# *Altered Thyroid Hormone Regulation and Behavioural Change in a Sub-population of Rats following Injury*

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March 2012

*A thesis submitted to the University of Sydney in fulfillment of the requirements for the degree Doctor of Philosophy*

## *Declaration*

The work presented in this thesis was undertaken in the Department of Anatomy and Histology and in the Brain and Mind Research Institute, the University of Sydney, Australia. I carried out all the work recorded in the thesis except where the contribution of others is noted. Specifically, for the procedures documented in Chapter 5, tissue from a tissue bank was used; I had performed all procedures required to obtain this tissue on other groups of animals. To the best of my knowledge it contains no material written by another person except where due reference is made in the text.

Parts of this work have been published and presented at conferences. A copy of the publication is included in the appendix.

Kilburn-Watt E, Banati R and Keay KA, Altered Thyroid Hormones and Behavioural Change in a Sub-population of Rats following Chronic Constriction Injury, *Journal of Neuroendocrinology,* 2010; **22**(8): 960-70.

Elisabeth Kilburn-Watt
March 2012

## *Acknowledgments*

I would like to thank Professor Richard Banati for his support for the concept of the work contained in this thesis, for finding me a home in which to explore the problem and for his continuing support throughout the process.

I would like to thank Associate Professor Kevin Keay for giving me the opportunity to work in his laboratory and to benefit from the many learning opportunities that became available to me as a result. I would like to thank him for his guidance, support and overall vision for completing the story.

I would like to thank the many fellow students who have contributed to my learning during the process of undertaking this work; in particular Eszter Kalman, Karmina Sosa, Alison Bembrick, David Mor and Jonathan Hakim. I would also like to thank Dr Paul Austin.

I would like to thank Dr Jenny Cox for her friendship, support and advice.

I would like to thank David for his love and his patience.

## *Overview Of This Thesis*

This thesis documents an investigation of thyroid hormones and the regulation of thyroid hormones in an animal model of neuropathic pain. The technique used in this model, Chronic Constriction Injury (CCI), produces an injury that creates an inescapable mechanical and inflammatory stress for the rat. Various forms of stress, and in particular unavoidable stress, have been shown to alter thyroid hormones in both rats and humans.

This rat model has been developed to more closely resemble the neuropathic pain state in humans where a proportion of patients develop a syndrome of social and behavioural changes, which has been termed 'disability'. As well as demonstrable sensory dysfunction, as a result of the nerve injury, a sub-population of the rats exhibit altered patterns of complex social behaviours.

The results of three experiments are presented. In each case a cohort of rats was subjected to the same procedure – Chronic Constriction Injury – and changes in social behaviour measured using a Resident-Intruder test. Rats were assigned to one of three groups based on the results of the behavioural test: No Disability (ND), Transient Disability (TD) or Persistent Disability (PD).

In experiment 1, documented in Chapter 4, peripheral thyroid and corticosteroid hormones were measured pre and post-CCI together with Thyroid Stimulating Hormone (TSH) levels. Results for the three different behavioural groups were compared.

In experiment 2, documented in Chapter 5, RNA was extracted from the hypothalamuses of rats sacrificed six days post CCI. Relative gene expression was determined for a number of factors involved in thyroid hormone and corticosteroid regulation.

In experiment 3, documented in Chapter 6, at the end of the experimental period, seven days post-CCI, the rats were perfused to remove blood and the brains

preserved in paraformaldehyde. The hypothalamuses were examined using immunohistochemical techniques for evidence of differing protein expression for molecules involved in thyroid hormone regulation.

***Summary of Findings***

***Behavioural Testing (Chapter 3)***

The results of behavioural testing for the experimental groups of animals used in this study conform to those previously found using this model (Monassi et al., 2003). Based on previously derived criteria 70% were classified as 'No Disability' (ND); 10% of the rats and were classified as 'Transient Disability' (TD) and 20% of rats were classified as 'Permanent Disability' (PD) animals. Rats classified as PD have significantly decreased levels of Dominance behaviour on days 1-3 and 4-6 post-CCI when compared to ND rats whereas TD rats have decreased Dominance behaviour on days 1-3 only. Pre-CCI behaviour is not predictive of post-CCI behavioural change.

***Peripheral Thyroid Hormones (Chapter 4)***

When compared to pre-injury levels, the mean total thyroxine (TT4), total triiodothyronine (TT3) and free thyroxine (fT4) levels were significantly decreased in the PD rats whereas the post-injury levels of these hormones did not change in the ND and TD rats. There was no difference between groups in mean free triiodothyronine (fT3) and thyroid stimulating hormone (TSH) levels pre and post-injury. The pattern of secretion of hormones differed in the ND and PD rats over the six days post-injury. In the ND rats, there was a surge in TSH post-injury and thyroid hormone levels were maintained at pre-injury levels. However, in the PD group, the TSH response was delayed, blunted and inadequate as thyroid hormone levels decreased. There was a significant correlation between decreased Dominance behaviour and both TT4 and fT4 but no correlation with TT3 or fT3.

***Peripheral Corticosteroids (Chapter 4)***

There was an increase in corticosterone in all animals post-CCI. The Transient Disability rats showed the greatest increase in corticosteroid and this was

significantly different to the other two groups of rats. There was no difference in post-CCI corticosterone levels between the PD and ND rats.

***Hypothalamo-pituitary-thyroid (HPT Axis) regulation (Chapters 5 & 6)***

RT-qPCR measurement of relative mRNA expression in the hypothalamus showed that there was no difference between No Disability, Transient Disability and two groups of control rats (Behavioural Controls (BC) and naïve group caged controls) for Thyrotrophin Releasing Hormone (TRH), Thyroid Hormone Receptor β (TR β), Deiodinase 2 (Dio2) and Deiodinase 3 (Dio3). Compared to the BC rats, the Permanent Disability rats had significantly decreased relative expression of TRH and Deiodinase 2 and significantly increased expression of Deiodinase 3 mRNA.

Immunohistochemical techniques used to label TRH, TR β, Deiodinase2 and Deiodinase 3 protein distribution in the Paraventricular Nucleus (PVN) of the hypothalamus showed significantly fewer immunoreactive profiles for Deiodinase 3 and TRβ in the group with changed behaviour (PD group) compared to the Behavioural Control rats. These differences were shown to be specific for the sections of the PVN where the hypophysiotrophic TRH secreting neurons are known to be primarily located.

***Hypothalamo-pituitary-adrenal (HPA axis) regulation (Chapter 5)***

RT-qPCR measurement of relative mRNA expression in the hypothalamus showed that there was no difference between ND, TD, PD and Behavioural Controls rats for Corticotrophin Releasing Hormone (CRH), Glucocorticoid Receptor (GR) and Vasopressin. Compared to the Behavioural Controls, group caged Control animals had significantly increased relative expression of CRH and Vasopressin mRNA.

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*List of Abbreviation*

**AACE:** American Association of Clinical Endocrinologists

**AHN:** anterior hypothalamic nucleus

**AN:** arcuate nucleus of the hypothalamus

**AN-ME:** arcuate nucleus-median eminence.

**AVP:** Arginine vasopressin

**BC:** behavioural control

**C:** control

**CAD:** coronary artery disease

**CCI:** chronic constriction injury

**CFS:** chronic fatigue syndrome

**CNS:** central nervous system

**CRH:** corticotrophin-releasing hormone

**Ct:** threshold cycle

**D:** dominance behaviour

**DAB:** 3-3'-diaminobenzadine

**Dio1:** type1 5' deiodinase enzyme

**Dio2:** type 2 5' deiodinase enzyme

**Dio3:** type 3 5 deiodinase enzyme

**DNA, cDNA:** deoxyribonucleic acid, complementary deoxyribonucleic acid

**EEG:** electroencephalographic

**EEM:** electromyographic

**fT3:** free triiod0thyronine

**fT4:** free thyroxin

**fx:** fornix

**GAPDH:** glyceraldehyde-3-phosphate dehydrogenase

**GR:** glucocorticoid receptor

**HPA:** hypothalamo-pituitary-adrenal

**HPT:** hypothalamo-pituitary-thyroid

**IFNδ:** interferon gamma

**IL:** interleukin

**IL-1β:** interleukin-1beta

**IL-6:** interleukin 6

**LPS:** lipopolysaccharide

**MBH:** mediobasal hypothalamus

**MCTs, MCT8:** monocarboxylate transporters, monocarboxylate transporter 8

**Md:** median

**NACB:** National Academy of Clinical Biochemistry

**ND:** no disability

**NS:** non-social behavior

**NTI:** non-thyroidal illness

**OATPs, OATP1C1:** organic anion transporters

**PAG:** periaqueductal grey

**PaDP:** dorsal parvocellular paraventricular nucleus

**PaLP:** lateral parvocellular paraventricular nucleus

**PaM:** magnocellular paraventricular nucleus

**PaMP:** medial parvocellular paraventricular nucleus

**PaV:** ventral parvocellular paraventricular nucleus

**PBHS:** phosphate buffered horse serum

**PBS**: phosphate buffered saline

**PCR**: polymerase chain reaction

**PD**: permanent disability

**PTU**: propylthiouracil

**PVN:** paraventricular nucleus of the hypothalamus

**REM:** rapid eye movement

**RIN:** RNA integrity number

**RNA, mRNA:** ribonucleic acid, messenger ribonucleic acid

**RT:** room temperature

**RT-qPCR:** reverse transcription real time polymerase chain reaction

**rT3:** reverse triiodothyronine

**S:** social behaviour

**Sub:** submissive behaviour

**SCH:** sub-clinical hypothyroidism

**SES:** sick euthyroid syndrome

**SWS:** slow wave sleep

**T3, TT3:** triiodothyronine, total triiodothyronine

**T4, TT4:** thyroxin, total thyroxin

**TBG:** thyroid-binding globulin

**TD:** transient disability

**TFT:** thyroid function tests

**TgAb:** antibodies to thyroglobulin

**TH, THs:** thyroid hormone, thyroid hormones

**TNFα:** tumour necrosis factor alpha

**TPOAb:** antibodies to thyroperoxidase

**TR, TRs:** thyroid receptor, thyroid receptors

**TRα:** thyroid receptor alpha

**TRβ:** thyroid receptor beta

**TRH, ppTRH:** thyrotrophin releasing hormone, prepro-thyrotrophin releasing hormone

**TRE:** thyroid response elements

**TSH:** thyroid stimulating hormone, thyrotrophin

**3V:** third ventricle

**VMH:** ventromedial hypothalamic nucleus

**ZI:** zona incerta

# *CHAPTER ONE*

# *Introduction and Literature Review*

## *Introduction*

It has long been known that individuals respond differently to changes in their environment, which can be collectively labeled “stressors”. This individual difference in response is clearly applicable across species and is implicated in the survival of individuals and/or species in evolutionary terms (Selye, 1976).

Stressors, whether extrinsic or intrinsic, interact with an organism via the nervous system allowing for rapid response. The endocrine system, utalising chemical messages, mediates more prolonged homeostatic responses. The nervous and endocrine systems are most closely structurally and functionally related in the Hypothalamus where control of secretion of many of the major metabolic hormones is regulated (Greenspan and Gardner, 2001).

It has been recognised that thyroid function is sensitive to stressful life events in some individuals. More recently the alteration in thyroid regulation seen in acute critical illness has been documented and animal models have been useful in helping to elucidate the mechanisms involved. The situation in chronic illness and conditions is less clear but increasingly some individuals with chronic conditions are reported to have a “low thyroid syndrome” (Fliers et al., 2006a) (Adler and Wartofsky, 2007).

Alterations to thyroid function are associated with mood and behavioural change. Specifically, when thyroid hormones are inadequate, in addition to well-documented systemic symptoms, symptoms related to the central nervous system are reported. These symptoms include fatigue associated with disturbed sleep, low mood and depression, cognitive impairment and memory loss (Esposito et al., 1997) (Gold and Pottash, 1986).

In an animal model of chronic stress, persistent behavioural change can be demonstrated in a sub-group of the animals. This model provides the opportunity to examine thyroid hormone regulation in this sub-population relative to animals that do not change their social behaviours under the same conditions of stress (Monassi et al., 2003).

# *Literature Review*

## *1.1 Behavioural Change/Disability in Chronic Conditions/Diseases*

A proportion of individuals with chronic health problems report a syndrome of other symptoms, in addition to the core disease state, which can be collectively termed 'disability'. The features of this syndrome are primarily behavioural in nature leading to the use of terms such as 'sickness behaviour', with the implication that these individuals are somehow less able 'to cope' than others. However the syndrome results in increased burden for the individual and for the health system and society in general.

### *1.1.1 Sickness behaviour in acute illness*

With the onset of febrile illness a pattern of behavioural change is seen in animals and also in humans. The common features displayed are lethargy, depression, anorexia and a reduction in grooming. One view is that this is a maladaptive response, the result of debilitation from the disease process and the inability to obtain food and water. An alternative view is that sickness behaviour is, in acute illness, an adaptive response that facilitates the role of fever in combating acute infections (Hart, 1988) (Konsman et al., 2002). In patients with acute viral infections psychological and somatic symptoms were correlated with biological markers for inflammation. Features of sickness behaviour including fever, malaise, pain, low mood and poor concentration were positively related to levels of pro-inflammatory cytokines (Vollmer-Conna et al., 2004).

### *1.1.2 Sickness behaviour in chronic illness*

While features of sickness behaviour are adaptive to surviving the life-and-death scenario of acute infection, does it have a role in chronic illness? Sickness behaviour is described across the chronic disease spectrum although the specific symptom of depression is the focus of many reports. The incidence of depression

in patients with chronic disease is higher than for the general population (Moussavi et al., 2007). Conditions in which the syndrome is reported include cardiovascular, pulmonary, musculo-skeletal, malignancy and others that could be collectively called 'inflammatory conditions' (Qiu et al., 2010) (Yohannes et al., 2010) (Provinciali and Coccia, 2002) (Pincus et al., 2002) (Gatchel, 2004) (Herrman and Chopra, 2009) (Benton et al., 2007). Patients diagnosed with Chronic Fatigue Syndrome (CFS) subsequent to an acute infectious illness were tested for cognitive function and assessed for mood disturbance and fatigue. In all three areas of assessment their scores were lower than for healthy controls: their cognitive performance was lower than for patients with acute viral infections and while both groups reported mood disturbance this was not as great as in a group of patients diagnosed with depression (Vollmer-Conna et al., 1997).

However depressed mood is a feature of chronic disease in a minority of patients. Data from a WHO world health survey showed that, depending on the chronic condition, between 9 and 23% of survey participants had both depression and a chronic disease (Moussavi et al., 2007). Using mean health scores, 'co morbidity' of depression and chronic disease was found to be detrimental for the patient. Health scores for patients with angina, arthritis, asthma and diabetes with depression were all found to be worse than for depression alone or any of the chronic diseases alone or in combination, without depression (Moussavi et al., 2007). A recent review of the literature revealed that in chronic heart and lung disease the reported incidence of depression may be as high as 70-80% but varying methods of assessing depression make true evaluation of the prevalence of the co morbidity difficult (Yohannes et al., 2010).

