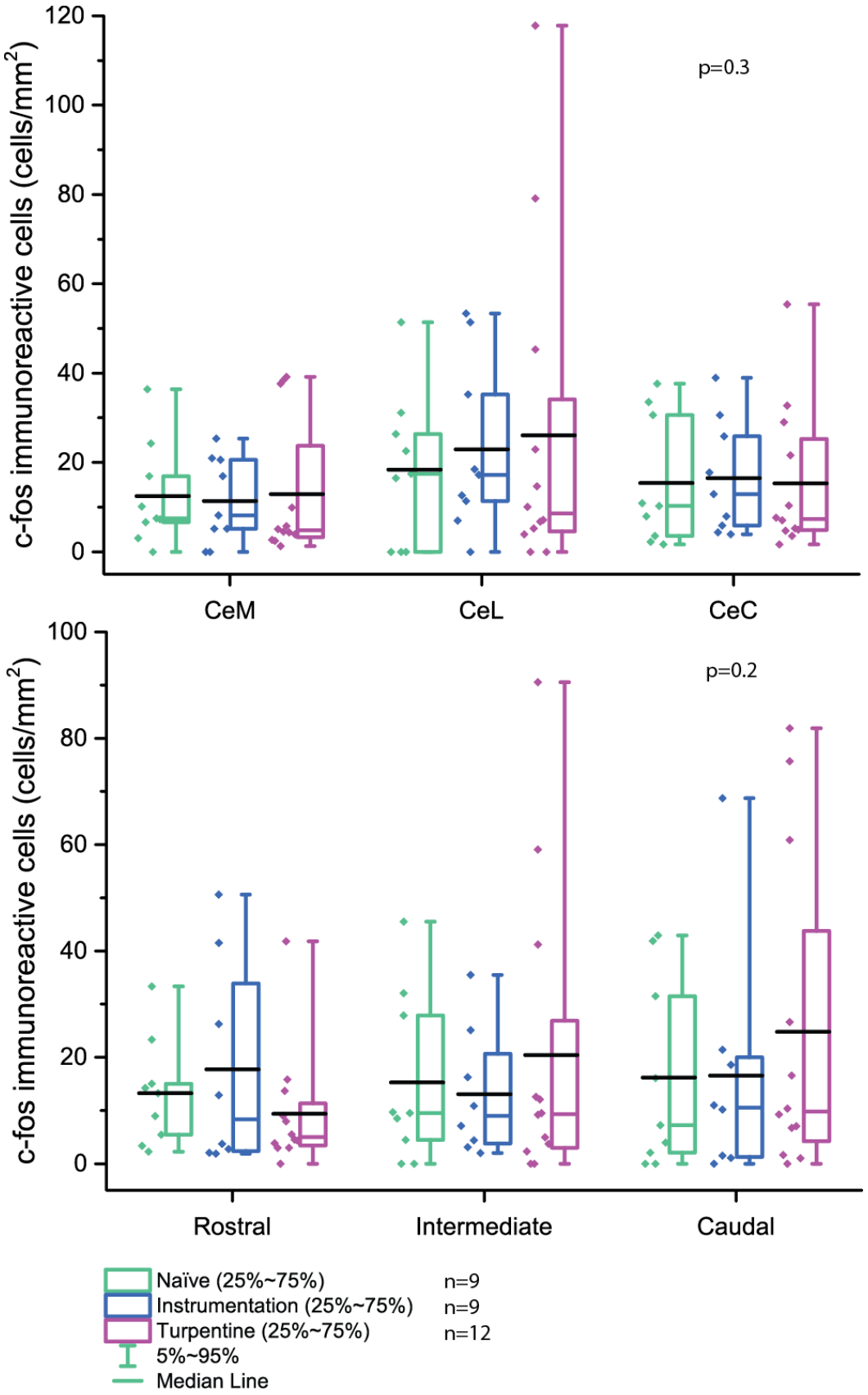


Figure 5-10: c-Fos immunoreactivity in the three subdivisions of the central amygdala (Medial, CeM; Lateral, CeL; Capsular, CeC), and along the rostro-caudal axis (acute bladder inflammation). Analysis conducted using 2-way ANOVA.

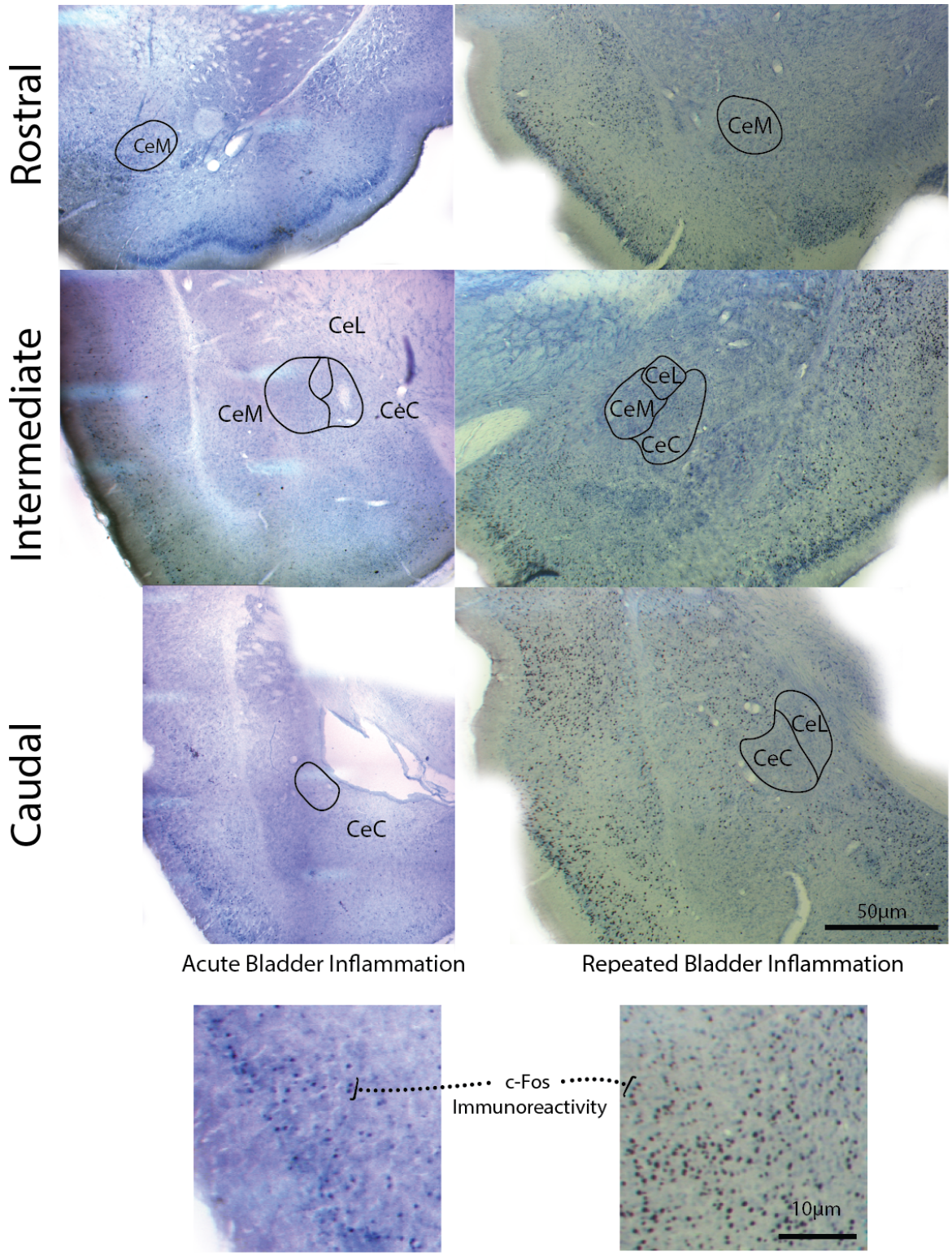


Figure 5-11: Examples of c-Fos immunoreactivity in the central amygdala following acute (left) and repeated (right) bladder inflammation. Examples from rostral (1st row) to caudal (3rd row) x50 magnification (x100 used for quantification purposes), with magnified sections to show examples of positive c-Fos staining (4th row). Toluidine blue counterstain used, with nickel-enhanced DAB visualisation of c-Fos protein.

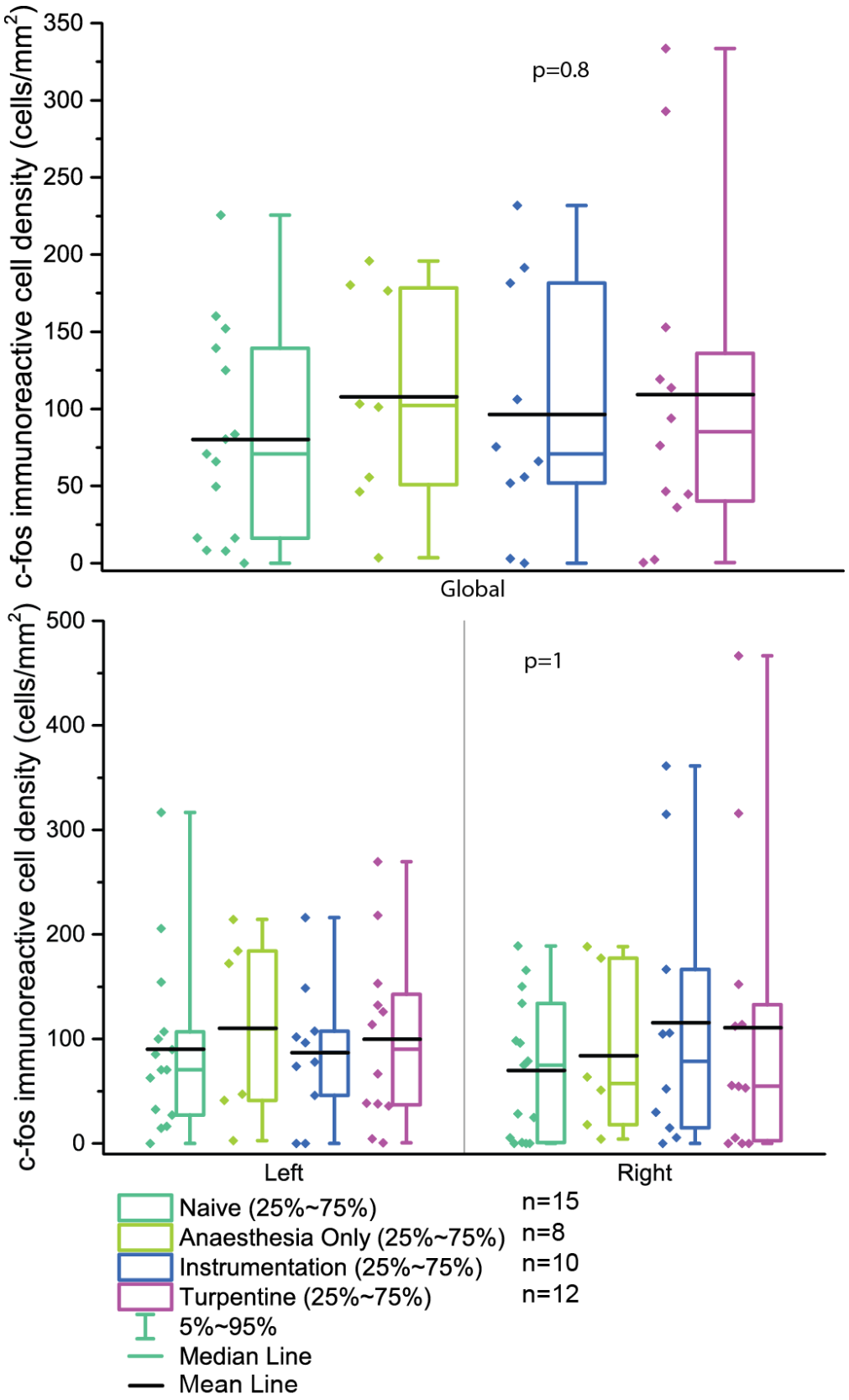


Figure 5-12: c-Fos immunoreactivity in the central amygdala and left/right hemispheres following repeated bladder inflammation. Analyses conducted using 1-way and 2-way ANOVA for global and hemispheric respectively.

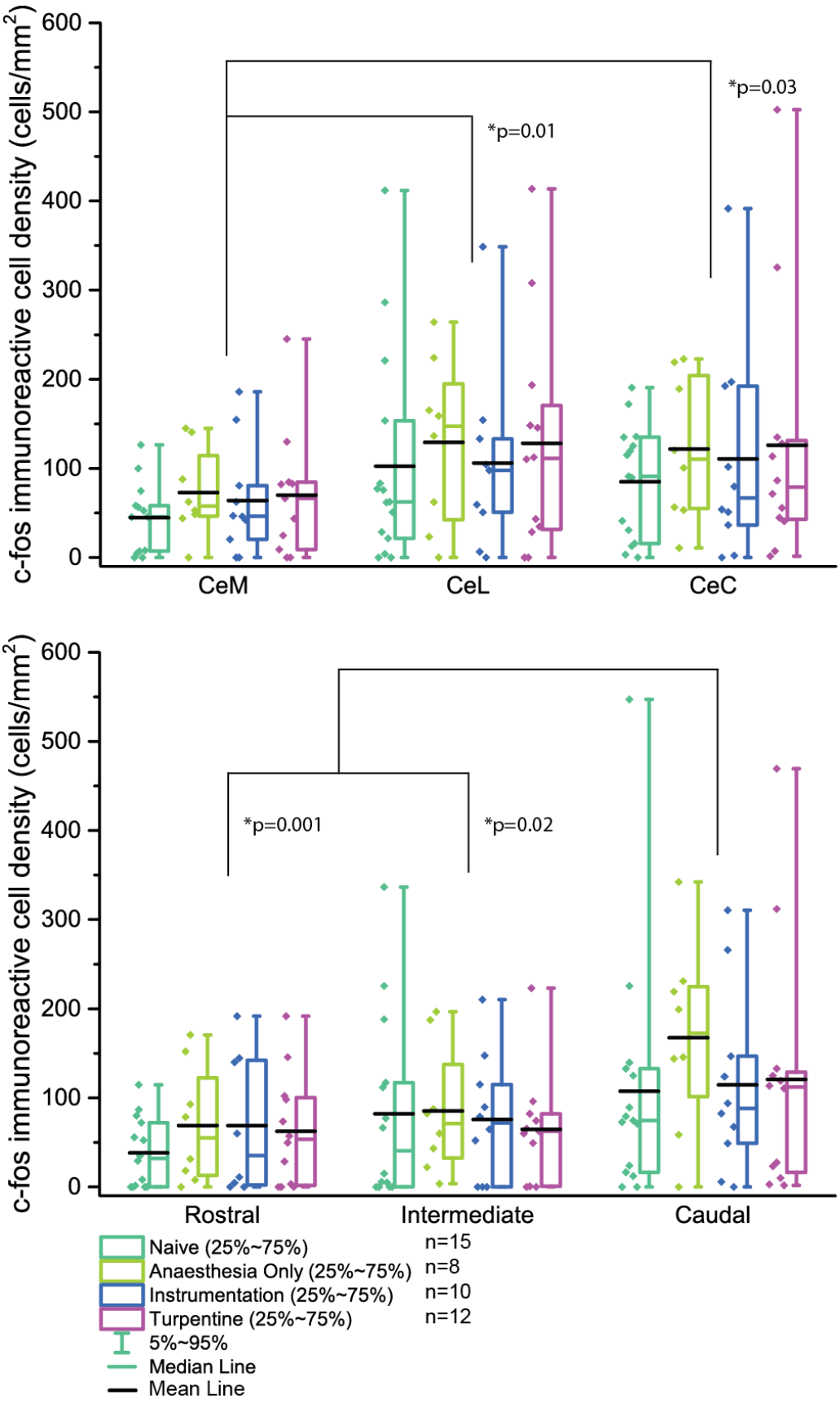


Figure 5-13: c-Fos immunoreactivity in the central amygdala within the three subdivisions of the amygdala (medial, CeM; lateral, CeL; capsular, CeC), and along the rostro-caudal axis following repeated bladder inflammation. Analysis conducted using 2-way ANOVA.