### *1.1.3 Disability in Chronic Pain*

Patients with chronic pain present a significant burden to health systems and state economies. Incidence of chronic non-malignant pain as high as 15% of the population is reported: these patients report poor quality of life and use large resources within health systems (Fredheim et al., 2008). They are also a financial burden to the state: in a study of neuropathic pain patients (said to occur in 1% of the population and to constitute at least 25% of patients attending pain clinics) 34% of patients under 65 years were on a disability pension and unable to work;

another 18% were working less than they wanted because of their condition (Meyer-Rosberg et al., 2001b) (Meyer-Rosberg et al., 2001a).

Many chronic pain patients experience not only sensory disturbance but also changes in complex behaviours, mood states and cognition. These changes can include altered social behaviours, disturbed sleep, fatigue, altered appetite, reduced interest in the environment, and very often depression (Menefee et al., 2000, Meyer-Rosberg et al., 2001b) (Fredheim et al., 2008). Chronic neuropathic pain, the most common form of chronic pain in patients attending pain clinics, is defined as pain that persists despite the healing of an injury to peripheral nerves, the spinal cord, or less frequently, higher brain regions. It is diagnosed clinically by the presence of sensory signs, which include hyperalgesia (increased sensitivity to noxious stimuli), allodynia (the experience of pain triggered by non-noxious stimuli) and spontaneous pain and dysaesthesias (Jensen et al., 2001). Neuropathic pain is reported in a heterogeneous group of conditions including diabetes, immune deficiencies, malignant diseases, traumatic and ischemic disorders (Jensen et al., 2001). Depending on the aetiology, significant percentages of chronic neuropathic pain patients also report ‘disability’, which is described by many who experience it as having a greater impact on quality of life than the pain itself (Meyer-Rosberg et al., 2001a). The health related quality of life scores for such patients are significantly lower than for the general population and also lower than for cancer patients in palliative care (Meyer-Rosberg et al., 2001b) (Fredheim et al., 2008). In one study, the most frequently reported problems were difficulty in sleeping (88%) lack of energy (86%) difficulty concentrating (76%), and drowsiness (71%) (Meyer-Rosberg et al., 2001b).

Interestingly, paralleling reports of emotional and behavioural change in patients with chronic conditions are reports of a “low thyroid syndrome” in similar groups of patients. There are similarities between the symptoms of hypothyroidism and the changes seen in the disability syndrome. However, these symptoms are non-specific. These patients are not diagnosed as hypothyroid because the levels of peripheral hormones do not support that diagnosis.

### *1.1.4 Summary*

Changes in mood and cognition as well as lethargy and sleeping disturbance are reported in numerous chronic diseases and in chronic pain conditions, which occur in diseases of many different aetiologies. This syndrome of disability is an additional burden to the patient, as well as to the health care system, affecting both physical and mental well-being. While ‘sickness behaviour’ appears to be an adaptive syndrome in acute illness, assisting in survival, in chronic illness it appears to be maladaptive as it is associated with negative outcomes for the individual.

## 1.2 Behavioural Change in Thyroid Dysfunction

Thyroid dysfunction is a common condition in adults affecting females to a much greater degree than males. Over production of thyroid hormones resulting in symptoms of hyperthyroidism is less common than thyroid deficiency. In countries where iodine deficiency is not endemic, overt hypothyroidism has a spontaneous presentation of 1-2% of individuals and is 5-8 times more common in females; mild hypothyroidism is thought to be extremely common affecting 10% of the population and up to 20% of women over 50 years of age (Tunbridge et al., 1977) (Vanderpump and Tunbridge, 2002b) (Ladenson et al., 2000). Mild thyroid dysfunction (also called subclinical thyroid disease) is therefore very common, but its management remains controversial (Gharib et al., 2005). Aside from iodine deficiency worldwide, the commonest cause of hypothyroidism is Hashimoto's Thyroiditis, an autoimmune condition causing progressive destruction of the thyroid gland. Hypothyroidism results in abnormalities of function in most organ systems: there is evidence that brain function is also affected.

### 1.2.1 Overt thyroid dysfunction

It has long been recognized that overt thyroid dysfunction is associated with disturbance of cognitive and emotional function, which adversely affects quality of life (Whybrow and Hurwitz, 1976) (Gold and Pottash, 1986) (Esposito et al., 1997) (Boelaert and Franklyn, 2005). Graves' Disease, the commonest form of hyperthyroidism, is associated with nervousness and emotional lability, easy fatigability, hyperexcitability, short attention span, impaired recent memory, and in severe illness, frank psychosis with hallucinations (Whybrow and Hurwitz, 1976) (Greenspan and Gardner, 2001) (Gold and Pottash, 1986) (Esposito et al., 1997).

Central nervous system (CNS) symptoms that are common in hypothyroidism include chronic fatigue, lethargy, inability to concentrate, apathy and loss of interest, paucity of speech, a general slowing of mental function and depression (Esposito et al., 1997) (Greenspan and Gardner, 2001) (Gold and Pottash, 1986). Appropriate treatment of either hyper or hypo-functioning thyroid states generally results in resolution of CNS symptoms (Whybrow and Hurwitz, 1976) (Greenspan and Gardner, 2001) (Esposito et al., 1997). However, in the treatment of

hypothyroidism there is ongoing controversy regarding what is appropriate treatment for all patients. Treatment of CNS symptoms requires higher doses of replacement hormone than treatment of systemic symptoms (Baskin et al., 2002) (Samuels et al., 2007). While treatment with l-thyroxine is considered more appropriate due to its long half-life and stability, some patients appear to have greater resolution of CNS symptoms when treated with combined thyroxine (T4) and triiodothyronine (T3) medication as in the original sheep thyroid extract (Bunevicius et al., 1999) (Danzi et al., 2005) (Klein and Klein, 2005). This implies that there may be individual differences in the regulation of thyroid hormone metabolism in the brain.

### *1.2.2 Mild and Subclinical Hypothyroidism (SCH)*

There is a great deal of controversy in the literature regarding this condition. The current most common view is that these individuals are not yet hypothyroid but may progress to hypothyroidism in the future and that the consequences of this condition are minimal and treatment is not warranted (Surks et al., 2004). Others argue that some individuals have symptoms and so should be treated (Vanderpump and Tunbridge, 2002a) (Gharib et al., 2005). Others have shown that symptoms may not be readily identified but none-the less are significant and warrant treatment: these include decreased cardiac contractility, elevated triglycerides and various cognitive deficits including memory loss. Significant percentages of individuals with this condition are diagnosed with depression (Hickie et al., 1996) (Esposito et al., 1997). Treatment with thyroxine has been shown to be beneficial in reversing both cardiac and CNS symptoms (Cooper et al., 1984) (Nystrom et al., 1988) (Monzani et al., 1993) (Cooper, 2001) (Monzani et al., 2001) (Davis et al., 2003) (Ladenson et al., 2000). The percentage of patients showing improvement in CNS function however, may depend on selection of patients as only approximately one in four patients show improvement (Cooper et al., 1984) (Nystrom et al., 1988) (Cooper, 2001) (Davis et al., 2003). It may also depend on the form of treatment and level of replacement therapy as discussed above. Thirty-eight percent of patients being treated for depression and with TSH levels in the upper half of the normal reference range were found to have hypothyroidism when given a TRH stimulation test (Kraus et al., 1997). Treatment of patients with chronic fatigue showing evidence of thyroiditis indicated that the favourable response to treatment with thyroxine was

independent of thyroid function tests (Wikland et al., 2001). Such reports call into question the ability of thyroid function tests to identify levels of thyroid dysfunction which present primarily as mood and cognitive change.

### *1.2.3 Animal models of thyroid deficiency*

Animal models have been invaluable in demonstrating the role of thyroid hormone in the development of the brain (Bernal, 1995) (Anderson, 2001) (Bernal, 2009). Inducing hypothyroidism in pregnant rats results in developmental abnormalities of the foetal brain (Morreale de Escobar et al., 1985). Rats with congenital tertiary hypothyroidism exhibit gross abnormalities of motor function from 14 days of age, which result in early death by 30 days. Post mortem studies reveal progressive neuronal morphological and biochemical changes (Stoica et al., 2007). To contribute to the understanding of the role of thyroid hormone in adult neurological functioning, and in particular aspects of emotional behaviour, animal models need to address the issue of measuring animal behaviours that can be extrapolated to behaviours in humans.

#### *1.2.3.1 Behavioral models of thyroid deficiency*

The recent literature indicates that investigators are applying measurements of animal behaviours used in models of depression to evaluate the effect of thyroid hormones on behaviour. The forced swim test was developed as an animal model of depression ("behavioural despair") and is used in screening for efficacy of anti-depressant drugs (Kulikov et al., 1997). In this behavioural paradigm, rats are given a 'pre-test' forced swim for 15 minutes and after 24 hours a five-minute 'post-test' forced swim. With each successive five minutes during the 15 minute pretest, rats spend increasing time in an immobile state ("behavioural despair"): compared to the first 5 minutes, rats spend twice the time in an immobile state during the 2nd and 3rd 5-minute intervals of the pre-test. The absence of leg movements while floating is used as the measure of 'immobility'. Compared to sham operated animals, severely hypothyroid rats (thyroidectomised) as well as mildly hypothyroid rats (low iodine diet) both showed increased immobility in the post-test situation: control animals spend a similar time in the immobile state as in the first 5 minutes of the pre-test whereas hypothyroid rats spend about twice this time in the immobile state (Kulikov et al., 1997). The change in response was not due to a difference in activity as pre-test scores were similar across groups. The forced swim test results support, in objectively measuring a change in behaviour,

that deficiency of thyroid hormone decreased the ability of animals to cope with an inescapable stress.

Similar results were obtained using escape deficit/active avoidance as a measure of changed behaviour in rats (Levine et al., 1990): this test is also used as an animal model of depression. In this behavioural paradigm, rats can terminate an electric shock by pressing a lever. With progressive trials over a number of days, sham operated rats showed progressive ability to be able to escape the shock. Thyroidectomised rats, however, showed a decreased number of successful attempts in avoiding the shock over successive days.

The effect of a mutant thyroid receptor alpha 1 was assessed in mice by applying a number of behavioural testing paradigms used in animal models of depression (Pilhatsch et al., 2010). The mutant animals had a receptor which has ten-fold lower thyroid hormone binding capacity than normal receptors and is suggested as a model for central hypothyroidism. The behavioural tests included active avoidance as described above (a measure of 'learned helplessness'), an open-field test for testing locomotion (number and distance of movements), a light/dark box for testing anxiety (number of movements between light and dark and duration remaining in the light box) and a startle response to an acoustic stimulus (measured by movement of the floor of the cage). Compared to wild type mice, mutant mice showed deficits in all the testing paradigms. Treatment of the mutant mice with thyroid hormone decreased the deficits in the depression/anxiety tests but not in the test of locomotion (Pilhatsch et al., 2010).

### *1.2.4 Summary*

It is known that deficiency of thyroid hormones can affect neurological function in adult humans, causing cognitive deficits and mood disturbance. There is evidence that these changes can also occur in mild thyroid hormone deficiency and in individuals not optimally treated with replacement hormone. Studies reporting response to thyroid hormone treatment suggest that there is a sub-population that benefit in terms of CNS function.

Using objective measurements of behaviours that are used as measures of inability to deal with stress, the affect of thyroid hormone deficiency can be assessed in

animals. There is evidence that behaviours associated with coping with stress are altered in animals with thyroid dysfunction. Changes in such behaviours have been demonstrated in severe and mild hypothyroidism.

# 1.3 Thyroid Hormone Metabolism and Regulation

## 1.3.1 Historical perspective – Function

While the thyroid was early recognised as a gland that could cause swelling of the neck and was given its present name by 1656, it was not until the 19$^{th}$ century that the treatment of goiter with iodine established the requirement of the gland for this trace element. Hypothyroidism or myxoedema, recognised as a clinical syndrome but thought to be a neurological or skin condition, was first successfully cured with extract of sheep thyroid in the late 19$^{th}$ century. Hyperthyroidism, first described by Robert Graves in young women with goiter, tachycardia and exophthalmos, was thought to be a cardiac condition (Werner and Ingbar, 1971) (Boelaert and Franklyn, 2005). Thus, early in the recognition of thyroid dysfunction, connections were made between the thyroid gland, the heart and the brain.

The physiologically active compound found in thyroid tissue was isolated, crystallized and purified by Kendall in 1915. The isolated 'iodin-containing' organic compound was tested in dogs and humans to confirm that "it produced the same effects as those found typically after the administration of desiccated thyroid – a rapid increase in pulse rate and vigor, increase in metabolism with loss of weight and increase in nervous irritability" (Kendall, 1983). The function of the compound in increasing the oxygen consumption by various tissues was demonstrated in 1952 by Barker and Klittgaard and the role of the thyroid in controlling cellular metabolism dominated the view of the function of this endocrine gland (Werner and Ingbar, 1971) (Schwartz and Oppenheimer, 1978). The essential role of thyroid hormone in foetal development in humans, in particular of the brain, was established, although the influence of the hormone was thought to be limited to the neonatal period (Schwartz and Oppenheimer, 1978). Subsequently the role of thyroid hormone in promoting growth and development as well as regulation of homeostatic functions has been well recognised (Greenspan, 2001).

Over the 100 or more years since the discovery of the role of thyroid gland extract in the control of metabolism there has been an explosion in knowledge regarding

the secretion of the gland, the mechanism of action of the hormones, their metabolism and regulation (Boelaert and Franklyn, 2005).

### *1.3.2 Thyroid Hormones*

Thyroxine, the thyroid hormone first described and characterized is an amino acid derivative containing four atoms of iodine, 3,5,3’,5’-tetraiodo-L-thyronine, T4. While the great majority of iodothyronine produced by the gland is T4, it is now recognized that this is a storage form of the molecule or prohormone. T4 is converted to the much more metabolically active and short-lived 3,5,3’-triiodo-L-thyronine, T3 (Kohrle, 1999) (Greenspan, 2001). A representation of the primary thyroid hormones (THs) is given in Figure 1.3.1.

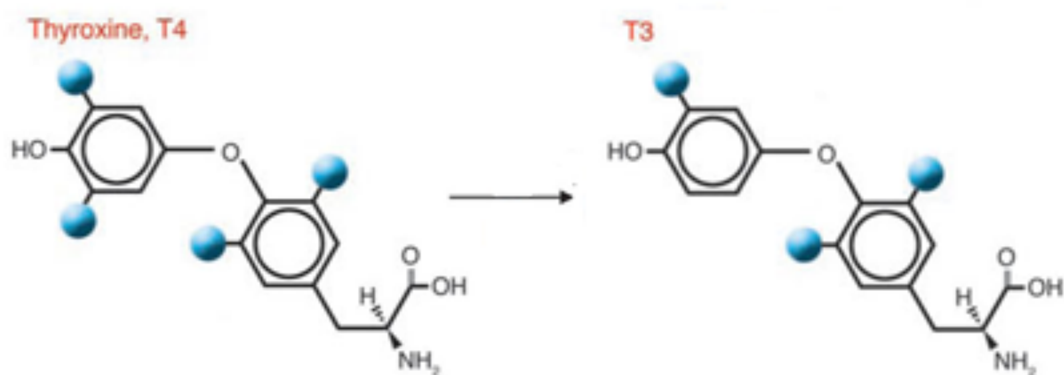



**Figure 1.3.1:** Molecular structure of l-thyroxine, 3,5,3’,5’-tetraiodothyronine, T4 and the metabolically most active l-iodothyronine, 3,5,3’-triiodothyronine, T3. Iodine atoms are shown in blue. Adapted from Bianco *et al.,* 2006.