Repeated Bladder Inflammation

There was no overall difference between left and right hemisphere, and no effect of group on c-Fos immunoreactivity (Figure 5-12).

A rostro-caudal gradient was present with significantly higher levels of activation observed in the caudal regions (Overall: 123.1 (86.0-127.1), N: 107.3 (31.6-182.9), AO: 167.5 (78.5-256.4), I: 114.6 (40.9-188.2), T: 120.6 (31.4-209.8)) when compared to both rostral ($p<0.001$; Overall: 56.9 (38.475.3), N: 38.2 (15.7-60.8), AO: 68.9 (13.9-124.0), I: 68.9 (3.5-134.3), T: 62.5 (22.7-102.3)), and intermediate ($p=0.005$; Overall: 76.8 (52.4-101.1), N:82.0 (21.6-142.4), AO: 85.3 (25.4-145.2), I: 75.8 (26.4-125.2), T: 64.8 (22.7-106.8)) as shown in Figure 5-13.

c-Fos immunoreactivity in the CeM (61.1 (43.7-78.5)) was significantly lower than that detected in both the CeL ($p=0.007$; 115.1 (81.3-148.9)) and the CeC ($p=0.005$; 108.1 (76.7-139.5)).

To check reliability of measurements, delineated amygdala area was analysed. No group differences were observed in measured area of central amygdala nuclei (data not shown).

To control for the effects of repeated anaesthesia, an additional control group was introduced for the repeated model. These AO animals showed no significant effect of repeated isoflurane anaesthesia on c-Fos immunoreactivity in the central amygdala.

Examples of c-Fos staining can be seen on the right panel of Figure 5-11 showing c-Fos immunoreactivity and amygdala delineation in sections from rostral, intermediate, and caudal areas.

Key findings

- Bladder inflammation has no effect on c-Fos immunoreactivity in the central amygdala
- Increased caudal activation of the central amygdala was observed in the repeated model only
- Some lateralisation was observed following acute inflammation, with immunoreactivity of the left hemisphere higher than right

5.5 Relationship between Thigmotaxis and Central Amygdala Activity

Responder Analysis

When behaviour was analysed by responder phenotype, significant differences between high and low activity animals was seen in frequency and duration ($p<0.001$ for both acute and repeated models, Table 5-6).

A significant difference between high and low responders was also seen for distance travelled in the repeated group ($p=0.009$), with an effect of group ($p=0.012$), but no interaction ($p=0.732$, Figure 5-7).

In the acute model, high/low thigmotactic phenotype influenced c-Fos immunoreactivity in the right intermediate central amygdala (p=0.05). In the repeated model, thigmotaxis showed an inverse relationship with findings of increased c-Fos immunoreactivity in the medial central amygdala (global: p=0.03, rostral: p<0.001, intermediate: p=0.006), and in the intermediate region of the lateral central amygdala (p=0.03) in low thigmotaxis animals. Multiple comparison procedures identified significant differences between c-Fos immunoreactivity of high and low thigmotaxis animals in the turpentine (Global CeM: p0.03, rostral CeM: p=0.004, intermediate CeM: p=0.04) and instrumentation groups (Rostral CeM: p=0.003, intermediate CeM: p=0.01).

Table 5-6: Responder data for thigmotactic outcomes in both models
** denotes p<0.05 between high and low thigmotactic groups*

	n	Low Thigmotaxis		High Thigmotaxis	
Acute	hi-lo	Frequency	Duration (s)	Frequency	Duration (s)
Naïve	9-4	19 (10.5-22)*	18.2 (11.5-36.2)*	9 (7-9)	5.7 (4.4-6.8)
Instrumentation	2-8	11 (10-12)*	7.6 (6.7-8.5)*	6.5 (2.5-7.5)	5.5 (2.5-13.4)
Turpentine	8-5	12.5 (11.5-14.5)*	13.8 (10.5-15.6)*	2 (1-2)	2.1 (1.6-2.7)
Repeated					
Naïve	9-6	14 (13-16)*	22.9 (12.5-31.8)*	6.5 (3-9)	8.6 (4.2-13.8)
Instrumentation	4-6	15 (13.5-16)*	28 (20.2-30)*	5 (2-6)	8 (1.4-15.7)
Turpentine	3-9	12 (11-12)*	17.28 (8.3-45.4)*	3 (1-9)	4.3 (1.1-9.1)

Key findings

- Possible existence of distinct behavioural phenotypes based on frequency of entry to inner zone, which show significant differences across the other open field outcomes measures
- Increased c-Fos immunoreactivity observed in the medial subdivision of the central amygdala in animals exhibiting a low thigmotaxis phenotype in the repeated model, with the reverse observed in the right intermediate central amygdala in the acute model

Correlations

Acute Bladder Inflammation

Overall

There were no significant correlations between c-Fos immunoreactivity and behaviour in the open field at the overall level.

Within Groups

In naïve animals, duration in the inner zone significantly correlated with c-Fos immunoreactivity in the intermediate CeL (ρ=0.76, p=0.05).

In the instrumentation group, rearing behaviour was correlated with c-Fos immunoreactivity in the CeC (overall ρ=0.73, right ρ=0.76, rostral ρ=0.80; p<0.05) and CeM (caudal ρ=0.71 p=0.03), and distance travelled was correlated with immunoreactivity in the caudal levels of the left central amygdala (ρ=0.80, p=0.02).

There were no significant correlations seen in the turpentine group.

Table 5-7: Responder data for locomotor activity and rearing in both models
No significant differences in rearing or distance travelled were detected

	n	Low Thigmotaxis		High Thigmotaxis	
Acute	**hi-lo**	**Distance (cm)**	**Rearing**	**Distance (cm)**	**Rearing**
Naïve	9-4	9267.74 (8196.73-10338.75)	58 (36-61)	8283.24 (6585.08-9981.39)	50.5 (41-65.5)
Instrumentation	2-8	7339.10 (2836.33-11841.86)	59.5 (59-60)	6963.91 (5919.82-8307.99)	44.5 (27.5-47)
Turpentine	8-5	8031.84 (6881.98-9181.70)	46.5 (33-53)	3280.72 (1199.09-5362.35)	26 (4-46)
Repeated					
Naïve	9-6	8779.38 (8165.49-9393.28)	58 (55-71)	7912.40 (6872.44-8952.35)	58.5 (46-68)
Instrumentation	4-6	8403.06 (6602.87-10203.25)	46.5 (41.5-48)	5726.77 (4901.66-6551.88)	55 (47-56)
Turpentine	3-9	6516.65 (5475.62-7557.69)	53 (2-54)	5793.12 (3985.70-7600.64)	56 (44-68)

Correlations Based on Thigmotactic Phenotype

No significant correlations were observed in low thigmotactic animals. In those showing higher levels of thigmotaxis, duration in the inner zone was significantly correlated with intermediate CeL c-Fos immunoreactivity (ρ=0.61, p=0.034), distance travelled correlated with c-Fos immunoreactivity in the caudal regions of the left central amygdala (ρ=0.62, p=0.031), and rearing was significantly correlated with central amygdala activation in the left hemisphere (ρ=0.52, p=0.045).

Correlations were seen between central amygdala activation and bladder inflammation (Table 5-7) and behavioural phenotype (Table 5-8). No overall patterns were observed.

Table 5-8: ***Correlations between behavioural outcomes and c-Fos immunoreactivity in the central amygdala following acute bladder inflammation R: Right, L: Left; glob: Global***

		Naïve				Instrumentation				Turpentine			
		Distance travelled	Inner Zone Freq.	Inner Zone Duration*	Rearing	Distance travelled*	Inner Zone Freq.	Inner Zone Duration	Rearing*	Distance travelled	Inner Zone Freq.	Inner Zone Duration	Rearing
LCeM*	ρ	-0.05	0.16	-0.07	0.14	0.28	0.15	-0.24	**0.66***	0.34	0.17	0.05	0.19
	Sig.	0.90	0.68	0.87	0.71	0.47	0.71	0.54	**0.05**	0.25	0.59	0.87	0.53
LCeL	ρ	0.41	-0.13	0.22	-0.07	0.60	0.55	0.57	0.13	0.26	0.25	0.37	-0.14
	Sig.	0.27	0.74	0.58	0.86	0.09	0.12	0.11	0.74	0.41	0.44	0.24	0.66
LCeC	ρ	-0.21	-0.31	-0.43	-0.42	0.12	-0.07	-0.26	0.48	0.23	0.17	0.22	0.26
	Sig.	0.58	0.42	0.25	0.26	0.76	0.85	0.51	0.19	0.45	0.58	0.48	0.38
Left	ρ	0.14	-0.14	-0.06	-0.13	0.50	0.42	0.16	0.49	0.39	0.32	0.35	0.06
	Sig.	0.71	0.72	0.88	0.73	0.17	0.26	0.68	0.18	0.18	0.29	0.24	0.85
RCeM	ρ	-0.34	-0.15	-0.34	-0.27	0.23	-0.33	-0.40	0.38	0.06	0.02	0.08	0.24
	Sig.	0.37	0.69	0.36	0.48	0.55	0.39	0.29	0.31	0.86	0.95	0.79	0.42
RCeL	ρ	-0.09	-0.33	-0.37	-0.39	-0.13	-0.07	-0.37	0.23	0.30	0.11	0.15	0.16
	Sig.	0.81	0.39	0.33	0.29	0.73	0.85	0.33	0.55	0.32	0.71	0.63	0.60
RCeC*	ρ	-0.34	-0.18	-0.43	-0.18	0.52	0.32	-0.22	**0.76***	0.22	0.06	0.02	0.30
	Sig.	0.37	0.64	0.25	0.65	0.15	0.40	0.57	**0.02**	0.47	0.86	0.94	0.32
Right	ρ	0.09	-0.10	-0.28	-0.14	0.33	-0.10	-0.43	0.62	0.24	0.08	0.11	0.29
	Sig.	0.82	0.79	0.47	0.71	0.39	0.80	0.25	0.07	0.42	0.81	0.71	0.33
CeM	ρ	-0.10	0.12	-0.12	0.08	0.28	-0.10	-0.36	0.58	0.25	0.12	0.08	0.27
	Sig.	0.80	0.77	0.76	0.84	0.46	0.79	0.35	0.10	0.41	0.71	0.78	0.36
CeL	ρ	0.27	-0.24	0.01	-0.22	0.56	0.53	0.45	0.21	0.38	0.28	0.39	0.01
	Sig.	0.48	0.53	0.99	0.56	0.12	0.14	0.22	0.59	0.20	0.36	0.19	0.97
CeC*	ρ	-0.29	-0.34	-0.52	-0.44	0.40	0.17	-0.27	**0.73***	0.23	0.12	0.13	0.29
	Sig.	0.44	0.37	0.15	0.23	0.29	0.66	0.48	**0.02**	0.44	0.70	0.68	0.34
Global	ρ	0.12	-0.15	-0.14	-0.18	0.51	0.29	-0.07	0.65	0.35	0.22	0.27	0.18
	Sig.	0.75	0.70	0.72	0.65	0.16	0.46	0.87	0.06	0.24	0.46	0.37	0.56

Table 5-8 (continued): Correlations between behavioural outcomes and c-Fos immunoreactivity in the central amygdala following acute bladder inflammation
r: Rostral, i: Intermediate, c: Caudal; glob: Global

		Naïve				Instrumentation				Turpentine			
		Distance travelled	Inner Zone Freq.	Inner Zone Duration*	Rearing	Distance travelled*	Inner Zone Freq.	Inner Zone Duration	Rearing*	Distance travelled	Inner Zone Freq.	Inner Zone Duration	Rearing
Rostral	ρ	-0.21	-0.01	-0.21	-0.08	0.23	-0.08	-0.34	0.53	-0.06	-0.13	-0.09	0.32
	Sig.	0.59	0.98	0.58	0.84	0.55	0.84	0.37	0.14	0.84	0.66	0.76	0.29
rLeft	ρ	-0.23	-0.13	-0.32	-0.19	-0.16	-0.15	-0.32	0.39	-0.25	-0.17	-0.15	0.22
	Sig.	0.54	0.74	0.40	0.62	0.68	0.70	0.40	0.29	0.41	0.58	0.63	0.47
rRight	ρ	0.03	0.48	0.22	0.40	0.35	-0.03	-0.34	0.52	0.02	-0.09	-0.05	0.30
	Sig.	0.94	0.19	0.57	0.28	0.35	0.94	0.37	0.15	0.94	0.77	0.88	0.31
rCeM	ρ	-0.02	0.28	0.01	0.26	0.23	-0.28	-0.18	0.12	-0.21	-0.12	-0.01	-0.16
	Sig.	0.97	0.46	0.98	0.50	0.56	0.47	0.64	0.75	0.54	0.72	0.97	0.63
rCeL	ρ	0.20	-0.22	-0.04	-0.23	-0.20	-0.08	-0.48	0.39	-0.01	-0.14	-0.10	0.24
	Sig.	0.64	0.60	0.93	0.58	0.61	0.83	0.19	0.30	0.97	0.64	0.76	0.43
rCeC*	ρ	0.14	0.49	0.00	0.40	0.36	0.13	-0.37	**0.80***	0.00	-0.10	-0.10	0.32
	Sig.	0.72	0.18	0.99	0.29	0.34	0.74	0.33	**0.01**	1.00	0.75	0.75	0.29
Inter.*	ρ	0.13	-0.11	0.21	-0.18	0.44	0.35	-0.05	**0.66***	0.31	0.18	0.28	0.14
	Sig.	0.74	0.79	0.59	0.65	0.24	0.36	0.91	**0.05**	0.30	0.56	0.35	0.66
iLeft	ρ	0.22	0.05	0.40	-0.01	0.34	0.41	0.09	0.50	0.23	0.13	0.22	-0.09
	Sig.	0.58	0.91	0.29	0.99	0.37	0.27	0.81	0.17	0.47	0.70	0.50	0.79
iRight	ρ	-0.27	-0.42	-0.49	-0.48	0.27	-0.22	-0.43	0.52	0.18	0.07	0.12	0.28
	Sig.	0.47	0.26	0.18	0.19	0.49	0.57	0.25	0.15	0.57	0.83	0.68	0.35
iCeM	ρ	-0.45	-0.18	-0.57	-0.16	0.09	-0.26	-0.39	0.62	0.04	-0.02	-0.01	0.36
	Sig.	0.26	0.66	0.14	0.70	0.82	0.49	0.30	0.07	0.89	0.94	0.98	0.23
iCeL*	ρ	0.33	0.16	**0.76***	0.05	0.44	0.49	0.17	0.47	0.25	0.11	0.25	-0.25
	Sig.	0.47	0.74	**0.05**	0.92	0.24	0.19	0.66	0.20	0.46	0.74	0.46	0.46
iCeC	ρ	0.29	-0.10	-0.10	-0.07	0.38	0.19	-0.18	0.61	0.15	-0.02	0.02	0.22
	Sig.	0.44	0.80	0.81	0.86	0.31	0.62	0.64	0.08	0.64	0.95	0.96	0.50