Metabolic activity of the iodothyronines is defined in terms of affinity for thyroid hormone nuclear receptors (TRs) where binding of T3 results in transcriptional change related to stimulating growth, brain maturation, heat production and oxygen consumption. The stimulatory effects on metabolism are due to increased production of $Na^+$-$K^+$ ATPase and increased beta-adrenergic receptors. However, it is also recognised that iodothyronines have non-nuclear receptor-mediated effects at the cell membrane, cytoskeleton, or other intracellular structures including the mitochondria. Thus while the position of the iodine atoms defines biological potency, biological activity is found in a number of iodothyronine

molecules as well as various metabolites and investigations in this area are ongoing (Greenspan, 2001) {Kohrle, 2007 #60).

Once released from the gland, thyroid hormones are highly bound to plasma proteins, providing yet another level of storage as only the 'free' or unbound hormones are metabolically active. While levels of binding proteins (thyroxine-binding globulin, thyroxine-binding prealbumin and albumin) may vary in different disease states and conditions, the level of unbound hormone remains normal in euthyroid individuals. Although the thyroid gland produces a small amount of T3 (and this can increase in iodine deficiency), the majority of T3 found in the circulation in humans is not produced by the gland but is derived from T4 by peripheral deiodination (Greenspan, 2001) (Boelaert and Franklyn, 2005).

### *1.3.3 Metabolism of Thyroid Hormones*

The metabolism of thyroxine has been shown to be complex, involving multiple deiodinase enzymes and differing isoenzymes in the same tissues. Deiodination is the major metabolic pathway in normal individuals but other, minor mechanisms are responsible for the metabolism of T4 including glucuronidation and sulphonation in the liver and these may have relatively greater importance in illness (Visser et al., 1983) (Visser, 1994) (Kohrle, 2002) (Greenspan, 2001). There are at least three deiodinase enzymes, all selenoproteins: type 1 5' deiodinase (Dio1) and type 2 5' deiodinase (Dio2) which catalyze deiodination of the outer ring, converting T4 to T3 and metabolizing reverse T3 (rT3); and type 3 5 deiodinase (Dio3) which removes an inner ring iodine atom and thus is responsible for metabolizing both T3 and T4 to less metabolically active molecules. While there are differing opinions as to the relative roles and functions of respective enzymes, the following diagram, Figure 1.3.2, represents a consensus in an evolving field (Kohrle, 2002) (Bianco and Kim, 2006) (Kohrle, 2007).

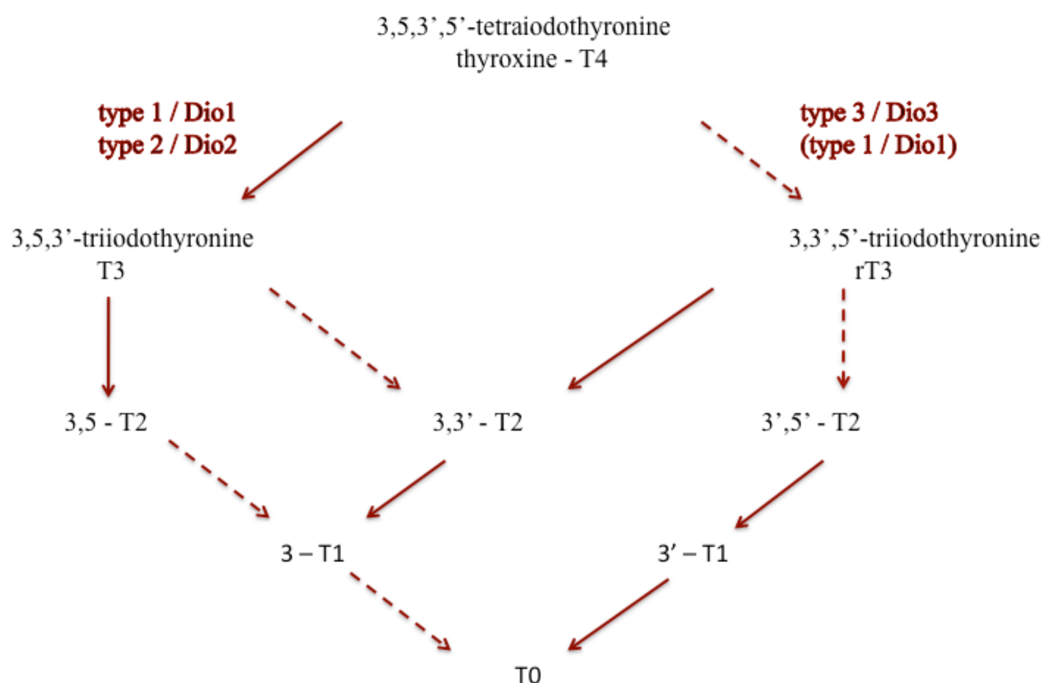



**Figure 1.3.2:** Schematic representation of the sequential deiodination of L-thyroxine (T4) and its iodothyronine metabolites to L-thyronine (T0). Iodine atoms are removed from the 5'-position (or the chemically equivalent 3'-position) from the phenolic or outer ring of iodothyronines by two 5'deiodinase enzymes (solid arrows), type 1 5'-deiodinase (Dio1) and type 2 5'-deiodinase (Dio2). The third deiodinase enzyme, type 3 5-deiodinase (Dio3) removes iodine from the tyrosyl or inner ring (broken arrows). T2-diiodothyronine; T1-monoiodothyronine. Adapted from Kohrle (2002).

The tissue distribution of the three enzymes varies. Accepted opinion is that Dio1 is expressed in liver, kidney, muscle and thyroid tissue and is responsible for providing T3 to plasma and metabolizing rT3; Dio2 is expressed in the central nervous system (CNS), pituitary and brown fat and provides intracellular T3 while Dio3, responsible for metabolizing T3 and T4, is found in CNS, placenta, skin and fetal liver (Greenspan, 2001). Dio2 and Dio3 have now been shown to have a more widespread tissue distribution and the role of Dio1 in normal homeostasis has been questioned. Dio2 is now thought to be more important in determining plasma T3 levels. Generalisation across species is also questioned as investigations in human and rodent tissues have provided conflicting evidence (Bianco and Kim, 2006) (Kohrle, 2007).

However, there is consensus that these enzymes play a vital role in providing active thyroid hormone to the tissues and that the balance of generation and metabolism of T3 is altered in disease states because of alterations in the activity of these enzymes. Moreover, over many years there has been consensus as to the tissue-specific regulation of thyroid hormone concentrations such that plasma levels do not necessarily reflect the concentration found in different tissues (Escobar-Morreale et al., 1997) (Kohrle, 2000) (Bianco and Kim, 2006) (Kohrle, 2007) (Gereben et al., 2008). Specific information related to what is known about the deiodinase enzymes found in nervous tissue and in specific brain regions together with evidence related to the regulation of these enzymes in the brain will be covered in greater detail in Section 1.8.

### *1.3.4 Thyroid Hormone Receptors*

Thyroid hormone receptors (TRs) are nuclear receptors belonging to a family of similar receptors (for retinoic acid, oestrogen and vitamin D). The TRs, when bound by their ligand, T3, influence transcription of target genes through interaction with corepressor or coactivator molecules**.** In contrast to steroid hormone receptors, thyroid hormone receptors are thought to bind DNA at specific thyroid response elements (TRE) in the absence of hormone, usually leading to transcriptional repression. Binding of T3 with the TR is thought to result in dissociation of co-repressor molecules and recruitment of co-activator molecules. The T3-TR binding is associated with conformational change in the receptor that causes it to function as a transcriptional activator (Greenspan, 2001) (Boelaert and Franklyn, 2005). A schematic representation of the mechanism of action of TRs, when bound by their ligand T3, is given in Figure 1.3.2.

Two genes encode the TR, $\alpha$ and $\beta$ and there are two isoforms of each; TR$\alpha$1 and TR$\alpha$2; TR$\beta$1 and TR$\beta$2. In humans, TR$\alpha$1 and TR$\beta$1 are thought to be the dominant isoforms with varying tissue distributions although both are found in the CNS. Most of the developmental and thermogenic effects of thyroid hormone in humans are thought to occur through the binding of T3 with these receptors (Lazar, 1993) (Greenspan, 2001).

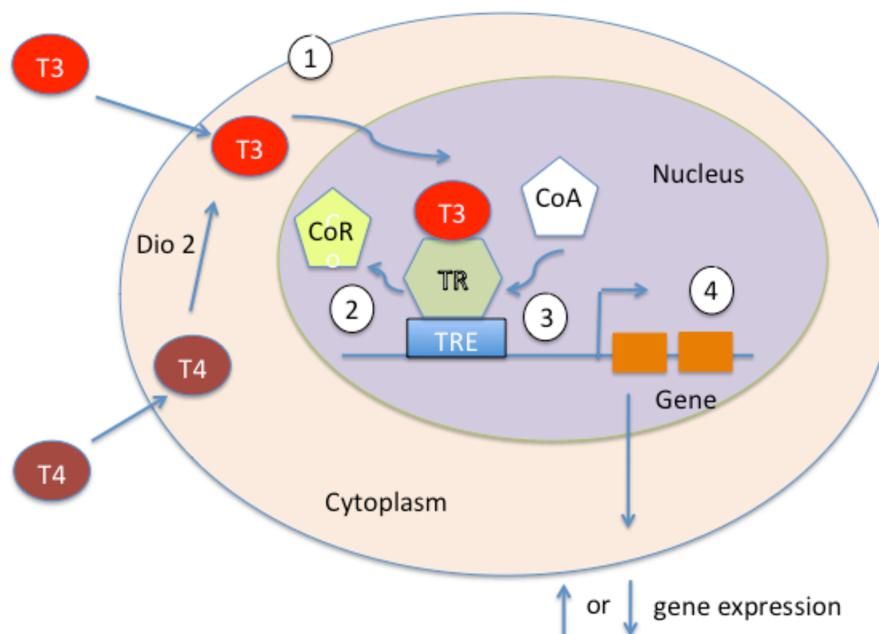



**Figure 1.3.3:** Simplified schematic representation of the mechanism of thyroid hormone receptor action. The thyroid hormone receptor (TR) binds specifically to thyroid hormone response elements (TRE) in the promoter regions of target genes. In the absence of hormone, TR binds co-repressor (CoR) proteins that suppress gene expression. When T3 enters the nucleus and binds to the TR (1), the CoR becomes dissociated from the receptor (2), co-activators (CoA) are recruited to the T3-bound receptor (3) and gene expression is altered (4). Adapted from mcgraw-hill.com/sites/dl/free/0071402357/156720/figure320_3.html.

### *1.3.5 Central Regulation of Thyroid Hormones*

The level of thyroid hormone in the plasma, and subsequently available to the tissues, is kept within a narrow range due to the action of TH in regulating its own production via negative feedback to the hypothalamus and anterior pituitary. Adaptation to temperature change is a known stress that requires altered thyroid hormone production, yet under conditions of large variations in ambient temperature, thyroid hormones remain very constant due to the regulatory control by the hypothalamo-pituitary-thyroid (HPT) axis (Bianco and Silva, 1988) (Andersen et al., 2003).

#### *1.3.5.1 Anterior Pituitary*

All aspects of the function of the thyroid gland, including the trapping of iodine, synthesis and release of T3 and T4 as well as the size and vascularity of the gland, are controlled by thyroid stimulating hormone (TSH) secreted by the anterior pituitary (Greenspan and Gardner, 2001). TSH is a glycoprotein, consisting of two sub-units, produced by the thyrotrophs of the anterior pituitary. TSH acts directly on membrane receptors on follicular cells of the thyroid to activate adenylyl cyclase and increase intracellular cAMP, which stimulates the activity of enzymes involved in the synthesis and release of thyroid hormones (Greenspan and Gardner, 2001).

Synthesis of TSH is directly controlled by binding of T3 to TRs, which regulate the expression of the genes that code for the TSH sub-units. T3 levels in the pituitary in turn depend on the activity of deiodinase enzymes, as previously discussed, as well as thyroid hormone transporter molecules. There is an inverse relationship between plasma levels of free T4 (fT4) and TSH in humans. TSH secretion is pulsatile in nature and there is a peak of secretion a few hours after onset of sleep in humans: rats show a similar peak in TSH secretion a few hours after the start of the light phase (when rats are less active) (Aron et al., 2001) (Fisher, 1996) (Campos-Barros et al., 1997) (Chiamolera and Wondisford, 2009).

#### *1.3.5.2 Hypothalamus*

The secretion of the thyrotrophs is also controlled by thyrotrophin releasing hormone (TRH) from the hypothalamus. TRH is a tripeptide derived from a large precursor protein (prepro-TRH (ppTRH)) that is secreted by specific neurons in the paraventricular nucleus (PVN). TRH secretion sets the “set point” which controls TSH secretion and injection of TRH causes rapid rise in TSH secretion within minutes. The TRH stimulation test is used to test the responsiveness of the thyrotrophs to stimulation and thus diagnose secondary hypothyroidism (Greenspan and Gardner, 2001) (Chiamolera and Wondisford, 2009). The TSH response to TRH is very sensitive to exogenous THs, which inhibit the normal response (Aron et al., 2001). While this can be explained by negative feedback by THs at the level of the pituitary, THs have also been shown to act at the level of the hypothalamus. In hypothyroid rats, relative to controls, a two-fold increase in ppTRH mRNA in the PVN was observed and in situ hybridization of ppTRH

showed that this response occurred exclusively in medial parvocellular neurons of the PVN (Segerson et al., 1987). These changes did not occur when rats were treated with thyroid hormone. Thus low levels of TH induced both transcription and translation of the TRH prohormone. Recent studies with knockout mouse models have confirmed the primacy of the TRH neuron for both TSH and TH synthesis and the role of normal TRs in the regulation of TRH and TSH by THs (Nikrodhanond et al., 2006) (Chiamolera and Wondisford, 2009).

### *1.3.6 Other regulators*

Other physiological molecules, some used as therapeutic agents, are known to alter the HPT axis activity. Somatostatin and dopamine are both known to inhibit TSH secretion (Van den Berghe et al., 1998b) (Greenspan, 2001). Somatostatin blunts the early morning TSH surge and inhibits high levels of TSH in hypothyroid individuals. Dopamine also inhibits TSH secretion and blunts the TSH response to TRH: dopamine agonists act in a similar way while antagonists stimulate TSH in euthyroid individuals. The immune system, through the synthesis of TSH receptor antibodies by B-lymphocytes, can have a regulatory effect on thyroid hormone synthesis. TSH receptor antibodies can either block the action of TSH or mimic TSH activity depending on the site of binding with the receptor (Greenspan, 2001). Two specific physiological stresses are known to have profound effects on the activity of the HPT axis: starvation and inflammatory illness. These, together with the known inhibitory effects of glucocorticoids will be discussed further in Section 1.5.

### *1.3.7 Thyroid Hormone Transporters*

Thyroid hormones act in the intracellular space and thus need to be transported across cell membranes; T4 must be transported into cells to be metabolized, as deiodinases are membrane proteins with their active sites located in the cytoplasm (Bianco et al., 2002). While both T4 and T3 are lipid soluble and passive diffusion has been assumed as the means of intracellular localisation, transport molecules have recently been identified (Jansen et al., 2005). These include organic anion transporters (OATPs), L-type amino acid transporters and monocarboxylate transporters (MCTs). One anion transporter, OATP1C1, has been identified as having a high affinity for T4 while MCT8 shows a preference for T3. These transporters have been identified in a variety of tissues and the

activity of these transporters is believed to determine the access of the iodothyronines to their sites of action and metabolism (Jansen et al., 2005).

### *1.3.8 Summary*

Since the early recognition of the function of the thyroid gland there has been continuing investigation into the normal physiological pathways involved with increasingly sophisticated techniques. The basic elements of the regulatory control of the thyroid gland were determined more than fifty years ago but refinement of the understanding of the mechanisms concerned, for example the recognition that the hypothalamus rather than the pituitary is the major centre of feedback control and establishment of the set-point role of the hypothalamus, has been fairly recent.

Investigations that have and continue to elucidate the role of the numerous iodothyronines, TRs, deiodinases and transport molecules have revealed the immense complexity of the functional control of these iodinated molecules which are essential for growth, development and normal cognitive and emotional function. The outcome of this plethora of information is the recognition of the immense scope available for individual genetic variation within the various elements comprising the components and regulation of the HPT axis. This variation provides the basis for the possibility of differing individual responses when various stresses interact with the HPT axis.

## 1.4 "Low Thyroid Syndrome"

The description of this syndrome varies but in general patients present with low or low normal peripheral T3 and T4 with a TSH level in the normal range. Investigations have shown that there is a blunting of the TSH response to TRH and decrease or abolition of the early morning TSH surge (McIver and Gorman, 1997) (De Groot, 2006) (Adler and Wartofsky, 2007). Controversy continues over the thyroid status of these patients, with some arguing that the TSH level is inappropriately low for the thyroid hormone levels and that this is evidence of altered hypothalamic regulation (De Groot, 1999) (Wartofsky et al., 1999) (De Groot, 2006).

### 1.4.1 Thyroid Function indices

The decision as to whether an individual's thyroid function is normal can be made on clinical criteria and/or based on biochemical measurements. Thyroid function tests (TFT), which are used as a measure of thyroid function, are based on peripheral indices with wide normal ranges. Selection of appropriate normal (reference) populations for thyroid function tests has long been recognised as posing many problems, due in part to the high frequency of undiagnosed thyroid dysfunction in the general population (Evered et al., 1978). Moreover, individuals seldom have thyroid indices measured prior to presenting with disease or injury and so change from previous levels cannot be evaluated. Based on the results obtained, patients are said to be euthyroid (have normal thyroid function) or to be hyper or hypothyroid (increased or decreased thyroid function). Thyroid hypofunction can be further categorised as primary (etiology is in the thyroid gland); secondary (dysfunction of the pituitary) or tertiary (dysfunction of the hypothalamus) (Greenspan, 2001).

### 1.4.2 The TSH Test

According to the American Thyroid Association, the serum level of TSH is the single most reliable test to diagnose all common forms of hyper and hypothyroidism. It is noted that while a normal TSH test excludes the diagnosis of hypothyroidism in all patients with primary hypothyroidism, it will not reliably identify those with central hypothyroidism; hence, if there is suspicion of pituitary or hypothalamic disease, fT4 should also be measured (Ladenson et al., 2000). The underlying basis for the use of TSH/fT4 as the diagnostic strategy is that

individuals have a narrow intraindividual variation in fT4 and small changes are reflected in logarithmic changes in TSH (Keffer, 1996).

The upper reference limit for TSH has steadily declined over the last few decades due to the development of more sensitive assays and a more rigorous screening of individuals when determining normal reference ranges (Baloch et al., 2003). In a consensus paper published in 2004, a panel of experts decided that the normal range was appropriate at 0.5–4.5 μIU/ml (Surks et al., 2004). At that time the panel did not accept the proposal by the National Academy of Clinical Biochemistry (NACB) that, since 95% of the normal population had TSH values below 2.0 μIU/ml, the normal range should be 0.5-2.5 μIU/ml (Baloch et al., 2003). The NACB based their recommendations on a review of previous normal limits after individuals with thyroid antibodies were excluded. Others question the exclusion criteria and point to methodological issues when establishing normal reference ranges, in particular the importance of time of day when sampling (Jensen et al., 2007). Yet others argued that the range should be as suggested by the NACB and that the evidence for a narrower reference range is compelling (Dickey et al., 2005) (Wartofsky and Dickey, 2005). The American Association of Clinical Endocrinologists (AACE) in 2002 practice guidelines used a reference range with an upper limit of 5 μIU/ml (Baskin et al., 2002). The AACE has since updated these guidelines and a normal range of 0.3-3.0 μIU/ml is suggested with values between 3.0 and 5.0 μg/ml requiring further investigation (Gharib et al., 2005).
The adjustment of the upper reference limits for TSH is useful for those individuals who have mild or developing hypothyroidism and can now benefit from earlier diagnosis and treatment. However, the range is still wide relative to significant biological effect. NACB guidelines suggest that a magnitude in difference in TSH values of 0.75 μg/ml would be clinically significant when monitoring a patient's response to therapy (Baloch et al., 2003). A change in TSH level within the normal range could therefore have significance for the individual. Intraindividual variation has been shown to be very narrow, not only for fT4 but also for TSH, allowing for circadian variation (Andersen et al., 2002) (Andersen et al., 2003). Thus individuals have a set point or 'thyrostat', which does not vary within the range of normal values unless disease or dysfunction occurs. The lowering of the upper reference range does not address the issue of the possibility

of hypothalamic dysfunction (rather then disease) as the cause of inappropriate TSH levels in individuals (De Groot, 2006).

### *1.4.3 Thyroid Hormones*

Based on guidelines, as stated above, thyroid hormone levels are usually not measured unless thyroid pathology is indicated based on an abnormal TSH level or central hypothyroidism (considered rare) is suspected (Ladenson et al., 2000). Because of the variability in peripheral THs due to variations in plasma binding proteins, measurement of total T4 (TT4) and total T3 (TT3) levels are usually now replaced by measurements of the metabolically active free T3 and T4 (fT3 and fT4). The reference ranges for all of the hormone measurements are wide. Typically, the difference between the upper and lower reference levels for fT4 and TT4 is more than 250% and for fT3 and T3 about 220%. It is argued that for the individual, movement within these wide limits can have significant clinical effect (Danzi et al., 2005). This viewpoint is supported by the demonstration that intraindividual difference is small and differences in fT4 of 6 pmol/L (range: 9-23 pmol/L) and in fT3 of 1.5 pmol/L (range: 3.5-7.7 pmol/L) are greater than the combined effects of individual variation and analytical variability and could be clinically significant (Andersen et al., 2002) (Andersen et al., 2003) (Baloch et al., 2003). The understanding, that a decrease in fT4 and/or fT3 to a value that is still within a normal reference range, may not be normal for a patient, has also assisted in the earlier recognition of mild or developing hypothyroidism. This knowledge does not however address the issue of the possibility of hypothalamic dysfunction, resulting in inappropriate TSH levels, as the cause of low thyroid hormones in individuals (De Groot, 2006).

### *1.4.4 Thyroid Antibodies*

Antibodies to proteins normally found only in the thyroid gland can be assayed in serum and indicate inflammatory damage to the thyroid follicular cells. Above normal levels of antibodies to thyroperoxidase (TPOAb) are diagnostic of Hashimoto’s Thyroiditis. This autoimmune disease, which other than iodine deficiency is the commonest cause of hypothyroidism, results in progressive destruction of the gland. Significant titers of antibodies to thyroglobulin (TgAb), the intra-follicular protein involved in TH synthesis, is also indicative of autoimmune thyroid diseases of different aetiologies (Greenspan, 2001). One half

of patients with TSH values in the 3.0- 4.0 μIU/ml range were reported to have moderate to high titers of thyroid antibodies and thus autoimmune thyroid disease with metabolic evidence of subtle thyroid dysfunction (Lindstedt et al., 2001). Tests for thyroid antibodies have become more sensitive over the years. Direct biopsy of the gland, moreover, has been useful in confirming evidence of inflammatory change in the thyroid not evident in blood tests (Wikland et al., 2001).

### *1.4.5 Sick Euthyroid Syndrome/Non Thyroidal Illness*

The earliest descriptions of a "low thyroid syndrome" related to critically ill patients in hospital intensive care units and post-operative surgical patients. Acute critical illness or trauma induces alterations in thyroid hormones within hours. Peripheral levels of T3 fall rapidly whereas TSH levels and T4 remain in the normal range. Reverse T3 (rT3) levels are elevated (Wartofsky and Burman, 1982) (Chopra, 1997) (Baloch et al., 2003). Clinical dilemmas ensued in distinguishing this state from true hypothyroidism and deciding whether treatment should be implemented (Wartofsky and Burman, 1982) (Chopra, 1997) (Utiger, 1995) (Stathatos and Wartofsky, 2003) (De Groot, 2006) (Fliers et al., 2006a) (Adler and Wartofsky, 2007).

The consensus of clinical opinion was that these patients were in fact euthyroid; that this response to acute critical illness is an adaptive mechanism akin to plunging the body in ice that, in lowering metabolic rate, facilitates the healing process. The terms Sick Euthyroid Syndrome (SES) and Non Thyroidal Illness (NTI) have been used to delineate this condition from true hypothyroidism. However the decrease in T3 has been correlated with poor prognosis and when patients also develop low levels of T4, correlation with mortality has been documented (Becker et al., 1980) (den Brinker et al., 2005) (Baloch et al., 2003). Figure 1.4.1 gives a diagrammatic representation of thyroid indices with progressive severity of the condition. Conversely, recovery has been shown to correlate with increasing levels of TSH and returning of peripheral indices to normal levels; controversy remains over whether on not these patients should be treated with replacement hormones (Utiger, 1995) (De Groot, 1999) (Wartofsky et al., 1999) (De Groot, 2006).

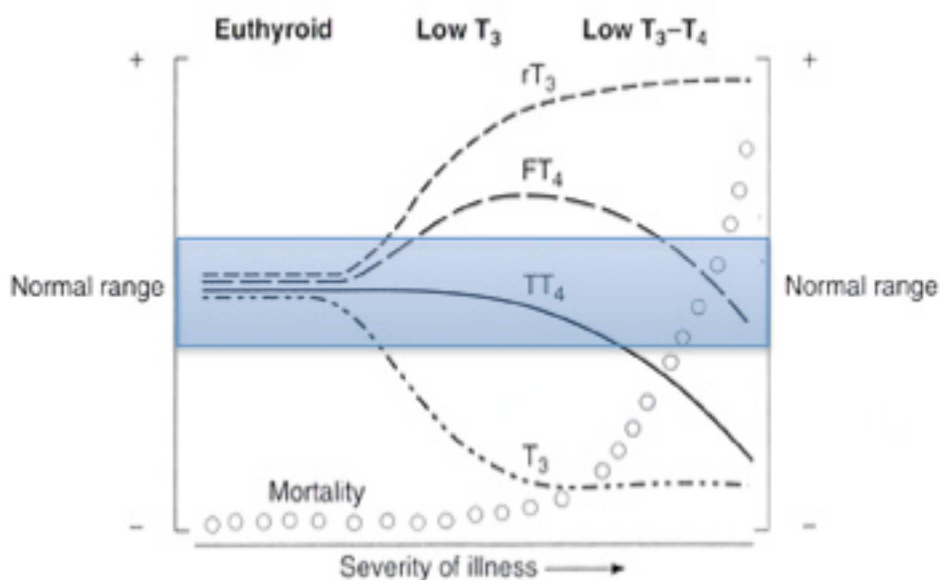



**Figure 1.4.1:** Changes seen in the peripheral thyroid hormone levels in patients with acute critical illness and trauma. Levels are represented relative to pre-illness levels and to the normal range. Shortly following onset peripheral T3 falls rapidly while rT3 levels rise, as do fT4 levels (low T3 syndrome). T4 levels initially remain steady but if illness is prolonged will fall (Low T3/T4 syndrome). Severity of the condition is correlated with the fall in T3 and mortality is correlated with falling T4 levels. (Adapted from Greenspan, 2001: original source Nicoloff, 1991).

It is now recognised that most hospitalized patients have abnormal thyroid indices (Baloch et al., 2003). In the acute situation where only T3 levels are suppressed, the suggested mechanisms are thought to involve the peripheral deiodinase enzymes. Decreased conversion of T4 to T3 has been shown either due to inhibition of this process or a decrease in deiodinase type 1 (Dio1) (Chopra et al., 1985) (Chopra et al., 1986). Other studies suggest an increase in deiodinase type 3 (Dio3), responsible for the metabolism of T4 and rT3, occurs (Den Brinker et al., 2005). A schematic representation of the suggested changes in T4 and T3 metabolism is shown in Figure 1.4.2.

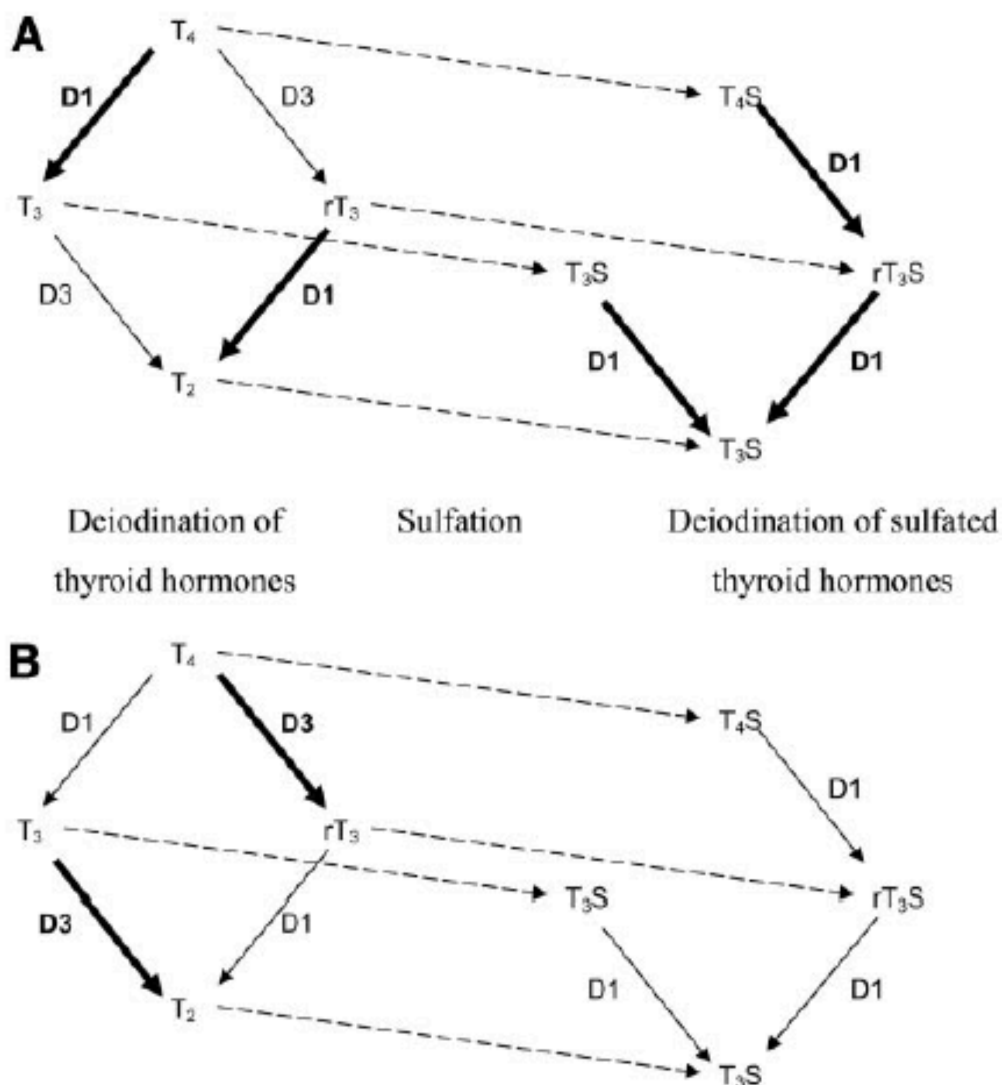



**Figure 1.4.2:** Peripheral thyroid hormone metabolism during normal homeostasis (A) and during critical illness (B). During critical illness deiodination is altered, with reduced Dio1 activity (thin arrows) and increased Dio3 activity (thick arrows), which both result in increased rT3 levels at the expense of TT3 levels (left side). Sulfated thyroid hormones, however, are exclusively deiodinated by Dio1 (right side) and reduced Dio1 levels will result in elevated levels of all sulfated thyroid hormones. Dotted arrows indicate sulfation of thyroid hormones. Adapted from Den Brinker *et al.,* (2005).