Table 5-8 (continued): Correlations between behavioural outcomes and c-Fos immunoreactivity in the central amygdala following acute bladder inflammation

r: Rostral, i: Intermediate, c: Caudal; glob: Global

		Naïve				Instrumentation				Turpentine			
		Distance travelled	Inner Zone Entry Frequency	Inner Zone Duration*	Rearing	Distance travelled*	Inner Zone Entry Frequency	Inner Zone Duration	Rearing*	Distance travelled	Inner Zone Entry Frequency	Inner Zone Duration	Rearing
Caudal*	ρ	0.29	-0.16	-0.15	-0.10	**0.66***	0.39	0.08	0.60	0.46	0.35	0.33	0.08
	Sig.	0.45	0.68	0.71	0.79	**0.05**	0.30	0.83	0.09	0.11	0.24	0.27	0.79
cLeft*	ρ	0.36	-0.22	-0.16	-0.10	**0.80***	0.64	0.39	0.34	0.46	0.38	0.36	0.08
	Sig.	0.43	0.63	0.74	0.83	**0.02**	0.09	0.34	0.41	0.14	0.22	0.25	0.79
cRight	ρ	0.24	-0.15	-0.23	-0.14	0.21	0.04	-0.34	0.65	0.23	0.10	0.04	-0.24
	Sig.	0.54	0.70	0.55	0.71	0.62	0.92	0.41	0.08	0.52	0.79	0.92	0.51
cCeM*	ρ	0.16	-0.13	0.07	-0.23	0.46	0.20	-0.22	**0.72***	0.41	0.19	0.07	0.09
	Sig.	0.72	0.78	0.88	0.62	0.22	0.60	0.58	**0.03**	0.16	0.54	0.81	0.77
cCeL	ρ	0.19	-0.28	-0.35	-0.16	0.68	0.12	0.66	-0.42	0.44	0.41	0.43	0.04
	Sig.	0.68	0.54	0.44	0.73	0.32	0.88	0.34	0.58	0.20	0.24	0.22	0.91
cCeC	ρ	0.34	-0.15	-0.24	-0.06	0.10	-0.44	-0.46	0.78	0.42	0.39	0.42	-0.09
	Sig.	0.45	0.75	0.60	0.90	0.90	0.56	0.54	0.22	0.20	0.24	0.20	0.79

*Table 5-9: Correlations between behavioural phenotype and c-Fos immunoreactivity following acute bladder inflammation * denotes parameters with significant correlation L: Left, R: Right; glob: Global*

		Low Thigmotaxis				High Thigmotaxis			
		Distance travelled	Inner Zone Entry Frequency	Inner Zone Duration	Rearing	Distance travelled*	Inner Zone Entry Frequency	Inner Zone Duration*	Rearing*
LCeM	ρ	-0.04	-0.16	-0.29	-0.01	0.22	0.29	0.01	0.38
	Sig.	0.87	0.54	0.27	0.98	0.43	0.30	0.98	0.16
LCeL*	ρ	0.14	0.17	0.25	-0.39	0.53*	0.40	0.35	0.36
	Sig.	0.60	0.52	0.34	0.13	0.05	0.15	0.22	0.20
LCeC	ρ	**-0.23**	-0.10	-0.16	-0.06	0.22	-0.01	-0.23	0.21
	Sig.	0.40	0.71	0.55	0.84	0.42	0.96	0.41	0.45
Lglob*	ρ	0.03	0.03	-0.02	-0.25	0.51*	0.38	0.12	0.52*
	Sig.	0.92	0.91	0.94	0.36	0.05	0.16	0.66	0.04
RCeM	ρ	-0.01	-0.06	-0.06	0.00	0.14	0.04	-0.16	0.41
	Sig.	0.97	0.82	0.82	1.00	0.61	0.88	0.58	0.13
RCeL	ρ	-0.09	-0.26	-0.22	-0.20	0.07	-0.22	-0.30	0.27
	Sig.	0.75	0.34	0.41	0.46	0.80	0.44	0.28	0.33
RCeC	ρ	0.21	0.16	-0.18	0.28	0.03	-0.10	-0.30	0.37
	Sig.	0.42	0.54	0.51	0.29	0.91	0.72	0.28	0.18
RGlob	ρ	0.03	-0.14	-0.25	-0.01	0.19	0.03	-0.21	0.46
	Sig.	0.90	0.62	0.36	0.98	0.50	0.91	0.46	0.09
GlobCeM	ρ	-0.04	-0.16	-0.26	-0.01	0.23	0.22	-0.08	0.47
	Sig.	0.88	0.56	0.34	0.98	0.42	0.44	0.79	0.08
GlobCeL	ρ	0.07	0.00	0.09	-0.42	0.43	0.18	0.11	0.44
	Sig.	0.80	0.99	0.75	0.10	0.11	0.53	0.71	0.10
GlobCeC	ρ	0.01	0.04	-0.19	0.14	0.17	-0.05	-0.29	0.30
	Sig.	0.97	0.87	0.49	0.61	0.55	0.85	0.30	0.27
Glob	ρ	0.06	0.00	-0.09	-0.16	0.32	0.16	-0.06	0.50
	Sig.	0.82	0.99	0.73	0.56	0.24	0.57	0.83	0.06

*Table 5-9 (continued): Correlations between behavioural phenotype and c-Fos immunoreactivity following acute bladder inflammation. * denotes parameters with significant correlation; r: Rostral, i: Intermediate, c: Caudal; glob: Global*

		Low Thigmotaxis				High Thigmotaxis			
		Distance travelled	Inner Zone Freq.	Inner Zone Duration	Rearing	Distance travelled*	Inner Zone Freq.	Inner Zone Duration*	Rearing*
rGlob	ρ	-0.06	0.05	-0.09	0.16	0.13	0.08	-0.17	0.39
	Sig.	0.82	0.86	0.74	0.55	0.65	0.79	0.54	0.15
rLeft	ρ	-0.32	-0.07	-0.18	0.22	0.12	0.08	-0.14	0.10
	Sig.	0.23	0.79	0.49	0.41	0.68	0.78	0.62	0.72
rRight	ρ	0.07	0.11	-0.05	0.12	0.11	0.03	-0.14	0.50
	Sig.	0.78	0.68	0.85	0.66	0.71	0.92	0.62	0.06
rCeM	ρ	-0.02	-0.13	-0.04	-0.10	0.17	0.36	0.08	0.29
	Sig.	0.94	0.66	0.88	0.74	0.54	0.19	0.78	0.29
rCeL	ρ	-0.22	-0.11	-0.28	-0.02	0.14	-0.05	-0.10	0.41
	Sig.	0.41	0.69	0.29	0.95	0.63	0.87	0.75	0.15
rCeC	ρ	0.02	0.15	-0.14	0.43	0.15	0.04	-0.21	0.47
	Sig.	0.95	0.58	0.59	0.10	0.59	0.88	0.46	0.08
iGlob	ρ	0.04	0.00	-0.07	-0.24	0.18	0.02	0.00	0.47
	Sig.	0.89	0.99	0.79	0.37	0.53	0.93	0.99	0.07
iLeft	ρ	0.07	0.12	0.04	-0.26	0.29	0.21	0.22	0.47
	Sig.	0.78	0.65	0.90	0.33	0.32	0.48	0.44	0.09
iRight	ρ	-0.03	-0.13	-0.15	0.02	0.12	-0.05	-0.25	0.40
	Sig.	0.92	0.64	0.58	0.95	0.68	0.87	0.38	0.14
iCeM	ρ	-0.16	-0.12	-0.13	0.22	0.09	-0.06	-0.27	0.42
	Sig.	0.56	0.67	0.62	0.40	0.75	0.84	0.36	0.13
iCeL*	ρ	0.08	0.01	-0.02	-0.31	0.24	0.25	0.61*	0.31
	Sig.	0.77	0.97	0.95	0.27	0.46	0.43	0.03	0.32
iCeC	ρ	0.09	0.12	-0.08	-0.09	0.12	-0.02	-0.19	0.43
	Sig.	0.74	0.66	0.77	0.75	0.67	0.95	0.51	0.12

*Table 5-9 (continued): Correlations between behavioural phenotype and c-Fos immunoreactivity following acute bladder inflammation. * denotes parameters with significant correlation r: Rostral, i: Intermediate, c: Caudal; glob: Global*

		Low Thigmotaxis				High Thigmotaxis			
		Distance travelled	Inner Zone Entry Frequency	Inner Zone Duration	Rearing	Distance travelled*	Inner Zone Entry Frequency	Inner Zone Duration*	Rearing*
cGlob	ρ	0.11	-0.02	-0.09	-0.16	0.41	0.25	0.01	0.27
	Sig.	0.67	0.93	0.75	0.56	0.12	0.37	0.97	0.33
cLeft*	ρ	0.09	0.06	0.03	-0.29	0.62*	0.41	0.05	0.48
	Sig.	0.74	0.83	0.93	0.30	0.03	0.19	0.89	0.12
cRight	ρ	0.04	-0.24	-0.29	-0.16	0.13	0.01	-0.09	-0.11
	Sig.	0.88	0.40	0.30	0.57	0.69	0.97	0.77	0.73
cCeM	ρ	0.16	-0.11	-0.23	-0.05	0.43	0.27	0.12	0.38
	Sig.	0.58	0.71	0.41	0.85	0.13	0.35	0.67	0.18
cCeL	ρ	0.04	-0.20	0.09	-0.39	0.42	0.22	-0.02	0.14
	Sig.	0.90	0.58	0.81	0.26	0.20	0.51	0.94	0.69
cCeC	ρ	-0.16	-0.41	-0.28	-0.21	0.40	0.36	0.02	0.06
	Sig.	0.64	0.20	0.40	0.54	0.23	0.28	0.96	0.87

Repeated Bladder Inflammation

Overall

Duration in the inner zone significantly correlated with c-Fos immunoreactivity in the left CeC (ρ=0.30, p=0.04).

Within Groups

In naïve animals, rearing activity was significantly correlated with c-Fos immunoreactivity in the rostral CeM (ρ=0.59, p=0.02).

Animals in the anaesthesia-only group showed significant negative correlations between duration and c-Fos immunoreactivity in the CeC (right: ρ=-0.73, p=0.04, intermediate: ρ=-0.73 p=0.04), CeL (caudal: ρ=-0.79, p=0.02), right hemisphere (overall: ρ=-0.79, p=0.02, intermediate: ρ=-0.80, p=0.02, caudal: ρ=-0.76, p=0.03), and in the intermediate (ρ=-0.71, p=0.05) and caudal (ρ=-0.76, p=0.03) levels of the central amygdala.

In instrumentation animals, duration in the inner zone was significantly correlated with c-Fos immunoreactivity in the CeM (right hemisphere: ρ=0.64, p=0.04), and rostral (ρ=0.64, p=0.04) regions of the central amygdala, with frequency also showing a correlation with activation in the rostral central amygdala (overall: ρ=0.75, p=0.01, right: ρ=0.68, p=0.03).

In turpentine animals, frequency in the inner zone was significantly correlated with c-Fos immunoreactivity in the left CeC (ρ=0.67, p=0.02), and caudal regions of the central amygdala (overall: ρ=0.68, p=0.01, left: ρ=0.72, p=0.01, CeL: ρ=0.65 p=0.02). Duration in the inner zone was also significantly correlated with c-Fos immunoreactivity overall (ρ=0.58, p=0.05), in the CeC (overall: ρ=0.60 p=0.04, caudal: ρ=0.60, p=0.04), CeL (overall: ρ=0.63, p=0.03, caudal: ρ=0.61, p=0.03), and in the rostral (right: ρ=0.60, p=0.04) and caudal (overall: ρ=0.78, p<0.001, left: ρ=0.68, p=0.01.

Looking at correlations within thigmotactic phenotypes, low thigmotactic animals showed significant negative correlations between distance travelled and left hemispheric c-Fos activation in the central amygdala (Caudal: ρ=-0.51, p=0.02, CeC: ρ=-0.56, p=0.006). In the high thigmotaxis animals, a negative correlation between rearing activity and c-Fos immunoreactivity in the CeC (ρ=-0.44, p=0.04).

Table 5-10 and Table 5-11 summarise the correlations detected in the repeated bladder inflammation model.

Key findings

- More correlations between behaviour and immunoreactivity were seen in the repeated compared to acute model of bladder inflammation
- The majority of correlations involved measures of thigmotaxis (frequency and duration in the inner zone) associated with activation in the CeC and CeL

*Table 5-10: Pearson Correlation Coefficients between behaviour and open field following repeated bladder inflammation * denotes significant correlation within parameter, † denotes significant correlation also seen in acute model*

		Naïve				AO				Instrumentation				Turpentine			
		Distance travelled	Inner Zone Entry Frequency	Inner Zone Duration	Rearing*	Distance travelled	Inner Zone Entry Frequency	Inner Zone Duration*	Rearing	Distance travelled	Inner Zone Entry Frequency*	Inner Zone Duration*	Rearing	Distance travelled	Inner Zone Entry Frequency*	Inner Zone Duration*	Rearing
LCeM	ρ	0.34	0.06	0.09	-0.02	-0.48	-0.34	-0.30	-0.70	0.12	0.19	0.24	0.16	-0.37	0.20	0.17	-0.19
	Sig.	0.22	0.83	0.74	0.94	0.23	0.41	0.47	0.12	0.75	0.59	0.51	0.65	0.24	0.53	0.59	0.56
LCeL	ρ	-0.03	0.12	-0.06	0.11	-0.25	-0.39	-0.47	0.29	0.30	0.45	0.52	-0.02	-0.17	0.52	0.36	-0.55
	Sig.	0.91	0.67	0.85	0.69	0.55	0.34	0.24	0.58	0.39	0.19	0.13	0.95	0.60	0.08	0.25	0.06
LCeC*	ρ	-0.39	0.12	0.22	0.19	-0.52	-0.44	-0.48	0.63	0.27	0.46	0.53	0.04	-0.24	0.67*	0.67*	-0.33
	Sig.	0.15	0.67	0.43	0.51	0.19	0.27	0.22	0.18	0.45	0.18	0.12	0.92	0.46	0.02	0.02	0.29
Left	ρ	-0.09	0.13	0.08	0.13	-0.61	-0.53	-0.56	0.59	0.28	0.44	0.51	0.06	-0.28	0.54	0.47	-0.47
	Sig.	0.75	0.64	0.79	0.64	0.11	0.17	0.15	0.22	0.43	0.20	0.13	0.87	0.37	0.07	0.12	0.12
RCeM*	ρ	-0.46	-0.13	0.10	-0.04	-0.56	-0.42	-0.67	-0.37	0.26	0.58	0.64*	-0.03	-0.29	0.50	0.56	0.10
	Sig.	0.08	0.64	0.72	0.88	0.15	0.29	0.07	0.47	0.46	0.08	0.04	0.93	0.36	0.10	0.06	0.76
RCeL	ρ	-0.01	0.04	0.38	0.04	-0.11	-0.18	-0.60	0.59	0.13	0.31	0.43	0.17	-0.45	0.36	0.57	0.09
	Sig.	0.96	0.89	0.16	0.89	0.80	0.67	0.12	0.22	0.71	0.38	0.22	0.63	0.14	0.25	0.05	0.78
RCeC*†	ρ	0.07	0.31	0.14	-0.08	-0.52	-0.40	-0.73*	0.55	0.58	0.44	0.10	-0.29	-0.52	0.15	0.28	-0.02
	Sig.	0.80	0.26	0.61	0.77	0.19	0.33	0.04	0.25	0.08	0.20	0.79	0.42	0.08	0.63	0.38	0.95
Right*†	ρ	-0.02	0.26	0.27	-0.07	-0.51	-0.42	-0.79*	0.68	0.55	0.49	0.24	-0.17	-0.50	0.26	0.44	0.03
	Sig.	0.95	0.34	0.32	0.79	0.20	0.30	0.02	0.14	0.10	0.15	0.50	0.64	0.10	0.42	0.16	0.93

*Table 5.10 (continued): Pearson Correlation Coefficients between behaviour and open field following repeated bladder inflammation. * denotes significant correlation within parameter, † denotes significant correlation also seen in acute model*

		Naïve				AO				Instrumentation				Turpentine			
		Dist.	Freq	Duration	Rearing*	Dist.	Freq	Durati	Rearing*	Dist.	Freq	Duration	Rearing*	Dist.	Freq	Duration	Rearing*
CeM	ρ	-0.21	-0.04	0.14	-0.07	-0.53	-0.39	-0.47	-0.62	0.13	0.36	0.46	-0.07	-0.05	0.42	0.48	-0.14
	Sig.	0.45	0.88	0.62	0.80	0.17	0.34	0.25	0.19	0.72	0.31	0.18	0.84	0.87	0.17	0.11	0.67
CeL*	ρ	0.04	0.28	0.27	0.03	-0.18	-0.29	-0.56	0.54	0.25	0.46	0.56	-0.23	-0.24	0.57	0.63*	-0.33
	Sig.	0.89	0.32	0.33	0.92	0.66	0.48	0.15	0.27	0.49	0.18	0.09	0.51	0.45	0.05	0.03	0.29
CeC*†	ρ	-0.08	0.33	0.21	-0.01	-0.55	-0.45	-0.64	0.69	0.50	0.52	0.32	-0.43	-0.13	0.52	0.60*	-0.31
	Sig.	0.79	0.24	0.46	0.96	0.16	0.27	0.08	0.13	0.14	0.12	0.37	0.21	0.69	0.08	0.04	0.32
Global*	ρ	-0.06	0.26	0.24	-0.01	-0.58	-0.50	-0.70	0.71	0.36	0.51	0.49	-0.31	-0.23	0.48	0.58*	-0.32
	Sig.	0.84	0.34	0.39	0.98	0.13	0.21	0.05	0.12	0.31	0.13	0.15	0.39	0.48	0.11	0.05	0.31
Rostral*	ρ	0.07	0.23	0.28	0.12	-0.51	-0.37	-0.43	-0.62	0.61	0.75*	0.64*	-0.31	-0.03	0.32	0.40	-0.03
	Sig.	0.81	0.40	0.31	0.67	0.19	0.37	0.28	0.19	0.06	0.01	0.04	0.39	0.92	0.31	0.20	0.92
rLeft	ρ	0.07	0.16	0.21	0.10	-0.53	-0.41	-0.36	-0.68	0.27	0.37	0.41	-0.08	-0.41	-0.29	-0.30	0.10
	Sig.	0.81	0.57	0.45	0.73	0.17	0.32	0.38	0.14	0.44	0.29	0.23	0.83	0.19	0.36	0.35	0.76
rRight*	ρ	-0.01	0.09	0.05	0.30	-0.44	-0.26	-0.52	-0.35	0.57	0.68*	0.50	-0.25	-0.13	0.56	0.60*	0.05
	Sig.	0.99	0.76	0.85	0.27	0.28	0.53	0.18	0.49	0.09	0.03	0.14	0.48	0.69	0.06	0.04	0.88
rCeM*	ρ	-0.21	0.09	-0.04	0.59*	-0.60	-0.43	-0.42	--	0.20	0.49	0.55	-0.04	0.05	0.31	0.39	-0.06
	Sig.	0.45	0.74	0.90	0.02	0.11	0.28	0.30	--	0.57	0.15	0.10	0.91	0.88	0.33	0.21	0.85
rCeL	ρ	0.30	0.29	0.30	-0.08	0.38	0.35	-0.02	-0.53	0.27	0.35	0.49	-0.12	-0.25	-0.12	-0.05	0.06
	Sig.	0.28	0.29	0.28	0.79	0.36	0.40	0.97	0.28	0.46	0.32	0.16	0.75	0.43	0.70	0.87	0.86
rCeC	ρ	-0.19	0.27	0.32	0.25	-0.54	-0.42	-0.48	-0.66	0.63	0.38	0.01	-0.36	0.02	0.01	-0.01	-0.08
	Sig.	0.49	0.34	0.25	0.38	0.17	0.30	0.22	0.15	0.05	0.28	0.97	0.31	0.96	0.98	0.98	0.80

*Table 5.10 (continued): Pearson Correlation Coefficients between behaviour and open field following repeated bladder inflammation. * denotes significant correlation within parameter, † denotes significant correlation also seen in acute model*

		Naïve				AO				Instrumentation				Turpentine			
		Dist.	Freq	Dur.	Rear *	Dist.	Freq	Dur.	Rear *	Dist.	Freq	Dur.	Rear *	Dist.	Freq	Dur.	Rear *
Inter.*†	ρ	-0.14	-0.01	0.11	0.31	-0.51	-0.51	-0.71*	-0.53	-0.03	0.01	0.16	-0.01	-0.03	0.36	0.37	-0.26
	Sig.	0.61	0.96	0.68	0.25	0.19	0.20	0.05	0.28	0.93	0.99	0.66	0.97	0.93	0.25	0.23	0.41
iLeft	ρ	0.01	-0.09	-0.03	0.17	-0.49	-0.54	-0.59	-0.68	0.06	0.09	0.21	0.11	-0.03	0.49	0.31	-0.55
	Sig.	0.96	0.76	0.91	0.54	0.22	0.16	0.12	0.14	0.87	0.80	0.57	0.76	0.92	0.11	0.32	0.06
iRight	ρ	-0.21	-0.08	0.23	0.24	-0.47	-0.40	-0.80*	-0.16	0.10	0.27	0.38	0.00	-0.54	0.25	0.34	0.15
	Sig.	0.46	0.77	0.41	0.38	0.24	0.33	0.02	0.76	0.77	0.46	0.27	0.99	0.07	0.43	0.28	0.64
iCeM	ρ	0.27	0.19	0.32	-0.09	-0.36	-0.24	-0.33	-0.67	-0.11	-0.11	0.02	-0.10	-0.46	-0.03	0.07	0.14
	Sig.	0.33	0.51	0.24	0.75	0.38	0.56	0.42	0.15	0.76	0.77	0.95	0.79	0.14	0.93	0.84	0.65
iCeL	ρ	0.05	0.02	-0.05	0.35	-0.46	-0.53	-0.64	-0.47	0.07	0.13	0.24	0.09	0.00	0.43	0.54	0.00
	Sig.	0.87	0.94	0.85	0.21	0.25	0.17	0.09	0.35	0.84	0.72	0.50	0.81	0.99	0.17	0.07	0.99
iCeC*	ρ	0.00	-0.08	0.13	-0.05	-0.47	-0.41	-0.73*	-0.43	-0.06	0.01	0.20	-0.15	0.12	0.21	0.21	-0.28
	Sig.	0.99	0.79	0.65	0.85	0.24	0.31	0.04	0.39	0.87	0.98	0.59	0.68	0.70	0.51	0.51	0.38
Caudal*†	ρ	0.05	0.29	0.27	-0.23	-0.65	-0.57	-0.76*	0.70	0.18	0.41	0.53	-0.19	-0.13	0.68*	0.78*	-0.25
	Sig.	0.86	0.29	0.33	0.42	0.08	0.14	0.03	0.12	0.63	0.24	0.12	0.59	0.68	0.01	0.00	0.44
cLeft*†	ρ	-0.10	0.20	0.10	0.16	-0.58	-0.57	-0.67	0.70	0.26	0.43	0.50	0.01	-0.15	0.72*	0.68*	-0.28
	Sig.	0.73	0.47	0.73	0.56	0.13	0.14	0.07	0.12	0.47	0.21	0.14	0.97	0.64	0.01	0.01	0.38
cRight	ρ	0.14	0.33	0.25	-0.19	-0.61	-0.53	-0.76*	0.57	0.05	0.17	0.23	0.17	-0.34	0.36	0.56	0.05
	Sig.	0.63	0.23	0.36	0.50	0.11	0.17	0.03	0.24	0.89	0.64	0.52	0.64	0.29	0.25	0.06	0.89
cCeM	ρ	-0.29	-0.26	-0.02	0.03	-0.66	-0.51	-0.63	-0.31	0.19	0.42	0.55	0.08	0.17	0.14	0.14	-0.18
	Sig.	0.30	0.34	0.94	0.90	0.08	0.20	0.09	0.55	0.60	0.23	0.10	0.83	0.60	0.67	0.66	0.58
cCeL*	ρ	0.10	0.32	0.36	-0.23	-0.59	-0.56	0.79*	0.51	0.12	0.23	0.22	-0.44	-0.19	0.65*	0.61*	-0.41
	Sig.	0.73	0.24	0.18	0.42	0.13	0.15	0.02	0.30	0.75	0.52	0.54	0.20	0.56	0.02	0.03	0.19
cCeC*	ρ	0.02	0.24	0.13	-0.13	-0.62	-0.55	-0.67	0.67	0.08	0.21	0.22	-0.27	-0.48	0.36	0.60*	0.04
	Sig.	0.94	0.38	0.64	0.64	0.10	0.16	0.07	0.14	0.83	0.56	0.55	0.45	0.11	0.25	0.04	0.90

5-11: Correlation coefficients between behavioural phenotype and c-Fos activation in the central amygdala

		Low Thigmotaxis				High Thigmotaxis			
		Dist.*	Freq	Duration	Rearing*	Dist.	Freq	Duration	Rearing*
LCeM	ρ	-0.05	-0.13	-0.03	0.13	0.03	0.04	0.12	-0.01
	Sig.	0.82	0.56	0.89	0.58	0.88	0.84	0.60	0.95
LCeL	ρ	-0.31	-0.08	-0.13	0.05	0.06	-0.13	-0.09	-0.04
	Sig.	0.16	0.74	0.55	0.81	0.77	0.56	0.70	0.86
LCeC*	ρ	**-0.56***	-0.04	0.13	0.16	-0.08	0.08	0.16	-0.02
	Sig.	0.01	0.85	0.55	0.48	0.71	0.72	0.47	0.94
Left	ρ	-0.41	-0.09	-0.03	0.15	-0.02	-0.03	0.07	-0.06
	Sig.	0.06	0.68	0.89	0.51	0.93	0.88	0.76	0.81
RCeM	ρ	-0.38	0.05	0.22	0.21	0.01	0.08	0.15	-0.05
	Sig.	0.09	0.81	0.33	0.35	0.98	0.71	0.49	0.83
RCeL	ρ	-0.25	0.01	0.32	0.21	-0.35	-0.40	-0.28	0.03
	Sig.	0.25	0.97	0.15	0.34	0.10	0.06	0.20	0.91
RCeC	ρ	0.12	0.11	-0.14	0.04	-0.14	-0.18	-0.05	-0.13
	Sig.	0.58	0.64	0.53	0.85	0.52	0.40	0.80	0.56
Right	ρ	-0.03	0.11	0.03	0.14	-0.24	-0.25	-0.10	-0.10
	Sig.	0.89	0.62	0.88	0.55	0.28	0.26	0.64	0.66
CeM	ρ	-0.24	-0.03	0.10	0.19	0.09	0.24	0.38	-0.3
	Sig.	0.28	0.90	0.65	0.38	0.70	0.26	0.08	0.18
CeL	ρ	-0.27	0.04	0.07	0.16	-0.14	-0.13	0.04	-0.36
	Sig.	0.22	0.87	0.75	0.47	0.53	0.54	0.85	0.11
CeC	ρ	-0.12	0.08	-0.07	0.11	-0.07	0.16	0.36	-0.44
	Sig.	0.58	0.74	0.76	0.63	0.74	0.47	0.09	0.04
Global*	ρ	-0.23	0.04	0.02	0.19	-0.11	0.04	0.26	**-0.43***
	Sig.	0.29	0.86	0.92	0.40	0.61	0.85	0.23	0.05

5-11 (continued): Correlation coefficients between behavioural phenotype and c-Fos activation in the central amygdala