A decrease in conversion of T4 to T3 and increased metabolism of T3 would explain decreased T3 levels while normal T4 levels, and elevated fT4, prevent a central response. As acute illness is prolonged and TT4 falls, a defect in hormone binding has been identified with fT4 levels remaining normal (Kabadi, 2001). However as severe disease persists and with falling fT4, dysregulation of the

accepted homeostatic control mechanism occurs (Kaptein et al., 1981) (De Groot, 2006) (Greenspan and Gardner, 2001) (Van den Berghe et al., 1998b).

### *1.4.6 Non Thyroidal Illness in prolonged critical illness*

Investigations in long-term intensive care patients provide evidence that the syndrome is different when critical illness is prolonged. There is a generalized decrease in peripheral hormones. In addition to low levels of T3 and T4, peripheral gonadal steroid levels are low. Pituitary trophic hormones are suppressed; as well as TSH, gonadotrothins, prolactin, growth hormone and corticotrophin levels are all low and fall with time in intensive care (Van den Berghe et al., 1998b). The exceptions are peripheral levels of cortisol which are elevated throughout the time of illness (Van den Berghe et al., 1998b) (Van den Berghe, 2002). A schematic representation of the endocrine changes seen in chronic critical illness is shown in Figure 1.4.3.

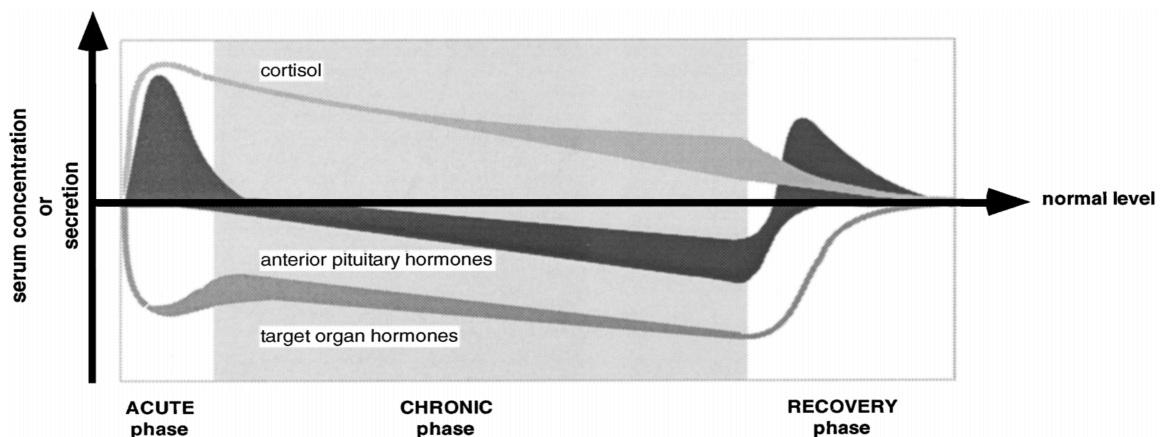



**Figure1.4.3:** Simplified concept of the pituitary-dependent changes during the course of critical illness. In the acute phase of illness (first hours to a few days after onset), the anterior pituitary hormones are maintained or amplified, whereas anabolic target organ hormones are inactivated. Cortisol levels are elevated as well as ACTH. In the chronic phase of protracted critical illness (intensive care dependent for weeks), the anterior pituitary appears uniformly suppressed in relation to reduced circulating levels of target organ hormones. Impaired anterior pituitary hormone secretion results in further target hormone decrease over time. Cortisol is the exception; the circulating levels remain elevated through a peripheral drive, a mechanism that ultimately may also fail. The onset of recovery is characterized by restored sensitivity of the anterior pituitary to reduced feedback control. Adapted from Van den Berghe *et al.,* (1998).

In the chronic critically ill, it appears clear that there is central suppression of trophic hormones that are not responsive to the accepted negative feedback regulation. Specifically for the HPT axis, TSH levels fall progressively and the pulsatile nature of secretion is diminished while both T4 and T3 peripheral levels remain low (Van den Berghe et al., 1997) (Van den Berghe et al., 1998a). A schematic representation of the possible mechanisms underlying the differences between the NTI seen in acute illness and those seen when critical illness is prolonged is shown in Figure 1.4.4.

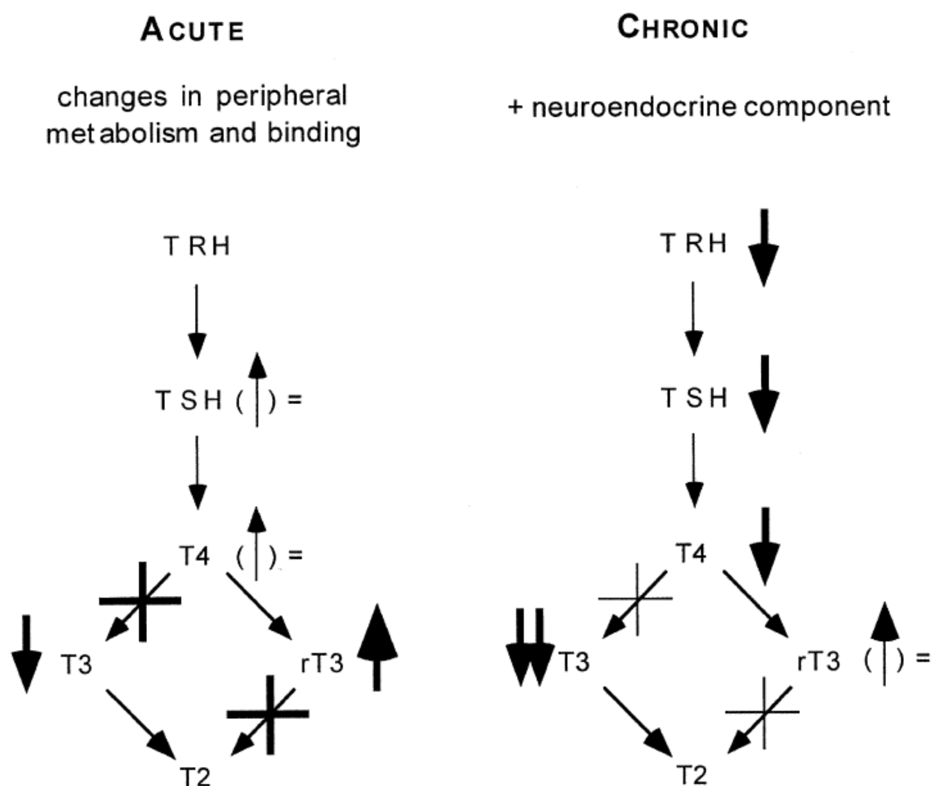



**Figure 1.4.4:** Simplified overview of the major changes occurring within the HPT axis during the acute and chronic phases of critical illness. In the acute phase, changes occur to systemic deiodinases altering the levels of the peripheral hormones: while T3 levels fall and rT3 levels rise, T4 levels remain unchanged. In the chronic phase there is central inhibition of TRH: this results in decreased TSH secretion and decreased levels of T4 as well as further decrease in T3. Adapted from Van den Berghe (2002).

The argument that central suppression of thyroid secretion is present in prolonged critical illness is strengthened by evidence that hypothalamic TRH gene expression, demonstrated with *in situ* hybridization histochemistry in post-mortem

specimens, is positively related to T3 and TSH levels measured in the 24 hours before death. In patients who died due to an acute condition TRH expression did not correlate with T3 (Fliers et al., 1997). Further, infusion of TRH has been shown to restore peripheral thyroid hormones to normal levels, although concurrent infusion of growth hormone releasing peptide is necessary for the restoration of the pulsatile TSH secretion (Van den Berghe et al., 1998a). In addition, onset of recovery from severe illness is marked by increase in peripheral TSH levels (Bacci et al., 1982).

Van den Berghe suggests that recognition that acute and prolonged critical illnesses represent different neuroendocrine paradigms explains some of the anomalies that are seen and that contribute to ongoing controversy (Van den Berghe, 2002) (De Groot, 2006). Clearly, severity and duration of illness, incidents of acute illness, such as infection, superimposed on ongoing prolonged conditions together with possible onset of recovery, could all contribute to confusing peripheral hormone indices. If it is accepted that the low thyroid state in prolonged critical illness is due to hypothalamic suppression, questions as to the need for replacement therapy are raised with ongoing controversy (Chopra et al., 1987) (De Groot, 1999) (Wartofsky et al., 1999) (Stathatos et al., 2001) (Van den Berghe, 2002) (De Groot, 2006).

To what extent decreased thyroid hormone tissue levels are involved in specific problems associated with prolonged critical illness such as diminished cognitive status, lethargy, somnolence and depression is still speculative and requires ongoing investigation (Vaughan et al., 1985) (Van den Berghe, 2002).

Various mechanisms have been suggested for the pathogenesis of hypothalamic suppression of TRH in critical illness. Fasting or starvation are often part of critical illness and have profound effects on central thyroid regulation; hypercortisolism has been well documented to be associated with suppressed TSH; cytokines have been implicated in cases of sepsis and dopamine, used therapeutically, is known to provoke or enhance central hypothyroidism (Van den Berghe, 2002) (De Groot, 2006) (Adler and Wartofsky, 2007). Animal models have been useful in elucidating the relative roles for these factors and will be discussed in detail in section 1.5.

### 1.4.7 The “low thyroid syndrome” in chronic conditions

The use of the terms “low thyroid syndrome”, SES and NTI, originally used with acute critically ill patients, has been extended to many clinical situations where thyroid indices show a similar pattern. These include a diverse range of chronic conditions. Investigations in different patient groups reveal the syndrome in some but not necessarily all or even a majority of patients, making if difficult to evaluate the incidence of the syndrome in chronic conditions or the elements of the disease condition that may contribute to the syndrome. As noted by Van den Berge with respect to critical prolonged illness, those with ongoing chronic illness can have exacerbations of their condition or concurrent other incidents, for example infection, complicating the picture. Severity and duration of the condition are other variables.

Reports vary, but typically thyroid hormone levels include normal or decreased TT4 and fT4, decreased total TT3 and fT3 along with usually normal TSH levels (Dimopoulou et al., 2001) (Adler and Wartofsky, 2007). As with the prolonged critically ill, the significance, if any, of low thyroid levels for the chronically ill patient in terms of cognitive and emotional factors and general well being has not been well investigated. In the reports of some chronic conditions a link can be made between low T3, inflammatory markers and depression.

#### 1.4.7.1 Chronic heart and lung disease

In chronic heart failure, the prevalence of NTI is reported to be approximately 20% (Adler and Wartofsky, 2007). Evidence of the early peripheral changes seen in NTI (lower T3 and higher rT3 compared to controls) was reported in a group of patients with compensated ischemic heart disease (Shanoudy et al., 2001). In moderate to severe cardiac disease, thyroid hormone levels correlated with the severity of the condition and T3 levels were reported to be the highest independent predictor of mortality in hospitalized chronic heart disease patients (Iervasi et al., 2003) (Iervasi et al., 2007). A study of patients admitted to hospital with coronary artery disease (CAD), in which thyroid hormones were measured and symptoms of depression assessed, showed that the patients with both CAD and depression had lower T3 levels and a higher prevalence of heart failure (Bunevicius et al., 2006). In other studies, levels of inflammatory markers and

measures of depression correlated with increased morbidity and mortality in chronic heart disease (Frasure-Smith et al., 2007) (Redwine et al., 2007). Evidence of abnormal thyroid hormone regulation, with absent or delayed TSH response to TRH administration, has been reported in some patients with chronic obstructive pulmonary disease (COPD) both in severe disease and acute exacerbations. In patients with stable severe COPD, hypoxia precipitated both central and peripheral HPT axis changes (Dimopoulou et al., 2001).

#### *1.4.7.2 Inflammatory conditions and connective tissue disorders*

A number of inflammatory conditions have been associated with abnormal thyroid function tests. Thyroid function tests were examined in a group of 170 patients with connective tissue disorders of varying etiologies. The authors concluded that undiagnosed thyroid disease was indicated in a third of patients and that an additional 54% had indices indicative of NTI (Arnaout et al., 1994). Patients with rheumatoid arthritis, an inflammatory condition, had lower TSH values than osteoarthritis patients (non inflammatory) but did not have higher T4 levels suggesting central depression of the HPT axis (Ilias et al., 1999). Thyroiditis has been associated with chronic fatigue syndrome (CFS). Fine needle biopsy of the thyroid gland identified thyroiditis in 40% of patients who had experienced chronic fatigue for longer than a year. Of these patients, 60% had TSH levels within the normal range and the clinical response to thyroxine was equally favorable irrespective of the initial TSH level (Wikland et al., 2001) (Wikland et al., 2003). These authors conclude that thyroid function tests do not necessarily identify hypothyroidism in this inflammatory condition, as the TSH levels are inappropriately low in a significant number of individuals.

#### *1.4.7.3 Mood disorders*

Depression is associated with abnormal thyroid function tests. For patients diagnosed with hypothyroidism, based on accepted TFT criteria, depression is often one of the presenting symptoms. However, a significant group of patients with mood disorder have peripheral thyroid indices that are suggestive of more subtle grades of thyroid dysfunction or evidence of HPT dysregulation as seen in NTI (Gold et al., 1981) (Gold et al., 1982) (Bartalena et al., 1990) (Wilson et al., 1992) (Hickie et al., 1996) (Esposito et al., 1997) (Musselman and Nemeroff, 1996) (Hendrick et al., 1998) (Fountoulakis et al., 2006). There is also a high

prevalence of anti-thyroid antibodies among patients with depression (Gold et al., 1982) (Musselman and Nemeroff, 1996). Thyroid hormone deficiency is implicated when patients respond to T3 augmented therapy (Sokolov et al., 1997) (Abraham et al., 2006).