		Low Thigmotaxis				High Thigmotaxis			
		Dist.*	Freq	Dur.	Rear*	Dist.	Freq	Dur.	Rear*
Rostral	ρ	-0.11	0.01	0.10	0.27	0.06	0.15	0.18	-0.31
	Sig.	0.63	0.98	0.67	0.22	0.77	0.49	0.41	0.17
rLeft	ρ	0.00	-0.09	0.04	0.41	0.03	-0.02	0.00	-0.21
	Sig.	0.98	0.69	0.84	0.06	0.88	0.91	0.99	0.35
rRight	ρ	-0.22	0.05	0.03	0.07	-0.06	0.15	0.04	-0.13
	Sig.	0.34	0.83	0.88	0.76	0.78	0.51	0.84	0.56
rCeM2	ρ	-0.33	0.01	0.06	0.33	-0.03	0.21	0.26	-0.13
	Sig.	0.13	0.96	0.79	0.13	0.88	0.33	0.23	0.58
rCeL2	ρ	0.08	-0.04	0.07	0.26	0.06	0.03	0.01	-0.34
	Sig.	0.72	0.86	0.76	0.25	0.78	0.91	0.97	0.13
rCeC2	ρ	0.14	-0.10	-0.18	0.14	0.02	0.17	0.20	-0.16
	Sig.	0.54	0.65	0.42	0.52	0.94	0.45	0.35	0.49
Interm.	ρ	-0.22	-0.18	-0.06	0.26	0.04	0.04	0.20	-0.13
	Sig.	0.33	0.42	0.81	0.24	0.85	0.87	0.36	0.59
iLeft	ρ	-0.15	-0.17	-0.07	0.06	0.16	-0.07	0.03	0.07
	Sig.	0.52	0.45	0.76	0.80	0.48	0.76	0.90	0.77
iRight	ρ	-0.22	-0.07	0.12	0.27	-0.22	-0.20	-0.05	0.26
	Sig.	0.32	0.76	0.59	0.23	0.32	0.36	0.81	0.25
iCeM	ρ	-0.01	-0.05	0.17	0.29	-0.09	0.00	-0.01	-0.34
	Sig.	0.95	0.82	0.46	0.19	0.68	1.00	0.95	0.13
iCeL	ρ	-0.13	-0.11	-0.06	0.17	0.00	0.01	0.13	0.11
	Sig.	0.58	0.62	0.80	0.44	0.98	0.97	0.54	0.63
iCeC	ρ	-0.15	-0.05	0.03	-0.13	0.07	0.17	0.32	-0.21
	Sig.	0.49	0.81	0.90	0.57	0.75	0.44	0.13	0.35
Caudal	ρ	-0.35	0.05	0.13	0.00	-0.09	0.12	0.31	-0.38
	Sig.	0.11	0.84	0.56	0.99	0.67	0.58	0.16	0.09
cLeft	ρ	-0.51	0.00	0.05	0.00	-0.03	0.16	0.16	0.30
	Sig.	0.02	0.98	0.83	0.99	0.88	0.47	0.46	0.19
cRight	ρ	-0.05	0.22	0.15	0.15	-0.14	-0.21	-0.09	-0.19
	Sig.	0.84	0.33	0.50	0.50	0.52	0.34	0.70	0.41
cCeM	ρ	-0.30	-0.15	0.02	-0.09	0.08	0.26	0.38	-0.09
	Sig.	0.18	0.50	0.92	0.69	0.73	0.24	0.08	0.69
cCeL*	ρ	-0.21	0.15	0.13	-0.01	-0.16	-0.04	0.08	**-0.43***
	Sig.	0.35	0.52	0.58	0.97	0.48	0.86	0.73	0.05
cCeC	ρ	-0.24	0.11	0.06	0.18	-0.29	-0.11	0.11	-0.31
	Sig.	0.27	0.62	0.80	0.41	0.18	0.63	0.62	0.18

6 Results - Study 3: Stavudine-associated Neuropathy in Female Rats

To induce anti-retroviral associated neuropathy, two intravenous tail vein injections of Stavudine (d4T) were given to adult female Wistar rats under isoflurane anaesthesia. Control animals received an equal volume of sterile 0.9% saline solution (B. Braun Melsungen AG, Germany), and naïve animals were untreated but similarly handled. Baseline evoked hypersensitivity measurements to mechanical, thermal, and cold stimuli were taken prior to d4T treatment, and on days 4, 7, 10, and 13 post-injection. Animals were subsequently exposed to the open field paradigm for 15 minutes on day 14 post-injection, and behaviour analysed for thigmotaxis.

Exclusions

One animal was excluded from the naïve group due to technical failure during video capture of open field behaviour. *A priori* exclusion criteria for evoked measures were discarded when no significant effect of treatment was observed, and results are reported for the entire data set.

6.1 Mechanical Hypersensitivity

Electronic von Frey apparatus was used to test hind paw hypersensitivity to mechanical stimuli. Each data point represents an average of five measurements.

No significant effect of d4T treatment on mechanical hypersensitivity was found (2-way RM ANOVA p=0.685 left, p=0.367 right), and there was no interaction between group and time-point (p=0.820 left, p=0.983 right). There was an effect of time-point (p<0.001), with all animals reducing baseline mechanical withdrawal from 48.42g (45.27-51.57), 50.90g (47.17-54.62), and 50.35 (47.05-53.63), to 40.79g (35.82-45.74), 42.33g (37.94-46.71), and 44.82g (40.34-49.30) at 4 days post-injection in naïve, saline, and d4T animals respectively (Figure 6-1).

6.2 Thermal Hypersensitivity

Hargreaves apparatus was used to investigate thermal withdrawal thresholds, with each data point representing an average of three measurements.

No significant differences in thermal hypersensitivity were detected due to group (p=0.351 left, p=0.867 right), time-point (p=0.380 left, p=0.090 right), or an interaction between the two (p=0.902 left, p=0.264 right). Baseline and day 13 withdrawal latencies were 11.0s (10.1-11.8) and 11.5s (10.112.8) for naïve animals, 11.9s (10.9-12.9) and 11.2s (10.2-12.2) for the saline group, and 11.1s (10.3-11.9) and 11.2s (10.2-12.3) in the d4T treated group (Figure 6-1).

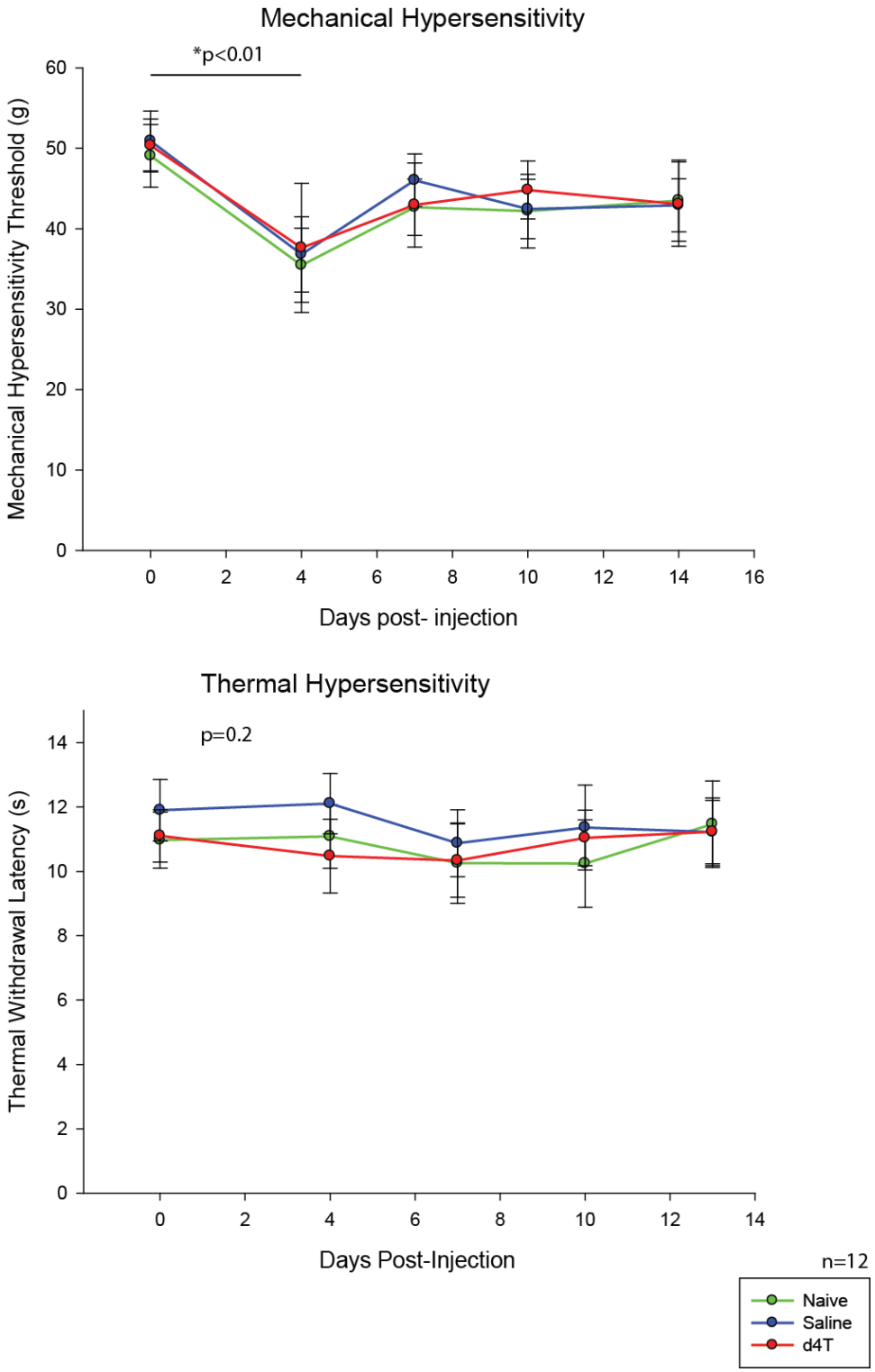


Figure 6-1: Mechanical and thermal hypersensitivity following d4T treatment

All groups showed reduced mechanical thresholds on the first day of testing (mean ± 95% CI). There were no differences in thermal hypersensitivity Data analyses conducted using 1-way Repeated Measures ANOVA

6.3 Cold Hypersensitivity

A drop of acetone was applied to each hind paw in turn, and flinch responses frequency noted.

Each data point represents an average of five measurements.

No flinch responses to acetone were observed (p=1 for all tests performed, data not shown).

6.4 Open Field Behaviour

No differences in open field behaviour were associated with d4T treatment in female rats.

Frequency of entry to the inner zone was 17 (8-21) in naïve animals, 12 (8-21) in saline vehicle, and 14.5 (12.5-20) in d4T (1-way ANOVA on ranks p=0.56). Duration in the inner zone was not significantly altered (1-way ANOVA p=0.607), with naïve animals spending 23.4s (9.8-25.6), compared to 7.1s (15.5-22.3) in vehicle, and 17.4s (22.16-26.24) in d4T groups (Figure 6-2). Rearing behaviour was also not significantly different between groups (1-way ANOVA on ranks p=0.174), with naïve animals rearing a median of 79 (61.5-82.5) times, compared to 66 (58-80) in saline, and 59 (52-71) in d4T treated animals. Distance travelled was 8847.99cm (7665.70-10030.28) in naïve animals, 8967.27cm (8063.93-9870.61) in saline vehicle, and 9254.44cm (8482.54-10026.34) in the d4T group (1-way ANOVA p=0.793) (Figure 6-3).

Key findings

- No evidence of mechanical, thermal, or cold hypersensitivity within 14 days of d4T antiretroviral
- No effect of d4T on open field behaviour
- Sex differences could explain unexpectedly negative result

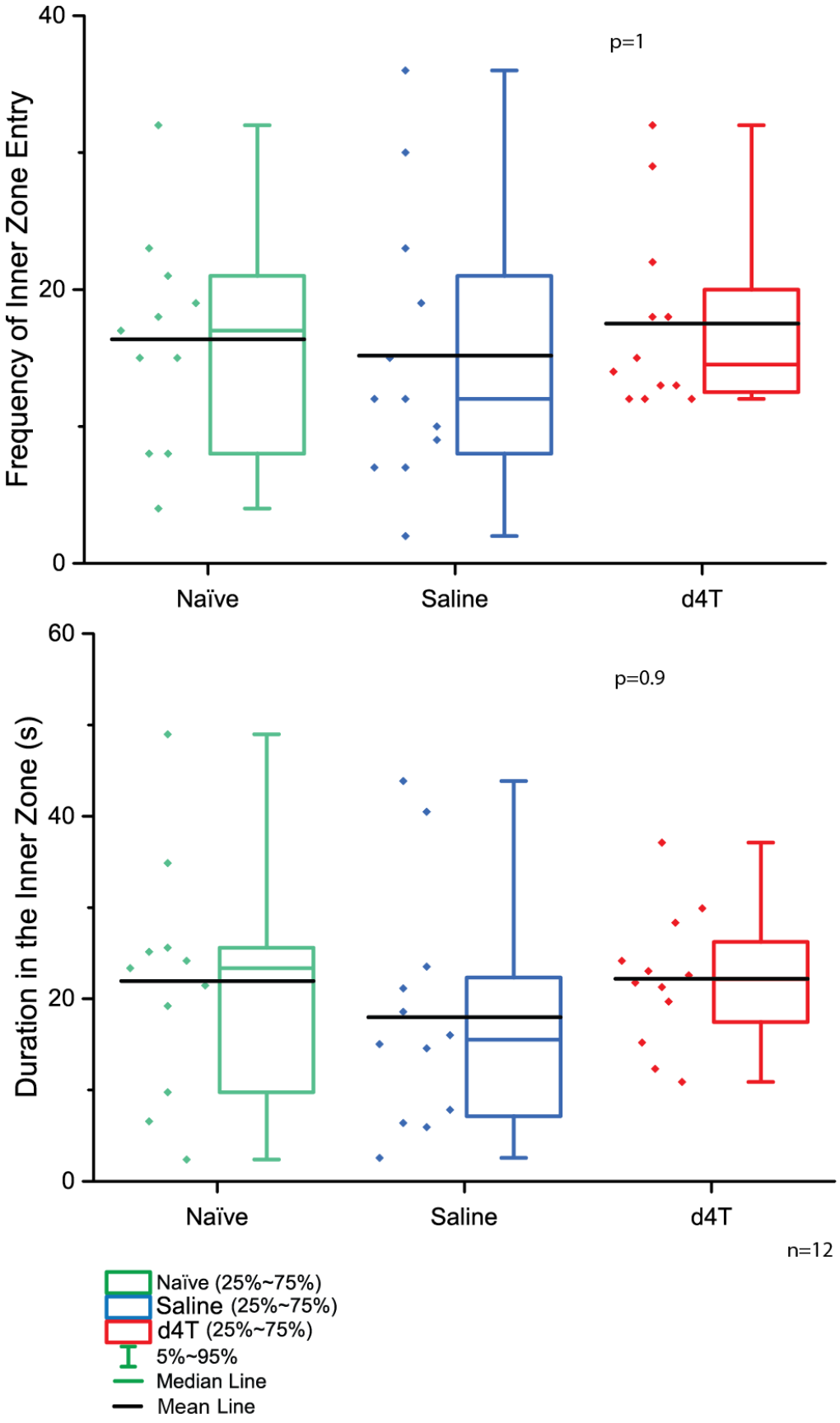


Figure 6-2: Inner zone activity (frequency and duration) in the open field following d4T treatment No significant differences were observed. Data analysed using 1-way Kruskal-Wallis ANOVA on Ranks (frequency) and 1-way ANOVA (duration)

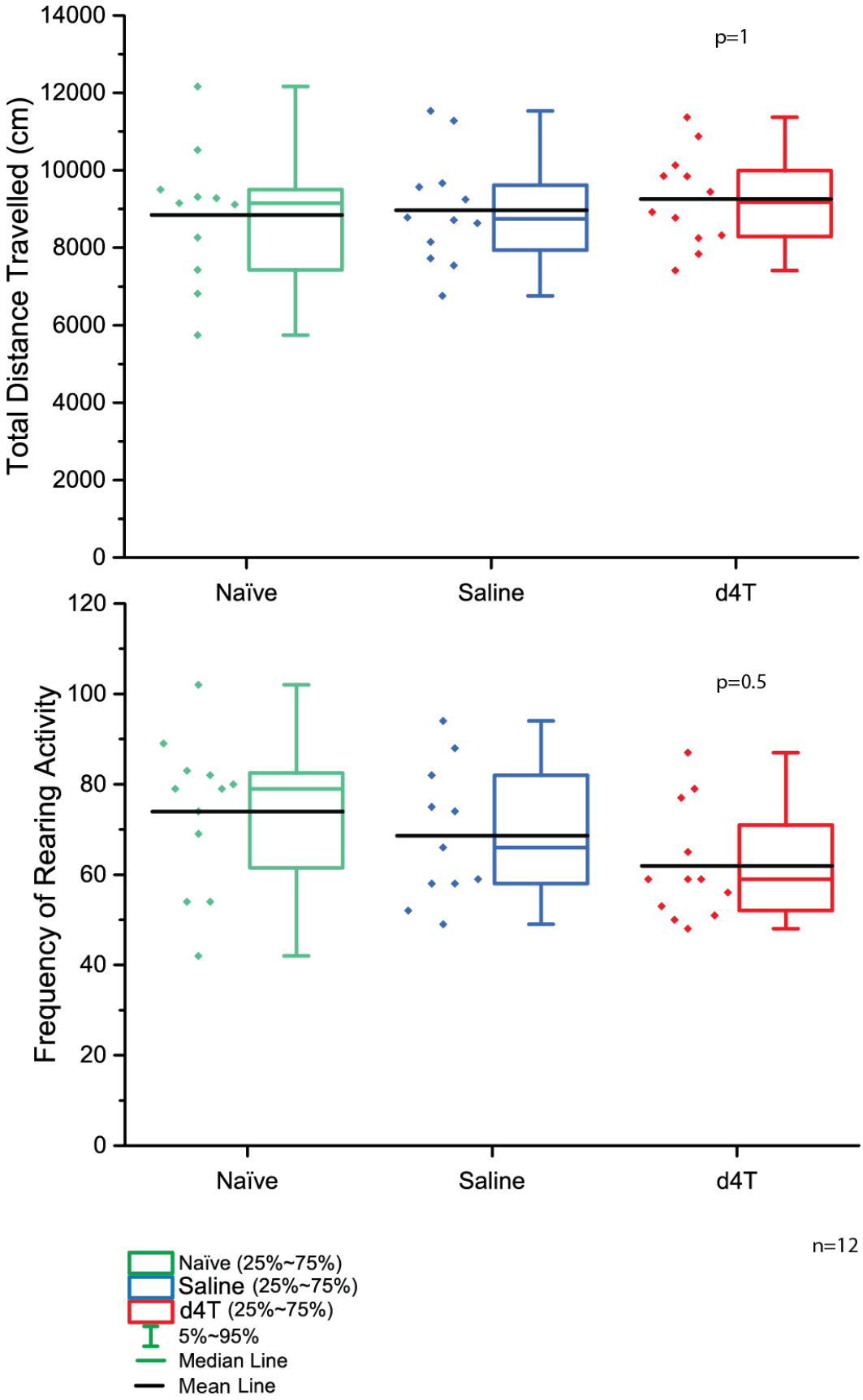


Figure 6-3: Total distance travelled and rearing activity in the open field following d4T treatment No significant differences were observed. Data analysed using 1-way ANOVA (distance) and 1- way Kruskal-Wallis ANOVA on Ranks (rearing)

7 Discussion

These studies have shown robust behavioural phenotypes are present in naïve rats in terms of open field behaviour. The data also indicates catheterisation, with or without turpentine-induced bladder inflammation is capable of inducing thigmotaxis, and this is not accompanied by clear changes in amygdala activation, but is associated with robust upregulation of inflammatory mediators. There was no effect of bladder inflammation on burrowing behaviour. Unexpectedly, Stavudine treatment in female rats did not cause the hypersensitivity and thigmotactic alterations observed in male rats.

7.1 Repeated Open Field

The Open Field paradigm was developed to detect behavioural alterations in the rat associated with emotionality (Walsh & Cummins, 1976). This study was designed to investigate how the behaviour of male Wistar rats changes with repeated exposure to the open field. The primary outcome of interest was thigmotaxis, a predation risk avoidance behaviour characterised by preference for movement close to the walls. Thigmotaxis is an ethological outcome measure, investigating the balance between exploratory drive and heightened risk awareness associated with a vulnerable physical state, e.g. pain. By looking at behaviour in naïve animals, it is possible to characterise naïve behaviour, and determine whether all animals behave in the same way, and if not, characterise behavioural phenotypes present.

As the Open Field has been used for over 80 years, there are a number of outcomes measures that have been used. Table 7-1 summarises the most common outcomes measures used in the open field.

Table 7-1: Summary of outcome measures commonly used in the Open Field Paradigm

Outcome		**Behaviour**	**Primary Detection Method**
General Field Activity	Distance Travelled	General Activity	Automated
	Velocity	General Activity	Automated
	Rearing	Exploratory/Escape / High Arousal	Manual
	Grooming	Arousal	Manual
	Freezing	Fear	Automated
	Sleeping/Immobility	Low Arousal	Automated/Manual
	Faecal Boli	'Emotionality'	Manual
Inner Zone activity	Distance Travelled	Thigmotaxis / 'Timidity' / 'Anxiety'	Automated
	Frequency of Entry	Thigmotaxis / 'Timidity' / 'Anxiety'	Automated
	Duration in Zone	Thigmotaxis / 'Timidity' / 'Anxiety'	Automated
	Velocity	Thigmotaxis / 'Timidity' / 'Anxiety'	Automated

In naïve animals repeatedly exposed to the open field, behavioural outcomes decreased with exposure, as previously reported (Walsh & Cummins, 1976). To minimise this effect, I designed this study to maximise time between exposures, and minimise the number of exposures required.

Four behavioural phenotypes were identified, based on initial thigmotactic phenotype, and how that changed over time. There were a minority of animals that consistently exhibited either high (n=5) or low (n=3) thigmotaxis, but in the majority of animals, thigmotaxis was initially low, increasing over time. The speed of this decrease distinguishes the two majority phenotypes as slow (n=8) or rapid (n=10) thigmotaxis, based primarily on thigmotactic activity during their second exposure to the open field. This distribution pattern, with a minority exhibiting extreme behaviours and the majority occupying an intermediate phenotype was also noted in much earlier open field studies into the non-specific excitability level of rats in the open field (Lát & Gollová-Hémon, 1969).

One of the purposes of conducting this study was to investigate the feasibility of utilising the open field paradigm in a test-re-test manner, allowing a 'baseline' session, during which behavioural phenotype can be assessed, prior to testing. This would improve understanding of how different stimuli influence open field behaviour, and whether all behavioural phenotypes react in the same way. Numerous studies have shown biochemical differences between animals with high and low innate activity levels, suggestive of these being trait rather than state characteristics (Cools *et al.*, 1993; Borta & Schwarting, 2005; Mällo *et al.*, 2007). The noted changes in phenotype during the first two open field sessions is in keeping with early work suggesting a change in factor loading of ambulation during initial exposure (Whimbey & Denenberg, 1967b), and based on this, it would be advantageous to test open field behaviour on two consecutive days prior to intervention, with a further two sessions occurring over the 48 hours following bladder inflammation. This time period is within the current Home Office project license permissions relating to induction of bladder inflammation, and would allow investigation of the time-course of the behavioural response to such stimuli. Baseline open field testing, preferably over 2 sessions, would allow prior behavioural phenotyping of the experimental animals, which could then be randomised to treatment in a balanced manner. This behavioural phenotyping would allow distinction of effects due to experimental pain from innate variation in trait behavioural phenotype, and enable researchers to investigate how individual differences influence behavioural outcome. This is important in pain research as the factors that predispose an individual to developing chronic pain are thought to involve complex interplay between physiological factors and psychological profile.

When behavioural phenotype based on frequency of inner zone activity was applied, this produced sub-groups that were different both in terms of thigmotaxis, and other behaviours such as total distance travelled, velocity, and rearing. These subgroups may reflect innate differences in trait phenotype.

Thigmotactic outcomes of frequency and duration in the open field showed greatest predictive value between weeks one and two, with the majority of animals exhibiting a consistent thigmotaxis phenotype. However, there was a group of animals ("rapid thigmotaxis") that dramatically reduced their inner zone activity between week 1 and 2, and this could skew attempts to interpret the effect of any intervention between open field exposures, as some animals would naturally show reduced thigmotaxis. However, as significant differences in thigmotaxis are detectable at an overall level, this is unlikely to be a deciding factor, although future studies into how experimental pain affects repeated open field exposure would increase understanding of how different behavioural phenotypes respond to nociceptive stimuli.

This study indicated duration in the open field showed the greatest consistency across re-testing, with strong correlations seen between consecutive testing, and also between initial and second exposure. However, experimental findings from this lab have shown frequency is more reliably modulated by experimental pain states (Wallace *et al.*, 2007b; Huang *et al.*, 2013), suggesting further investigation into the relationship between frequency and duration is warranted.

The fact that correlations were strengthened by repeated exposure to the open field strengthens the assertion that the behavioural phenotypes are indicative of trait differences.

Strong correlations were seen between both velocity and distance travelled, and duration and frequency in the inner zone, indicative of a link between these behaviours.

Additionally, rearing activity was shown to be robust and stable, with differences noted in freestanding and wall-supported rearing. It is likely that these two forms of rearing are representative of exploration and escape attempts respectively, particularly as wall-supported rearing was shown to be higher when the open field apparatus featured lower walls, i.e. increasing the potential for escape (Walsh & Cummins, 1976).

7.2 Bladder Inflammation

Bladder inflammation induced via instillation of a 50% turpentine solution has been shown to cause referred somatic hyperalgesia and bladder hyper-reflexia (Rice, 1995; Jaggar *et al.*, 1999), but until this study, investigations into affective alterations associated with this model of visceral inflammation were lacking. Therefore, this study was designed to investigate the presence of affective changes, such as increased thigmotactic behaviour in the open field, or decreased burrowing activity, and determine whether correlations between neural activation of the amygdala (as measured by c-Fos immunoreactivity) and behaviour are present. Levels of inflammatory mediators in the bladder were determined in order to provide a measure of insult severity, and

demonstrate the presence of nociceptive stimuli in the absence of measuring evoked hypersensitivity.

Bladder inflammation with turpentine, both acute and repeated, had effects on both inflammatory mediator levels in bladder tissue and activity in the open field. Additionally, there was a strong effect seen in the instrumentation group, with inflammatory changes and alterations in open field behaviour present, suggesting catheterization alone is responsible for part of the effect seen in the turpentine group.

Up-regulation of inflammatory mediators was observed in both acute and repeated models of visceral inflammation. Bladder weights were increased in both models, with the magnitude increase in total RNA higher following repeated bladder inflammation, likely associated with the extended duration, as changes were also seen in naïve animals. The magnitude of fold change, and the number of mediators affected was higher in the acute model compared to the repeated, suggesting a possible habituation to inflammatory stimuli occurs. The difference in expression could also reflect bladder re-modelling in response to repeated inflammatory stimuli (Tseng *et al.*, 2009), with remodelling seen in both urothelial (Lucon *et al.*,) and sensory innervation (Forrest *et al.*, 2013). Alternatively, it could simply be associated with the presence of a non-linear relationship between RNA and protein levels (Heumann *et al.*, 1984; Gygi *et al.*, 1999; Chen, 2002). In the rat, compared to a single acute treatment, repeated i.p. injections of LPS decrease the responsiveness of ACTH and corticosterone, decreased the corticosterone response to stress, and increased expression of vasopressin (AVP) in parvocellular neurons (Grinevich *et al.*, 2001), with similar suppression observed in studies on lung tissue following repeated ovalbumin, specifically decreased in inflammatory cell involvement (e.g. neutrophils & eosinophils) (Tigani *et al.*, 2007).

CCL-12 is up-regulated in both acute and repeated turpentine inflammation, and also following repeated instrumentation, suggesting involvement in a general inflammatory response, rather than irritant-specific (i.e. turpentine). It is also known as monocyte chemotactic protein (MCP-5) and acts during the early stages of inflammation to attract eosinophils, monocytes, and lymphocytes (Lam *et al.*, 2013a) as shown in Figure 7-1. Production by macrophages is stimulated by interleukin (IL)-13 (Cho *et al.*, 2006), and NGF (Susaki *et al.*, 1996), and it is strongly up-regulated by infection (Benítez-Hernández *et al.*, 2010; Li *et al.*, 2014a). IL-7 aggravation of collagen-induced arthritis is associated with CCL-12 up-regulation (Hartgring *et al.*, 2012), and in another study looking at thioglycollate-elicited peritonitis in mice, it was found that CCL-12 is up-regulated at three days post-insult, although it is worth noting CCL-12 was also one of the few mediators investigated

which had baseline detection levels above zero (Lam *et al.*, 2013a), suggesting the presence of constant low-level activation.