### *1.4.8 Summary*

In the "low thyroid syndrome" reported in chronic illness, the TSH level is not elevated despite peripheral hormone levels that are low. It is argued that these TSH levels are almost always inappropriately low for the observed serum T4. That the TSH level does not rise despite low peripheral hormone levels indicates that there is an altered control mechanism, that impaired hypothalamic function is the cause of the low TSH and thus low output from the thyroid gland (De Groot, 1999) (De Groot, 2006). Other features of the syndrome such as a blunting of the TSH response to TRH and suppression of the normal circadian surge of TSH at nighttime also implicate central elements of the HPT axis. Furthermore, in both acute and chronic conditions, serum hormone levels have been found to correlate with the severity of the underlying condition. While De Groot's views are controversial and far from universally endorsed, they have developed during the time that investigators have shown that the site of control in the HPT axis is in the hypothalamus, not in the pituitary as has been long believed (Chiamolera and Wondisford, 2009).

Mood disorder, fatigue, behavioural change and cognitive loss are reported in chronic conditions of many different etiologies as previously discussed. Underlying inflammatory disorders are common to many of these conditions. In similar groups of patients HPT axis dysfunction is reported in some individuals. While there is controversy as to the significance of the low thyroid syndrome, reports of behavioural change and low thyroid hormones in the same patients raises questions.

## 1.5 Stress and the Hypothalamo-pituitary-thyroid axis

The HPT axis, like other hormonal systems, allows for response to stress or change in the environment. The usual view of thyroid physiology as a static maintenance of basic metabolic function rather than a dynamic responsive system has been recently questioned (Flier et al., 2000). The classical feedback model allows for a change in peripheral hormone, for example as a result of increased utilisation, to be detected centrally. Hypothalamic stimulation leads to an increase in pituitary hormone, in turn stimulating target gland production, resulting in restoration of the peripheral hormone levels and homeostasis despite the changed circumstances. Change in environmental temperature is an example of a stressor activating the HPT axis. Thyroid hormone is essential for and is utilised in the maintenance of body temperature in mammals (Bianco and Silva, 1988) (Silva, 2005). Within reasonable limits thyroid homeostasis is maintained: an individual's thyroid indices do not change markedly with the seasons (Andersen et al., 2003). Growth and repair are other examples of stressors where HPT axis activation is implicated. A role for T3 in wound healing and muscle repair has been demonstrated (Safer et al., 2004) (Anderson et al., 1998). Specific tissue level changes to Dio2 and Dio3 gene expression following nerve injury suggest altered levels of tissue metabolism of T4 and T3 utilised in peripheral nerve repair (Li et al., 2001b) (Li et al., 2001a). Within normal physiological limits, homeostasis is maintained.

There are, however, many examples of stressors that result in disruption of the HPT axis. Many experimental stressors have been investigated in both humans and rats to determine their effect on thyroidal homeostasis. The results are very mixed. As summarized by Baumgartner *et al*: in humans, decreases, increases and no change in plasma TSH and peripheral thyroid hormones have been reported after different stressful stimuli (Baumgartner et al., 1998). In rats there is a similar range of experimental results. While the claim has been made that, in rats, all studies have unanimously reported a fall in the serum concentrations of TSH during stressful stimuli (Baumgartner et al., 1998), others have identified that the nature of the stressor is all-important in determining the effect on the HPT axis. Repeated tail shock in male Sprague-Dawley rats has been shown to have a

greater effect than single shock. Both T3 and T4 were significantly decreased only after repeated shock, while TSH did not rise after either shock regimen (Servatius et al., 2000). In an elegant paired experiment, T3 did not decrease in rats that were able to escape shock but did decrease in rats where the shock was inescapable (Helmreich et al., 2006). Thus repeated stress as well as inescapable stress, or the perception thereof, might have different effects on the HPT axis. Together with individual differences in ability to deal with perceived stressors, these factors might help explain the differing results obtained in human studies. A number of physiological stressors have been identified that cause disruption of the HPT axis and could play a role in the "low thyroid syndrome" seen in acute and chronic illness. Observations in clinical situations as well as the use of animal models have been useful in identifying the mechanisms involved.

### *1.5.1 Starvation or low carbohydrate diet*

Alterations to peripheral and central components of the HPT axis occur in acute and prolonged starvation. In acute starvation, the hormone picture is similar to that described in acute NTI. Peripheral T3 is low with normal T4 and elevated rT3 (Adler and Wartofsky, 2007). The response is generally interpreted as an attempt to reduce energy expenditure and thus as a response appropriate for survival, not warranting intervention (Van den Berghe et al., 1998b) (Adler and Wartofsky, 2007). Peripheral hormone alterations are thought to be due to altered levels of deiodinase enzymes, decreasing the conversion of T4 to T3 and the metabolism of rT3. Changes also occur centrally, and increasingly so with prolonged fasting, resulting in suppressed TSH levels. There is also a blunting of the TSH response to TRH (Adler and Wartofsky, 2007). Studies in rats confirm the changes seen clinically with starvation leading to decreased hypothalamic mRNA for TRH, decreased portal TRH and decreased pituitary TSH (Blake et al., 1991). Using *in situ* hybridization techniques, gene expression of Dio2 was up regulated in the arcuate nucleus (AN) in starvation; this would result in increased hypothalamic T3 concentrations, suggesting a mechanism for down regulation of mRNA for TRH. This suppression of mRNA for TRH was shown to be independent of the regulation of TRH by thyroid hormones (Diano et al., 1998). Furthermore, inhibition of hypothalamic Dio2 prevented the fall in mRNA for TRH seen in early fasting (Coppola et al., 2005). A rapid decrease in leptin, a hormone produced by adipose tissue, is thought to be responsible for these central

changes. While the investigations of Diano *et al.,* (1998) suggest involvement of the Dio2 enzyme others suggest that leptin acts either by direct stimulation of TRH neurons in the PVN of the hypothalamus or by acting indirectly via the melanocortin pathway (Ahima et al., 1996) (Kim et al., 2000) (Flier et al., 2000). These changes have been shown to be specifically due to lack of carbohydrate, as replacement of carbohydrate results in return of the hormones to normal while this does not occur with protein or fat replacement (Adler and Wartofsky, 2007).

Thus changes seen to the HPT axis in acute illness (NTI) are also seen in starvation. Of course some patients in acute illness also suffer malnutrition and so there may be more than one group of factors at work. In a carbohydrate deficient diet however, it appears that evidence supports the fall in leptin as the trigger for central (but not peripheral) changes to the HPT axis (Adler and Wartofsky, 2007) (De Groot, 2006).

### *1.5.2 Corticosteroids*

Corticosteroids are implicated in central suppression of the HPT axis. Administration of maintenance doses of glucocorticoids to patients with adrenal insufficiency or pharmacological doses to euthyroid patients resulted in suppression of plasma levels of TSH. Escape from this suppression occurred after 3 days and a role for corticosteroids in the diurnal variation of TSH proposed (Nicoloff et al., 1970).

Suppression of thyroid hormones and TSH has been noted in numerous studies of patients with Cushing's syndrome of varying etiologies; many authors also report that thyroid indices reverted to normal when the hypercortisolism was corrected (Luton et al., 1981) (Visser and Lamberts, 1981) (Benker et al., 1990) (Bartalena et al., 1991) (Adriaanse et al., 1994). Patients with incidentally discovered adrenal adenomas, but elevated urinary cortisol, demonstrated loss of TSH diurnal variation and blunted TSH response to TRH and thus mild impairment of the HPT axis even in mild hypercortisolism (Coiro et al., 2002).

It is generally accepted that pharmaceutical doses of glucocorticoid suppress the HPT axis (Greenspan, 2001). That this suppression occurs at the hypothalamic level was confirmed by the demonstration of decreased mRNA for TRH post

mortem in patients who were treated with glucocorticoids compared to a matched group of patients who did not receive glucocorticoid therapy (Alkemade et al., 2005).

The effect of physiological levels of endogenous glucocorticoids on the HPT axis is however less clear. The suggested role of cortisol in the diurnal variation of TSH was questioned when no correlation between the pulsatile and circadian TSH and cortisol rhythms was found (Brabant et al., 1989). Furthermore, acute stimulation of endogenous cortisol with CRH, in normal individuals, did not affect TSH levels; in contrast, dexamethasone resulted in a dose dependent suppression of TSH (Brabant et al., 1989). In another study, high cortisol levels, following either ACTH or CRH administration, resulted in no change to basal or TRH stimulated TSH in controls. The authors concluded that only prolonged hypercortisolism as seen in Cushing's syndrome interferes with TSH secretion (Rubello et al., 1992). Following studies in patients with adrenal insufficiency where the effect of changing cortisol levels on the HPT axis was investigated, Samuels (2000) concluded that the specific nature of the interactions between cortisol and TSH within the physiological range has yet to be fully clarified. While infusion of cortisol to establish a diurnal rhythm re-established a diurnal rhythm for TSH; shifting the cortisol cycle 12 hours did not effect the secretion of TSH (Samuels, 2000).

Numerous studies in rats have investigated the effect of elevated glucocorticoids on the HPT axis. Prolonged restraint in rats caused gastric ulceration after 8 hours and levels of corticosterone 30 times baseline levels. T3 was decreased, rT3 elevated while T4 remained constant. Deiodinase activity in liver and kidney was decreased and this was suggested as the explanation for changes in the peripheral hormone levels (Bianco et al., 1987). The time course of these changes was interesting; significant TSH suppression was demonstrated after 2 and 4 hours while corticosterone levels were rising and T3 and rT3 remained steady: deiodinase activity did not fall until 4 hours: T3 and rT3 levels were altered only at 6 and 8 hours of restraint when TSH levels were markedly suppressed. All of these alterations to the HPT axis, except for the initial TSH suppression, were prevented by adrenalectomy and basal replacement of corticosterone. The authors concluded that, in rats, corticosterone causes suppression of the HPT axis, but that

other mechanisms are also implicated in the response to stress; they note differences in these studies and those reported in humans (Bianco et al., 1987). In an experiment with intermittent rather than continuous stress levels, repeated foot-shock for 14 days, fT3 and fT4 were both decreased with no change in mRNA for TRH: there were negative correlations between peak corticosteroid levels and fT4 and mRNA for TRH. Peak corticosterone levels occurred at 40 minutes after foot shock, returning to baseline by two hours; thus shorter, repeated bursts of corticosteroids were also associated with suppression of the HPT axis. The authors noted the individual differences in response amongst the rats (Helmreich et al., 2005).

Thus while it is noted that physiological levels of glucocorticoids do not appear to affect the secretion of TSH, pathological levels and high secretion levels associated with prolonged and repeated stress have been implicated in HPT axis suppression. While other mechanisms may also be involved, a role for hypercortisolism in NTI seems clear, particularly in prolonged critical illness where cortisol levels remain abnormally high for prolonged periods of time (Van den Berghe et al., 1998b).

### *1.5.3 Infection and Inflammatory stress*

While most hospitalized patients show evidence of HPT axis disruption, this is most notable in patients with sepsis. The role of pro-inflammatory markers as causative factors in the syndrome of central and peripheral changes has been examined. There were greater falls in TSH and thyroid hormones in patients with sepsis when compared to those with acute myocardial infarction. The levels of a number of cytokines were markedly elevated in the septic patients relative to the cardiac patients; while the HPA axis was activated in both groups, there were no differences in hormone levels, suggesting that the two mechanism may be separate but additive in effect (Monig et al., 1999). A role for specific pro-inflammatory cytokines has been extensively investigated: while correlative studies in acutely sick patients suggest a major role for interleukin-6 (IL-6), experimental studies in humans suggest that other cytokines directly or indirectly lead to the peripheral changes seen in NTI (Papanicolaou, 2000). Numerous clinical reports confirm an association between cytokines and altered hypothalamic function, without direct evidence of causation, although evidence

suggests that the cytokines interleukin-1-beta (IL-1β), soluble interleukin-2 receptor, IL-6, tumor necrosis factor alpha (TNF-α), and nuclear factor kB have roles in the direct suppression of TSH in sepsis (De Groot, 2006) (Adler and Wartofsky, 2007).

In vitro studies have shown that the effects of cytokines can be demonstrated in multiple tissues including suppression of all aspects of thyroid follicular cell synthesis and regulation of deiodinase activity in thyroid, pituitary and liver (Papanicolaou, 2000). For example, in human thyroid cell culture TNF-α, interferon gamma (IFNδ) and IL-6 were shown to inhibit Dio2 activity and in cultured rat anterior pituitary cells, IL-1β stimulated Dio1 and Dio2 in a dose dependent fashion and Dio2 was stimulated by TNFα (Molnar et al., 2002) (Baur et al., 2000).

Papanicolaou (2000), in a review of the evidence for cytokine mechanisms in NTI, concludes that *in vitro* studies have yielded conflicting evidence both for effects on peripheral and central deiodinases and on pituitary thyrotrophs. While studies such as these confirm the sensitivity of the deiodinase enzymes to various cytokines, the situation in the intact animal may depend on the varying effect of these molecules in different tissues. The use of *in vivo* animal models is suggested as necessary to clarify the role of cytokines in this syndrome (Papanicolaou, 2000).

#### *1.5.3.1 Animal models of inflammatory stress*

A number of animal models have been developed to investigate the relationship between cytokines and the HPT axis changes seen in illness. The effect of the inflammatory process on the deiodinase enzymes is central to these investigations as local control of T3 concentrations depends on the activity of these enzymes, as previously discussed.

##### *1.5.3.1.1 Acute Illness*

A model for the NTI seen in acute critical illness was developed using sub-lethal injections of bacterial lipopolysaccharide (LPS) in rodents. Following LPS injection, mice developed systemic illness, which was accompanied by elevation in the serum levels of a number of cytokines. Within 4 hours there was a decrease in liver Dio1 mRNA, a decrease in T3 and T4 by 8 hours and fT3 and fT4 by 12

hours. TSH levels remained unchanged. Injections of a number of cytokines (IL-6, TNFα, IL-1α and IFNδ) demonstrated that some of the changes induced by LPS, but not all, could be induced by individual cytokines (Boelen et al., 1995). Using knockout mice in the same model these authors have subsequently shown that a number of different cytokines selectively effect peripheral and central components of the HPT axis changes (IL-6, IL-12, IL-18) (Boelen et al., 1996) (Boelen et al., 2004a) (Boelen et al., 2004b).

Systemic administration of LPS in rats has shown similar results in a number of studies and elements of possible causative mechanisms have been progressively demonstrated. Plasma T4, T3 and TSH were reduced but TRH gene expression in the PVN using *in situ* hybridization histochemistry did not show the expected increase which follows lowering of peripheral T4 in primary hypothyroidism. Infusion of IL-1β into CSF resulted in the same depression of T4 levels, TSH levels were only reduced after 7 days of infusion but TRH mRNA did not increase despite the low T4 level. The authors concluded that the inability of the PVN to respond to low T4 by increasing TRH gene expression was one of the factors contributing to inability of the pituitary to increase TSH output following inflammatory stimulation (Kakucska et al., 1994). Decreased PVN mRNA for TRH and pituitary mRNA for TSH with suppressed peripheral T3 have also been reported in LPS injected rats in which increased PVN mRNA for CRH and increased peripheral corticosterone levels were also found. Because of the possible role of corticosteroids in the HPT axis suppression, further studies were performed clamping corticosterone levels at morning baseline levels (adrenalectomy followed by corticosterone pellet implantation), which showed that the HPT axis effects were independent of the LPS induced increase in corticosterone (Kondo et al., 1997).

Changes have also been reported at the pituitary level. Following a single LPS injection in rats, there was a transient rise in Dio1 activity in the pituitary (at 1.5 hours) followed at 4 hours by a decrease in serum TSH levels and liver Dio1 activity. Pituitary Dio2 activity increased from 6 hours post injection to peak at 24 hours post injection during which time TSH levels returned to normal. Dio2 pituitary activity returned to baseline after 24 hours. Lower doses of LPS resulted

in 'similar effects to a lesser extent' indicating that LPS effects are dose dependent) (Baur et al., 2000).

Following on from their earlier work with LPS injected mice, Boelen *et al.,* showed that almost simultaneously with the peripheral hormonal changes there were changes in hypothalamus, pituitary, thyroid and liver tissues. Increased levels of mRNA for IL-1β were found in all tissues as well as multiple changes in gene expression for thyroid receptors and deiodinase enzymes. Differing results were found in the different tissues. For example, using RT-qPCR techniques, expression of mRNA for Dio1 and TRβ were reduced in the pituitary and liver while in the hypothalamus, expression of mRNA for Dio2 was increased and relative expression of TRβ and TRH did not change (Boelen et al., 2004c). Thus it seemed that a mechanism to explain the fall in peripheral T3 (a decrease in liver Dio1) occurred at the same time as a central change (increase in hypothalamic Dio2) was acting to increase local T3 production. This local hypothalamic thyrotoxicosis provides an explanation for the failure of the TRH neurons to respond to the lower peripheral hormones in this model of an acutely ill animal (Boelen et al., 2004c).

In the LPS rat model, similar hypothalamic changes have been demonstrated. After 24 hours, there were decreased T3, T4 and TSH levels. Over 24 hours Dio2 activity in the cortex and anterior pituitary steadily increased in line with the decreasing peripheral T4. In the mediobasal hypothalamus (MBH) however, Dio2 activity increased rapidly to peak at 12 hours post LPS injection. *In situ* hybridization using a radiolabelled cRNA probe complementary to the rat Dio2 gene showed specific localisation in the floor and infralateral walls of the 3$^{rd}$ ventricle in a distribution characteristic of tanycytes; LPS rats had 2.5 times the deposition of silver granules, significantly different to saline injected controls (Fekete et al., 2004). Subsequent data from these investigators, in a study in which corticosterone levels were clamped, demonstrated that this increase in Dio2 activity in the MBH is independent of the stress-induced increase in peripheral corticosteroid levels (Sanchez et al., 2008).

#### *1.5.3.1.2 Chronic inflammation*

A model that has been used to investigate changes to the HPT axis in chronic inflammation utilises turpentine injection to the hind limbs of mice. The

turpentine administration induces a sterile abscess and serious discomfort for the mouse without causing the level of illness of the LPS model and is more representative of a chronic condition as the animal can be followed over a longer period of time (five days) (Boelen et al., 2005) (Boelen et al., 2006). While peripheral thyroid indices fall in both cases, differences were found between the acute and chronic models: liver Dio1 and Dio3 activities and gene expression both decreased in the LPS model while muscle levels did not change when compared to control animals: liver deiodinase activities and mRNA expression did not change in the chronic model while Dio3 activity markedly increased in the muscle tissue containing the abscess (relative to controls). While liver mRNA expression of IL-1β increased markedly in the acute model, this was less marked in the chronic model but the increase persisted over 5 days. Compared to controls, mRNA expression for IL-1β and IL-6 mRNA were up regulated in the muscle/abscess in parallel with the increase in mRNA for Dio3. Immunohistochemistry demonstrated that the increased Dio3 protein was localised in inflammatory cells associated with the localised abscess (Boelen et al., 2005).

In the chronic model central changes also occurred. Compared to controls, in turpentine-injected animals expression of IL-1β and Dio2 mRNA was increased when whole hypothalamus extracts were used but no change in TRH and Dio3 mRNA expression was found (Boelen et al., 2006). Using RNA extracted specifically from the PVN however, relative expression of mRNA for TRH and Dio3 were both decreased at 24 hours relative to control animals. Gene expression for IL-1β was still up regulated in the PVN extracts whereas that of Dio2 and TRβ was not different to controls. It would appear from this work that central changes are similar in the chronic condition to those in acute illness and that the changes may be anatomically very specific. The demonstration of a specific decrease in Dio3 gene expression in the PVN, suggesting decreased degradation of T3, provides another mechanism to explain a localised thyrotoxicosis which may contribute to the failure of TRH neurons to be stimulated by low peripheral thyroid hormone levels (Koenig, 2008). Moreover, using pair-fed control animals, it was demonstrated that the decrease in TRH mRNA seen in the PVN was independent of food intake; the decrease in the mice with inflammation occurred 24 hours earlier than in the food restricted controls (Boelen et al., 2006).

In a rabbit model of prolonged critical illness (rabbits were euthanased seven days after burns to 15-20% of body surface) peripheral T3 and TSH were reduced (Mebis et al., 2009). *In situ* hybridization of TRH mRNA expression showed markedly reduced hybridization signal specifically in the PVN when compared to healthy animals. The ill animals received parenteral nutrition to control for the HPT axis changes due to malnutrition. *In situ* hybridization for Dio2 mRNA showed markedly increased signal in the floor and walls of the 3$^{rd}$ ventricle and mRNA expression of Dio2 using RT-qPCR on whole hypothalamus extracts was also significantly increased. Enzyme activity measurements of Dio2 and Dio3 in whole hypothalamus extracts however, while tending to be higher, were not different to controls. Moreover T4 and T3 levels in whole hypothalamus extracts were not elevated and indeed T4 levels were significantly lower: the authors concluded that the theory of localised hypothalamic hyperthyroidism as the mechanism responsible for TRH down regulation in prolonged critical illness was not supported by their data (Mebis et al., 2009). However, as shown in the turpentine-injected mice (Boelen et al., 2006), there can be changes that are confined to very specific anatomical regions and the measurements of Dio2 and Dio3 and T3 and T4 were all made on whole hypothalamus extracts in the rabbit model. Moreover, in the rabbit model, significant up-regulation of mRNA for thyroid hormone transporter molecules (MCT10 and OATPICI) was demonstrated with no change in gene expression of thyroid hormone receptors using whole hypothalamus extracted RNA (Mebis et al., 2009). Thus, there is evidence that there is a difference between the chronically ill rabbits and the control animals in a number of molecules involved with the regulation of the HPT axis and further investigations related specifically to the PVN in this model would have been of interest.

Each of the models used has contributed to the understanding of the changes to the HPT axis following inflammation. While decreased peripheral thyroid hormone levels, without the expected rise in TSH, are common to all experimental paradigms, there are marked differences in methodologies used, parameters measured and in the nature of the inflammatory stress. Since animals are euthanased or die within 48 hours, LPS models are examining the changes in acute illness only. In the model examining the situation in a local inflammatory

chronic condition, the injection of turpentine, reported changes had mostly normalized by five days (Boelen et al., 2006). The rabbit model of chronic prolonged illness examines a time point seven days after the initial trauma but these animals are severely ill and serve as a model for patients in prolonged intensive care rather than for those with chronic illness.

### *1.5.4 Summary*

In several different animal models examining dysregulation of the HPT axis following inflammatory stress, down regulation of TRH in the hypothalamus or specifically in the region of the hypophysiotrophic TRH neurons has been demonstrated. This down regulation occurred when peripheral thyroid hormones were low and TSH was not elevated. Pro-inflammatory cytokines are implicated as causative factors. Alteration of levels of deiodinase enzymes, which control the tissue concentration of T3, has been demonstrated. As a result of such studies, a hypothesis has been advanced in which increased Dio2, and possibly decreased Dio3, specifically in the PVN of the hypothalamus, create a localised T3-thyrotoxicosis explaining the inability of the hypophysiotrophic neurons to respond to decreased peripheral thyroid hormones (Fekete et al., 2005) (Fliers et al., 2006b) (Koenig, 2008).

Results may be dependent on the species used in the model, the methodologies used in the investigations and the specific anatomical structures examined. Differences between deiodinase mRNA determinations in preparations from the whole hypothalamus as compared to specific hypothalamic nuclei are examples of methodological differences contributing to differences in findings. The physiological stressors known to suppress HPT axis function, starvation and proinflammatory cytokines, appear to act independently of one another. In addition, the effects of cytokines seem to be independent of the HPT axis suppressing actions of glucocorticoids. While all three factors may occur simultaneously in the critically ill, the mechanisms involved appear to be able to be separated by careful experimental design. Animal models used to investigate the effect of inflammatory stress on the HPT axis have shown that the acute situation may well differ from the chronic and the systemic from the local inflammatory condition in terms of the effects on different tissues and the overall outcome for the animal.

Given the recognition that chronic disease is associated with the NTI syndrome and that mood and cognitive change has been reported in some individuals with chronic illness, animal models that examine the relationship between the HPT axis and behavioural change in a model of chronic inflammatory stress could be useful in elucidating changes in this sub set of individuals.

## 1.6 An Animal Model of Chronic Pain

Animal models have been used extensively to study chronic pain states resulting from nerve injury. Only by using such models have fundamental questions related to the pathophysiological changes in nerve injury been addressed (Bennett, 1993). The model that was developed by Bennett and Xie involves the tying of four chromic gut ligatures around the sciatic nerve resulting in a localised inflammatory response (Bennett and Xie, 1988). The inflammatory response, largely due to the presence of chromium, results in swelling of the nerve and mechanical constriction. The combined mechanical and inflammatory stimuli result in sensory dysfunction analogous to that seen in humans and can be demonstrated by various testing procedures (Bennett and Xie, 1988). Thus allodynia (painful response to a normally non painful stimulus) can be demonstrated using a cold plate (cold allodynia) and von Frey filaments (mechanical allodynia): hyperalgesia (increased sensitivity to noxious stimuli), using acetone for cold hyperalgesia and heat for heat hyperalgesia. Additionally, spontaneous pain behaviours such as non-weight bearing of the affected limb can be observed (Bennett and Xie, 1988) (Monassi et al., 2003).

The usefulness of an animal model depends on the extent to which it mimics the human condition. Most models however do not address the issue of behavioural change that is seen in a large percentage of neuropathic pain patients, as discussed previously. Monassi *et al.,* have shown that the model of Bennett and Xie has a strong resemblance to the human neuropathic pain condition by demonstrating that a sub-population of rats shows changes in complex social behaviours and in their sleep-wake cycle (Monassi et al., 2003) (Keay et al., 2004).

### 1.6.1 Chronic Constriction Injury (CCI)

The CCI model of Bennett and Xie is one of the commonest used pain models, largely due to the simplicity of the surgery and the consistency of the sensory disabilities produced (Bennett and Xie, 1988) (Monassi et al., 2003). The detailed procedure is given in Section 2.3.3. In brief, using male Sprague-Dawley rats, under general anaesthesia, the right sciatic nerve of the rat is isolated and four chromic impregnated ligatures are tied 1 mm apart just superior to the trifurcation

of the nerve. The ligatures are tied loosely so as not to impede the epineural blood flow so that the inflammatory response and subsequent oedema provides the mechanical stimulus (Bennett and Xie, 1988). The nerves can be examined post-mortem to ensure that the nerves of all animals are similarly inflamed and the sutures are all intact.

### *1.6.2 Sensory Disturbance following CCI*

Using the CCI model in our laboratory (Monassi et al., 2003), sensory testing pre and post-CCI has demonstrated pain responses (allodynia and hyperalgesia) in the injured limb and shown these responses to be no different to those reported by others using this model (Bennett and Xie, 1988). Spontaneous pain behaviours are also observed, such as guarding of the leg when sitting or standing and altered gait so as to avoid weight bearing on the injured limb. Moreover Monassi *et al.,* showed there was no difference in pain response between animals subsequently categorized into three different groups based on post-CCI behaviour as described below (section 1.6.3).

Figure 1.6.1, adapted from Monassi *et al.,* (2003), demonstrates the responses seen when rats undergo sensory testing, post-CCI, using procedures which evoke responses indicative of painful sensation as described above. Responses for both the injured and uninjured limb are shown. The finding, that there is no difference in pain sensation between behavioural groups, has been demonstrated consistently in our laboratory and reported on a number of occasions (Keay et al., 2004) (Russo et al., 2005) (Sosa, 2008) (Austin et al., 2010). In view of the reliability of this observation and to avoid additional testing burden on the rats, sensory disturbance was not evaluated in this study.

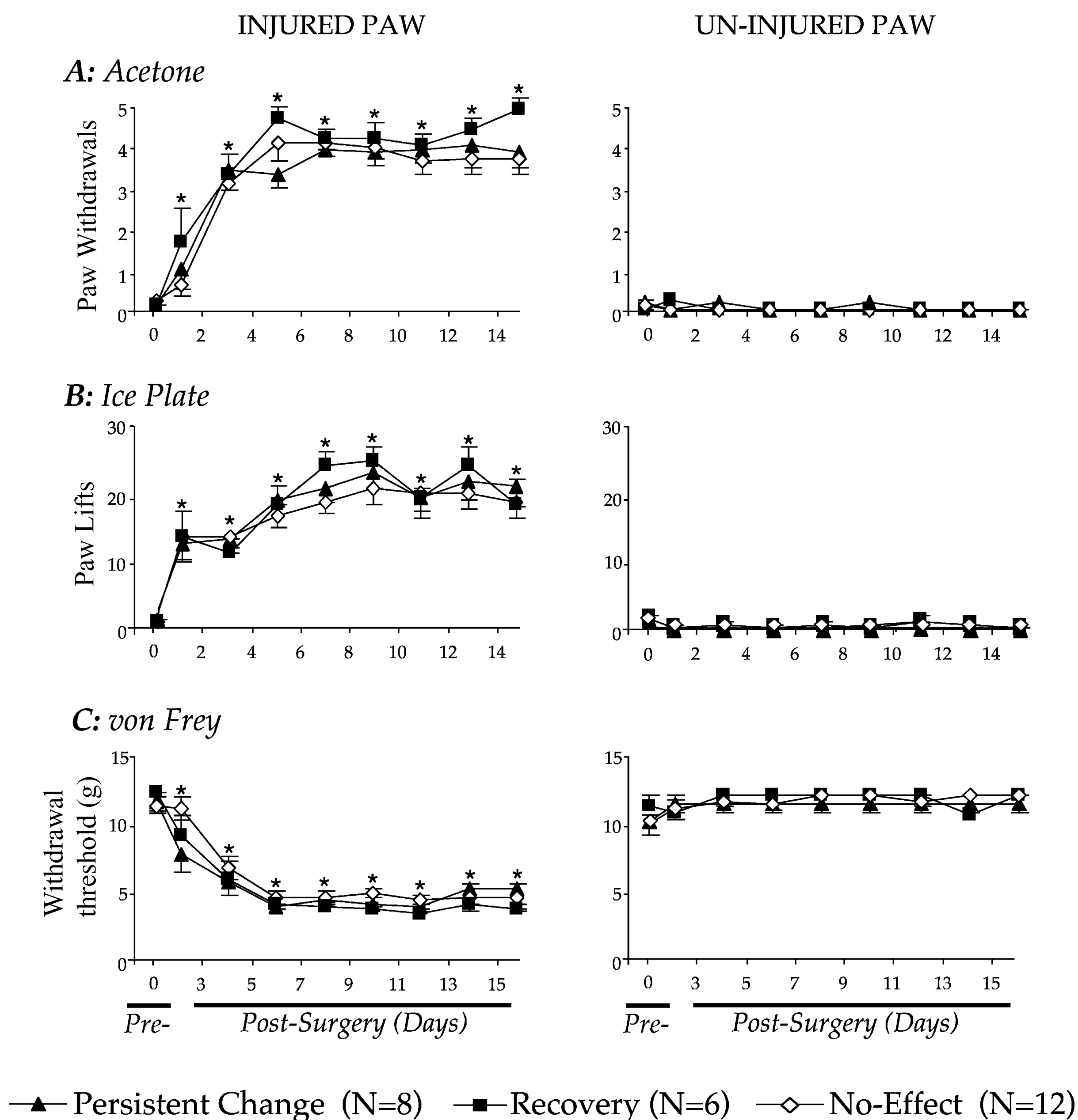



**Figure 1.6.1:** Graphs illustrating responses to sensory tests in the Persistent Change/Persistent Disability, Recovery/Transient Disability and No-Effect/No Disability groups: *$p<0.05$ with respect to pre-CCI levels.