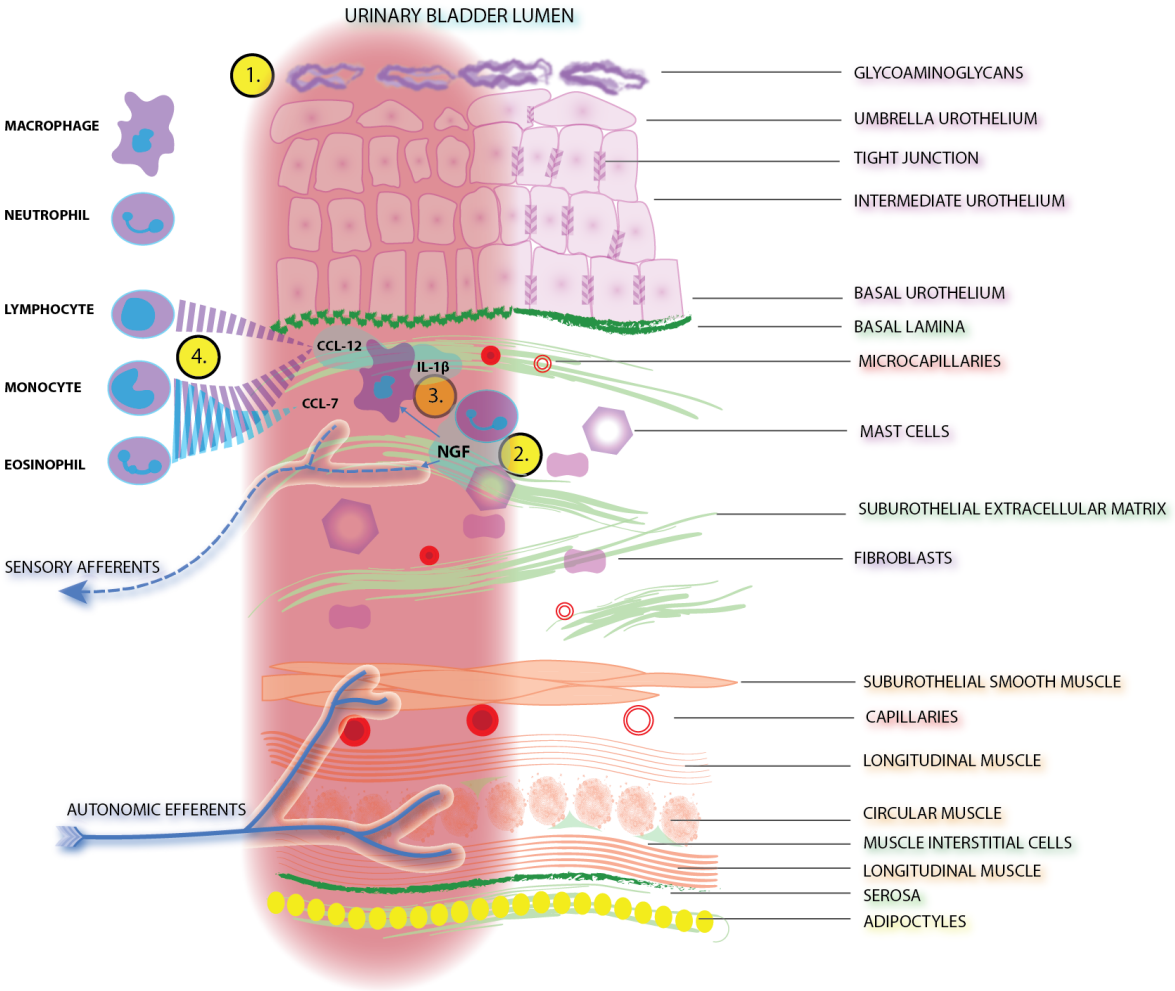


Figure 7-1: Inflammation in the bladder, comparing inflamed (red/left) and normal (right). 1) Integrity of tight junctions and basal lamina is lost; 2) NGF is produced by mast cells and neutrophils, which activates sensory nerve endings and 3) macrophages to produce IL-1β and CCL-12; 4) CCL-12 and CCL-7 attract monocytes, eosinophils, and lymphocytes (CCL-12 only).

In this study, CCL-12 was most highly up-regulated in the acute turpentine group (149 fold change, FC), although levels seen in the repeated turpentine (120FC) and repeated instrumentation (109FC) were very similar, and as the acute and repeated RT-qPCR array cards were not run simultaneously, it is likely that up-regulation of CCL-12 is not significantly different across all groups, and associated with a general inflammatory response to trauma due to catheterisation, or some other aspect of the surgical induction such as anaesthesia.

The other inflammatory mediators significantly up-regulated in both turpentine groups were CCL-7, and IL-1β.

CCL-7 is also a monocyte attractant (AKA MCP-3) as seen in Figure 7-1, and is associated with various conditions involving inflammatory and immune processes. It is one of a number of cytokines associated with a poor prognosis in gastric cancer patients (Chang *et al.*, 2014), is up-regulated serum from asthma patients (Pelikan, 2013), and found in increased levels in fibroblasts from patients with systemic sclerosis
(Distler *et al.*, 2009). CCL-7 has been suggested as a biomarker for IC/BPS (Corcoran *et al.*, 2013), and OAB (Ghoniem *et al.*, 2011). However, a study has also shown that a common treatment for nonmuscle invasive bladder cancer, bacillus Calmette-Guérin (BCG, from *Mycobacterium bovis*), upregulates a number of cytokines including CCL-7, which was still up-regulated 4 weeks following instillation (Seow *et al.*, 2008). Furthermore, it is also up-regulated in vaginal (Woo *et al.*, 2007) and urethral (Woo *et al.*, 2009) tissue following a simulated childbirth model involving vaginal distension. As cross-sensitisation has previously been observed between pelvic organs (Ustinova *et al.*, 2006), it is interesting that the first study (Woo *et al.*, 2007) also noted no up-regulation in bladder or rectal tissue.

The presence of significant up-regulation of CCL-7 following turpentine inflammation (acute: 18FC, repeated: 5FC), with very low levels detected in the repeated instrumentation group is in keeping with turpentine producing a cystitis-like state, and suggests the acute model is associated with higher levels of immune activation.

IL-1β is highly up-regulated following acute turpentine inflammation (273FC), and was less so but still significantly increased in the repeated turpentine group (7FC). It is an important mediator of the inflammatory process and is produced by macrophages in autoimmune disorders (Chen & Meckfessel, 2013), CRPS (Cooper & Clark, 2013), and neuropathic pain (Ramesh *et al.*, 2013). It is also associated painful bladder conditions such as IC/PBS (Schrepf *et al.*, 2014), is up-regulated in tissue from CYP treated mice (Girard *et al.*, 2008), and looking at other painful pelvic conditions, IL1- β is significantly up-regulated in menstrual blood from a group of primary dysmenorrhea patients compared to healthy control (Ma *et al.*, 2013). The increased levels observed following turpentine inflammation are in keeping with an intense inflammatory response, especially in the first twenty four hours.

Alterations in thigmotactic behaviour were only seen following acute bladder inflammation, but were seen in both turpentine and instrumentation groups', suggesting that the trauma associated with catheterisation is sufficient to induce behavioural alterations. This acute effect is in agreement with the majority of literature on bladder inflammation, which shows stronger acute effects of CYP compared to chronic (Juszczak *et al.*, 2010), and in the original turpentine inflammation studies by

McMahon & Abel, the greatest thermal responses were seen in the first few hours of inflammation, with tail and abdominal sensitivity showing more sustained sensitisation compared to the lateral hindpaw (McMahon & Abel, 1987).

Decreases in locomotor activity were seen in both intervention groups for acute and repeated models. Whether this can be interpreted as a sign of on-going nociception in these animals is unclear – decreased locomotor activity has been documented in other studies of experimental models of visceral pain and neuropathy, suggesting it is an effect worth understanding. It could be a pure 'locomotor inhibition effect' associated with non-nociceptive symptoms, but it seems likely that it could reflect increased vigilance, and an indication of increased environmental awareness expressed through reduction of 'unnecessary' movements akin to extended freezing noted following exposure to predators (Hofer, 1970). Furthermore, systemic inflammation is associated with constitutional symptoms such as decreased activity and general 'malaise', associated with both cytokine release and peripheral nerve activation (Poon *et al.*, 2013), meaning it is important when studying inflammation to fully characterise behavioural responses and inflammatory mediator levels to enable distinction of systemic inflammatory effects from behavioural depression associated with nociceptive stimuli.

Burrowing has been mooted as an ethological outcome measure sensitive to disruptions in general wellbeing (Deacon, 2009). Rats, being primary excavator burrowers (i.e. build their own burrows rather than occupying those built by other animals), readily burrow when given the opportunity, and decreases in burrowing activity have been documented in association with models of peripheral neuropathy (Andrews *et al.*, 2012), and somatic inflammation (Rutten *et al.*, 2014b), with reversal of deficits following analgesic treatment (Rutten *et al.*, 2014a). No studies to date have investigated the effect of visceral inflammation on burrowing outcomes, and the little data available on differences in burrowing activity between male and female rats comes from a largely observational study utilising small sample sizes, which found no significant differences (Stryjek *et al.*, 2012). Therefore, this study of burrowing behaviour following bladder inflammation in female rats provides useful information on both the effect of visceral inflammation, and also on whether burrowing shows sex-associated variation.

There was no significant effect of bladder inflammation on burrowing activity, in contrast with the reported decreases associated with acute somatic inflammation following intraplantar CFA (Rutten *et al.*, 2014a). However, this was the first study looking at burrowing activity in female rats, and it is possible that sex differences are present in this behavioural response. It could also be argued that abdominal sensitivity contributes less than hind-paw sensitivity to decreases in burrowing,

however this was not quantified in this study. So far, the majority of studies showing burrowing-related deficits have focused on conditions predominantly affecting the hindlimbs, such as peripheral neuropathy (Andrews *et al.*, 2012), or those involving central lesions such as prion disease (Cunningham *et al.*, 2005), so it is possible that conditions with less severe neurological implications, and those evoking non-peripheral pain and therefore not impairing limb function implicitly may not show such clear burrowing deficits. Burrowing is still a relatively new paradigm, and studies investigating the contribution of environmental factors are still on-going, so it is possible that future refinements may increase sensitivity and reduce the high levels of variation currently observed.

c-Fos is an immediate early oncogene which is frequently used as a marker of neural activation due to its rapid induction following stimuli including pain (Hunt *et al.*, 1987). To capture the peak of c-Fos activation, animals were sacrificed one hour and thirty minutes after exposure to the open field, and activity within the central amygdala (Ce) was determined.

There was no effect of turpentine inflammation on c-Fos immunoreactivity in the amygdala. Increased activation of the left amygdala was seen in both models, potentially indicative of left amygdala involvement in processing input related to open field exposure. Lateralisation has been seen in studies of amygdala activation, although there is no clear indication of generalisation, with both pain and social processing showing differential lateralisation, as shown in Table 1-5. However, there is evidence that lateralisation shows a time-dependent phenotype, with increased left activation in early stages of spinal nerve ligation (<day 6), and increased right Ce activation corresponding with expression of hypersensitivity (>day 14) (Gonçalves & Dickenson, 2012). Caudal activation of Ce, particularly CeL and CeC was observed in both models. Caudal activation has also been observed following acute CYP treatment in male rats, although higher intensities of staining were observed in the bed nucleus of the stria terminalis (Bon *et al.*, 1998), and following SNT, caudal activation of the CeL and CeC was also observed in male rats exposed to the open field using the same protocol this study was based on (Morland *et al.*, Manuscript in Preparation). Both studies observed increases of c-Fos in saline-treated animals, but as the former did not include a naïve control, and the later, as with this study, was without a "No Open Field" control, it is difficult to conclude whether this is due to the injection process/experimental neuropathy or some other element of the experimental experience such as handling or environmental factors.

The difference in c-Fos immunoreactive cell density between acute and repeated models is curious. That the levels are consistent within each model suggests it is not due to inconsistencies in counting, but it is unlikely that the difference in model duration (2 days compared to 15 days) could

be responsible for the differences. It is possible there was some other environmental factor present during the repeated model, which could increase baseline c-Fos activity in all animals.

To determine whether the changes in behaviour observed were related to activation of the Ce, correlations were performed between density of c-Fos immunoreactivity and open field behaviour.

Increased correlations between thigmotaxis in the open field and Ce c-Fos immunoreactivity, particularly the CeC and CeL, were seen in the repeated model, despite no significant differences in thigmotaxis being observed.

Interestingly, when open field behaviour following both acute and repeated bladder inflammation was compared and analysed using 2-Way ANOVA, overall differences in behavioural influence between turpentine and instrumentation group became clear – turpentine treatment was associated with decreases in all outcomes measures, whereas instrumentation only significantly decreased distance travelled and frequency of entry to the inner zone, emphasising the increased severity of the inflammation associated with turpentine. Additionally, in the overall analysis, rearing behaviour was the only outcome influenced by repeated bladder inflammation, showing an overall increase compared to the acute model, likely an effect associated with the longer experimental time-course due to increased handling. This analysis firstly suggests that much larger group sizes are required to detect the behavioural changes produced by turpentine and differentiate them from the effects of catheterisation. The group sizes were based on data from a model of traumatic peripheral neuropathy, and it is likely that the increased inflammatory component of bladder inflammation resulted in higher variation than that seen in nerve injury models.

Secondly, it shows that examining behavioural change as a whole, rather than individual outcome measures, can be more sensitive when determining the contribution from the control condition, in this case, catheterisation. Looking at data from both experiments, rearing activity and duration in the inner zone appear to distinguish the effect of turpentine from that of the catheterisation (i.e. distance travelled and frequency of inner zone entry).

7.3 d4T in female rats

The d nucleoside reverse transcriptase (dNRTI) family of anti-retroviral drugs such as ddC and ddI are associated with poor side-effect profiles, including peripheral neuropathy and lipodystrophy (Dubinsky *et al.*, 1989; Joseph *et al.*, 2004). d4T, or Stavudine, is another dNRTI which is still in widespread used as cheap an effective ART in the developing world but it is associated with a drug induced peripheral neuropathy (McGrath *et al.*, 2012), and to date, the few studies which

investigate the effect of such agents on behaviours such as thigmotaxis (Huang *et al.*, 2013), use exclusively male animals. This study investigated thigmotaxis following a previously validated treatment regime, aiming to determine firstly whether female rats respond in the same manner as male rats, and secondly, whether behavioural alterations correlate with sensory nociceptive measures of evoked hypersensitivity.

d4T, administered twice over 4 days via tail vein injection, failed to evoke spontaneous hypersensitivity, and had no effect on open field behaviour. This was unexpected as the protocol was previously validated (Huang *et al.*, 2013; Denk *et al.*, 2013), with the only difference from this study being the use of male, rather than female rats.

A pilot study was initially conducted to determine whether the dosing regimen of two intravenous injections was sufficient to induce peripheral neuropathy in female rats. After this pilot study showed no effect, small trials were conducted using both male and female rats and d4T from both a new batch, and a batch with proven efficacy in causing peripheral neuropathy in male Wistar rats of comparable weight (Huang *et al.*, 2013). The new batch appeared to be effective in male rats at comparable doses, with a mild decrease was seen in female rats, and as there was no literature suggesting differences in d4T metabolism between males and females, the study went ahead using the second batch of d4T.

However, in contrast with previous studies investigating d4T-associated hypersensitivity and thigmotaxis in the open field in male rats (Huang *et al.*, 2013), d4T treatment failed to significantly alter female behaviour. No deficits in open field behaviour were observed, and evoked measures were similarly unaffected, with all animals showing significantly decreased mechanical thresholds four days after the final d4T injection.

Previous studies have shown hypersensitivity following d4T treatment, although this used oral dosing via gelatin cubes, resulting in a higher dose of 87.5mg (assuming rat weight of 250g) at the start of testing, compared to 50mg in this study (Weber *et al.*, 2007). They also continued dosing throughout the testing period, which started 7 days after the start of dosing, and didn't see evoked hypersensitivity until 3 weeks, or 21 days of dosing. Therefore, it is possible that there is a sex difference in sensitivity to the neuropathic effects of d4T, with female rats requiring a chronic dosing regimen, compared to the more acute sensitivity seen in male rats.

d4T is thought to instigate neuropathic symptoms by disrupting mitochondrial activity leading to an increase in reactive oxygen species (ROS) (Velsor *et al.*, 2004; Lagathu *et al.*, 2007), although it has

also been shown that d4T has no effect on oxidative stress levels *in vitro* (Cui *et al.*, 1997; Kline *et al.*, 2009).

There is some evidence of increased mitochondrial resistance in women (Lagranha *et al.*, 2010; Guevara *et al.*, 2011), which could explain the lack of effect we observed, but as there are no long term studies of d4T effects in healthy individuals, it is difficult to compare this directly to the clinical situation as patients on d4T exhibiting peripheral neuropathy, will all have HIV infection, which in itself causes peripheral neuropathy, but may also have been previously treated with older antiretrovirals (e.g. AZT), making it very difficult to accurately determine the contribution d4T makes to neuropathic phenotype.

Further studies are required to determine whether the lack of effect observed represents increased resistance in females to d4T-induced neuropathy. It is possible that the differences between male and female metabolism result in a different time course, with peak behavioural and sensory alterations present at a later time point, and requiring higher dosages than tested in this study. Investigations into epidermal nerve fibre density in the hindpaw, and circulating levels of both d4T and of inflammatory cytokines would enable better interpretation of these findings. It is useful to note that distance travelled by these animals was comparable with naïve data from the bladder inflammation studies, suggesting activity levels were within the normal range and therefore not a factor in the results observed.

8 Limitations

There are a number of limitations present in the methods and data presented.

First, the c-Fos experiments could benefit from the inclusion of additional control groups. To determine whether c-Fos immunoreactivity seen following open field exposure is influenced by the inflammatory pain condition, an additional set of animals, with identical handling and treatment, but without open field exposure, could have been included. However, as this would have doubled the experimental group sizes and considerably increased the workload in terms of immunohistochemical analysis, this was omitted from the experimental design.

Immunohistochemical controls, including negative (lacking primary and/or secondary antibodies) and positive (including c-Fos antigen) controls were not conducted, as the protocol had been previously validated in the laboratory at Chelsea & Westminster Hospital (Morland *et al.*, Manuscript in Preparation; Farquhar-Smith *et al.*, 2002) and elsewhere (Moncho-Bogani *et al.*, 2005).

Due to cost constraints, inflammatory mediator levels were initially only assessed in bladder tissue from naïve and turpentine-inflamed animals following acute bladder inflammation, whereas instrumentation tissue was included in the repeated inflammation model. This restricts the conclusions that can be drawn from up-regulation in the acute model, as demonstrated by the differential up-regulation seen in the repeated instrumentation group compared to the repeated turpentine, with some markers in common, and some only seen in instrumentation and turpentine groups.

Male rats were chosen for the repeated open field investigation, despite female rats being used throughout the remainder of studies. They were selected to maximise comparison with the literature, however it would have also been informative for this series of experiments to use female rats. The initial plans were to investigate in both males and females, but time-constraints prevented this.

Measurement of oestrous cycle was not conducted, as it would have greatly increased the number of animals required, and involved an additional stressor not present in studies using male rats (i.e. vaginal swabs), reducing comparison with the published literature.

Dual-staining of sections, using a marker such as Galanin (Gray & Magnuson, 1987) or CGRP (D'Hanis *et al.*, 2007) would have aided identification of the amygdalar sub-divisions, in addition to providing insight into the classification of cells activated.

Behavioural alterations following d4t treatment failed to detect an effect. In hindsight, further pilot studies to determine the optimal dose to induce hypersensitivity would have been advisable. Hypersensitivity has been demonstrated in female rats following a more intense dosing regimen (Weber *et al.*, 2007). Oral dosing is not optimal, due to imprecision regarding the actual dose delivered, but additional d4T i.v. administration could have enhanced effects seen, in addition to providing valuable information regarding sex-related differences in sensitivity in peripheral neuropathy development.

In contrast with the other interventional studies, the effect of acute bladder inflammation on burrowing activity involved mixed housing, as opposed to the block design employed in other studies. This was a consequence of group randomisation based on baseline burrowing activity, and block randomisation would have required either ignoring baseline activity or re-distributing animals between cages, which would introduce undesirable social stress effects potentially associated with stranger interactions (Martin *et al.*, 2014). Further, it has been suggested that

mixed housing of naïve animals with those exposed to experimental nociceptive stimuli can influence open field outcome, although the effects are subtle (Legg, 2011).

Experimental design and laboratory capacity restricted the size of experimental batches, potentially introducing additional variation both in terms of seasonality, and time frame. Open Field exposure in study 1 was performed over the course of an entire day, introducing the potential for circadian variation, although as the time-frame involved did not cross between the light and dark phases, this effect is likely minimal (Eidman *et al.*, 1990).

9 Further Studies

There are numerous directions this research could now go in. The first studies required should address the limitations mentioned in the previous section. Investigation of c-Fos expression in animals without open field exposure would enable untangling of the activation due to bladder stimuli from those associated with open field exposure. Also, c-Fos expression in other amygdala nuclei such as the BL, Me, and ST, and extra-amygdala areas such as the insula, PAG, and RVM, which are also involved in response to nociceptive and environmental stimuli, would further elucidate the neural networks involved in the expression of thigmotactic behaviour.

A study examining inflammatory mediator up-regulation in acute instrumentation animals is also required, as many more mediators were affected in the acute turpentine, compared to repeated turpentine, and there was some degree of over overlap between repeated instrumentation and repeated turpentine. As behaviour was significantly different in these animals, it is likely that a high level of differential regulation would be observed in acute instrumentation.

Additionally, examination of inflammatory profiles associated with all animals in a study, rather than a selected subgroup, would enable identification of mediators whose expression correlates with behavioural changes, and provides the potential for identification of biomarkers for such changes, although this would currently be an expensive study to carry out. Studies looking at cytokines in urine or blood would also enable examination of changes over time, distinguishing mediators involved in initiation of inflammatory response from those responsible for maintenance of long-term inflammation.

Studies have shown systemic inflammation induces changes in central activation (Fan *et al.*, 2014), and that central changes can drive peripheral inflammation in a neurogenic model of cystitis (Jasmin *et al.*, 1998), and changes in inflammatory mediator expression have been observed centrally, including amygdala nuclei, following LPS-induced systemic inflammation (Skelly *et al.*,

2013; Araki *et al.*, 2014). It would therefore be interesting to examine central levels of mediators seen local to inflammation (e.g. CCL-2, IL-1β, and iNOS) in areas such as the amygdala. As it has been shown that isoflurane is capable of producing an inflammatory response in the brain independent of other stimuli (Wu *et al.*, 2012), it would also important to also include the inflammatory profile of anaesthesia-only animals to allow differentiation of effects due to anaesthesia from those associated with peripheral inflammation.

In this study, oestrous cycle was not considered, as identification of stage is not only a potentially stressful experience, but also it would necessitate larger group sizes to allow for analysis between stages. However, there is evidence of sex differences in the role of nitric oxide and transforming growth factor beta (TGF-β) following CYP-induced cystitis, with females showing higher baseline levels of NO2/3, compared to a greater TGF-β response in males (Tyagi *et al.*, 2009). Furthermore, oestrous cycle has been shown to influence centrally-mediated cross-sensitisation between the bladder, colon, and uterus (Winnard *et al.*, 2006). However, there is debate in clinical research regarding different methods of determining menstrual phase, with poor correlations between cell and hormonal markers, suggesting monitoring circulating hormone levels may yield more useful information, as demonstrated by greater correlation between hormonal levels and heart rate in healthy women, not seen when looking strictly at menstrual cycle (Leicht *et al.*, 2003). Furthermore, there is considerable variation in cycle length in healthy women, but a relatively restricted range of peak progesterone levels (Landgren *et al.*, 1980). Studies have shown decreased testosterone in a subgroup of women taking the combined oral contraceptive (COCP) show increased pain sensitivity compared to both women taking COCP with elevated testosterone levels, and those not taking COCP at all (Vincent *et al.*, 2013).

Comparison of the hyper-reflexia and referred hypersensitivity response to bladder inflammation in female rats with male rats would also yield valuable information on sex differences in behavioural responses. Female animals have been documented as having higher sensitivity to visceral stimuli (Aloisi *et al.*, 2010), higher activity and lower defecation in the open field (Slob *et al.*, 1981), and the opposite effect of social isolation on open field compared to males, with females showing reduced ambulation and increased 'anxiety' (Palanza, 2001).

The majority of animals in this study were randomised to group by the block method, meaning they were housed with cage-mates in the same experimental group. Studies have shown a degree of 'empathy' or altered social responses both between animals in experimental pain, and between control animals and those in experimental pain groups, with non-pain observers demonstrating hypersensitivity only if they were cage-mates in a model of acute bee venom pain (Li *et al.*, 2014b),

and although it has been suggested that there is no effect on naïve open field behaviour associated with mixed group housing, male rats with transection of L5 spinal nerve only show behavioural alterations when house in mixed groups (Legg, 2011). As sex-related effects have been demonstrated in expression of social behaviour, it is likely that female rats may exhibit a higher degree of 'empathic' behaviour (Langford *et al.*, 2010b), which could impact comparisons of behavioural outcomes dependent on whether housing is heterogeneous or homogeneous. The majority of animals in this study were randomised to group by the block method, meaning they were housed with cage-mates in the same experimental group. Studies have shown a degree of 'empathy' or altered social responses both between animals in experimental pain, and between control animals and those in experimental pain groups, with non-pain observers demonstrating hypersensitivity only if they were cage-mates in a model of acute bee venom pain (Li *et al.*, 2014b), and although it has been suggested that there is no effect on naïve open field behaviour associated with mixed group housing, male rats with transection of L5 spinal nerve only show behavioural alterations when house in mixed groups (Legg, 2011). Additionally, the concept of empathy or emotional contagion is controversial, as the use of words such as emotion/empathy can be misleading, as previously discussed in relation to 'anxiety' and 'allodynia'. Empathy and emotion are human constructs *per se*, and their use implies a degree of insight into subjective experience that is not currently possible when the subjects are unable to communicate their perspective. As sex-related effects have been demonstrated in expression of social behaviour, it is likely that female rats may exhibit a higher degree of 'empathic' behaviour (Langford *et al.*, 2010b), which could impact comparisons of behavioural outcomes dependent on whether housing is heterogeneous or homogeneous.

Increasing group sizes would facilitate investigation of behavioural phenotypes, such as those seen in the study of repeated open field exposure. This would allow full correlations to be conducted into expression of mediators implicated in protective pain-associated modulation of behaviour, with the potential for identification of useful biomarkers indicative of negative consequences in situations where the occurrence of pain can be predicted (e.g. post-operative pain).

Examination of morphological changes in the bladder would also facilitate better understanding of how turpentine bladder inflammation alters behaviour, particularly looking at the extent of mucosal denudation as this has been shown to correlate with increased sensitivity (Andersson *et al.*, 2008; Ikeda *et al.*, 2009). Infiltration of mast cells could also be quantified, as this is another factor known to contribute to the severity of inflammatory response (Sant *et al.*, 2007).

Furthermore, immunohistochemical investigations examining co-expression of neuromarkers such as those involved in endogenous endocannabinoid activity, fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) (Connell *et al.*, 2006), nociceptive activity (substance P receptor neurokinin-1 (NK1) (Singewald *et al.*, 2008), cytokine activity (calcitonin gene related peptide (CGRP)) (D'Hanis *et al.*, 2007), stress modulation (corticotrophin-releasing factor (CRF1)) (Ji *et al.*, 2007), and opioidergic receptors (Marchant *et al.*, 2007) with c-Fos would enable a full picture of the neurochemical response associated with amygdala activation under acute and repeated bladder inflammatory conditions. This is particularly important in a heterogeneous brain area such as the amygdala, as both inhibitory (GABA-ergic), and excitatory (glutamatergic) innervation is present (Cassell *et al.*, 1999), such that the presence of c-Fos alone can be taken as evidence for both activation and inhibition. Quantification of the stress response (e.g. via CRF1), would allow investigation into how much trait stress responses contribute to behavioural outcome, whereas receptors such as FAAH and MAGL, NK1, and opioid receptors would give insight into activation of pain circuits associated with endocannabinoids, substance P, and endogenous opioids respectively. Furthermore using only c-Fos and toluidine blue counterstaining made the task of precisely delineating the Ce and its subdivisions difficult – this process could be optimised by using fluorescence immunohistochemistry and combining c-Fos detection with CGRP (CeL and CeC specific) and Galanin (CeM specific) co-localisation (Olucha-bordonau *et al.*, 2015).

It is clear that understanding of behavioural alterations associated with pain are best detected when using a battery of ethological tests, allowing investigation of the different aspects of behaviour such as conditioned responses, analgesia-associated place preference, and at social and cognitive impairments. This would yield a thorough picture of which behaviours are more strongly impacted by pain of differing aetiologies.

The turpentine inflammation protocol could be refined by decreasing the interval between inflammation sessions in the repeated model, as a second inflammatory stimulus within twenty-four hours of the first shows heightened hypersensitivity (McMahon & Abel, 1987), although this was conducted in decerebrate animals. When we were developing the repeated model, we were looking to mimic the timeline seen in development of hypersensitivity in traumatic models of neuropathy, hence dosing so close might risk unnecessary suffering for the animals. However, investigation of the timeline effects following such dosing might enable modification of the repeated model, enhancing the pain phenotype and more reliably mimicking the clinical situation seen in chronic bladder pain conditions.

All experiments in this study were conducted during the light phase, with testing largely taking place between 9am and 2pm. However, as rats are nocturnal animals, it is likely that different results would be obtained were the behavioural studies performed between 7pm and 7am (i.e. the dark phase). Studies have shown increased ambulation and wall-supported rearing during the dark phase in male rats, although there was no difference between free-standing rearing activity (Gentsch *et al.*, 1982), and suggested stressful procedures of similar intensity have greatest effect during daylight (Paredes *et al.*, 2005).

10 Conclusion

In conclusion, the body of work included in this thesis has demonstrated bladder inflammation is associated with up-regulation of local inflammatory mediators in the urinary bladder, and acutely with an increase in thigmotactic behaviour. It is also clear that a high level of variation is present, suggesting larger group sizes and improved behaviour phenotyping prior to testing would be beneficial in improving the ability of the open field paradigm to detect changes in behaviour. Inclusion of detailed analysis rearing behaviour as demonstrated in the naïve repeated open field exposure study has the potential to provide useful information on how experimental pain affects arousal levels. The lack of observable effect of d4T on female rats was interesting, and suggestive of differential metabolism compared to males.

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