A: withdrawal responses to the application of acetone droplets. B: the number of paw withdrawal responses from a cold plate (10-11°C) over a six-minute period. C: the threshold force (using von Frey filaments) required to evoke paw withdrawal. Injured and uninjured limb responses shown (adapted from Monassi *et al.,* (2003).

### *1.6.3 Behavioural Disturbance following CCI*

Monassi demonstrated for the first time that although all rats had similar pain responses following CCI, they could be divided post-CCI into three distinct groups based on behavioural responses using the Resident-Intruder test as a measure of social interactions (Monassi et al., 2003).

#### *1.6.3.1 Resident-Intruder Test*

This test of social interactions is based on the well-documented drive in male rats to exhibit dominance over a stranger introduced to the living space (Blanchard et al., 1975) (Blanchard et al., 1977). The experimental animal (the resident) is 'at home' when a weight, age and sex matched animal (the intruder) is introduced into the cage. The behaviours of each animal are compared to its own established pre-injury baselines and thus are not subject to the normal variations in dominance found between male rats. The interactions can be videotaped for later analysis. For consistent and reproducible results, the 'resident', needs to have established ownership of the cage and also to learn the paradigm. Thus rats are placed in individual housing for three days prior to testing and the first two days of testing are not included in the analysis. Additionally, the testing is carried out during the dark phase (the rat's active time) and at approximately the same time each day (Monassi et al., 2003).

#### *1.6.3.2 Behavioural Analysis*

Monassi *et al.,* classified the behaviours of the resident following the introduction of the 'intruder" to the cage, according to criteria adapted from the work of Grant and Mackintosh (Grant and Mackintosh, 1963) and refined in our laboratory (Monassi et al., 2003).

Four distinct categories of behavioural response were identified:

- **Dominance (D):** behaviour that includes lying across or standing above or leaning on the intruder, aggressive grooming of the intruder's head, offensive upright or lateral attack postures and chasing, biting and fighting behaviours.

- **Social behaviour (S):** is investigation or sniffing of the partner, often focused around the ano-genital region.
- **Non-social behaviour (NS):** includes cage exploration and self-grooming.
- **Submissive behaviours (SM):** are defensive upright or supine postures to the approach or attack of the partner, defensive alerting or freezing.

Figure 1.6.2, adapted from Monassi *et al.,* (2003), illustrates the more common of the dominance behaviours towards the intruder partner. Typically, after adaptation to the paradigm, rats spend an average of 100 seconds in a 360 second period exhibiting Dominance (Monassi et al., 2003).

#### *1.6.3.3 Behavioural Classification*

Based on an analysis of post-injury relative to pre-injury behaviour, Monassi *et al.,* developed a behavioural classification which is used consistently in our laboratory. Although all 4 behavioural categories are examined, the classification is based on the duration and pattern of altered dominance behaviour (D). A rat is defined as showing decreased D on any one day if there was <70% of the mean D pre-injury. Sixteen days of post-CCI dominance behaviour were initially evaluated (Monassi et al., 2003).

Three distinct behavioural groups were defined: in the original study, behaviour was tested for 16 days post injury; since the change in behaviour was stable after 3 days, the initial criteria have been modified to the following:

- Rats with reduction of dominance on 2 or fewer than 2 days after injury are defined as No Disability (ND).
- Rats defined as having 'Disability' show decreased dominance on 3 or more days.
- This group is further divided into Persistent Disability (PD) and Transient Disability (TD) groups.
- The rats are assigned to the TD group if the dominance scores on days 5 and 6 return to pre-injury levels.
- Rats that are categorised as PD all have decreased dominance on at least one of days 5 and 6.

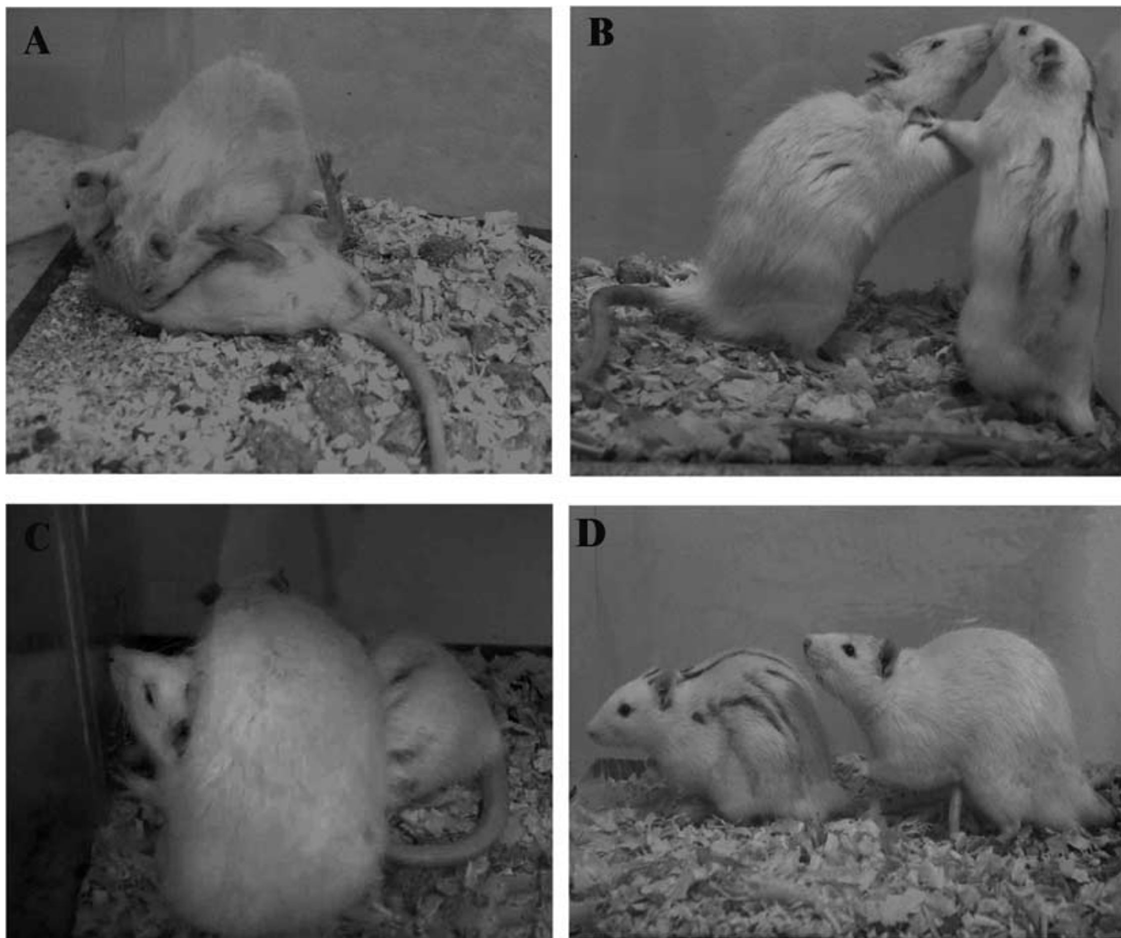



**Figure 1.6.2:** Video-stills (low-light, infra-red illumination) of dominance behaviours shown by resident rats to the presence of an intruder (identified by stripes) in the resident-intruder social interactions test. (A) Resident 'on top' of a submissive intruder; (B) offensive sideways/upright attack by resident leading to upright defensive posture by intruder; (C) biting targeted towards the neck/back of the intruder and (D) resident chasing intruder (adapted from Monassi *et al.,* (2003).

Using the defined criteria, rats were assigned to a 'Behavioural Group' based on the number and pattern of days when dominance behaviour was <70% the pre-injury baseline level. Monassi reported that 46% of the animals were classified using the above criteria as No Disability (ND), 23% as Transient Disability (TD) and 30% as Persistent Disability (PD) (Monassi et al., 2003). In subsequent publications from our laboratory, using the same criteria, similar percentages of animals in each category have been reported (Sosa, 2008) (Mor et al., 2010, Austin et al., 2010).

As shown in Figure 1.6.3, just as these groups could not be distinguished based on sensory testing, they could not be distinguished based on pre-CCI interactions either. There were no differences between groups for any of the four behaviours pre-CCI.

There were, however, significant differences in Dominance and Non Social behaviours for the TD and PD groups following injury. The TD group had significantly less D and more NS on days 1-4 post-CCI when compared to pre-injury levels whereas the group classified as PD had significantly less D and more NS on days 1-4 and days 5-16 post-injury. Thus the change in social behaviour, or disability, persisted. Since the reproducability of these findings has been consistently demonstrated in a number of studies, the testing time has been shortened to six days post-CCI with no change in the overall findings. (Sosa, 2008) (Austin et al., 2010).

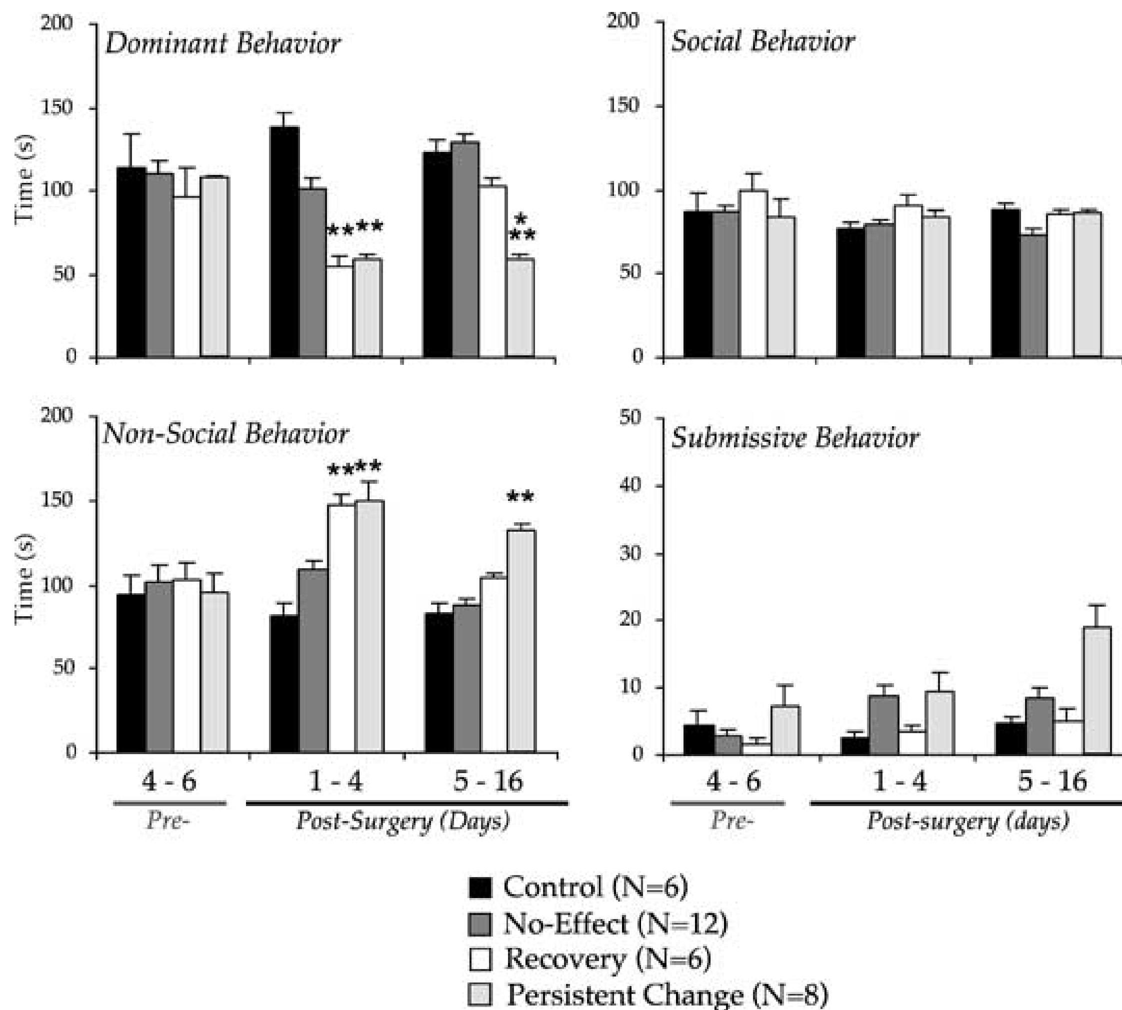



**Figure 1.6.3:** Histograms illustrating mean duration (± SEM) of four different types of behavioural activity, Pre-CCI and on days 1-4 and 5-16 Post CCI, for Control animals and each of the behavioural groups (No-Effect/No Disability, Recovery/Transient Disability and Persistent Change/Persistent Disabitity). *P<0.01, **P<0.001 with respect to Pre-CCI levels (adapted from Monassi *et al.,* (2003).

### *1.6.4 Sleep-Wake Disturbance following CCI*

In addition to the social disability, the PD group of animals exhibited significant changes in their sleep-wake cycle. Compared to pre-CCI behaviour and in contrast to the ND rats, their sleep pattern changed after injury. This group spent more time awake during both the light and dark phases of their day and this change was due to a decrease in slow-wave sleep (SWS) time rather than in rapid eye movement (REM) sleep. The ND rats did not change the amount of time spent in either SWS or REM in either phase of the light cycle whereas the TD group showed change in the light phase only (Monassi et al., 2003).

Figure 1.6.4, adapted from Monassi *et al.,* (2003) illustrates the differences found when sleep/wake patterns were analysed in the three behavioural groups following CCI. Recordings of brain and muscle activity were made following insertion of stainless steel screw electrodes via burr holes to record fronto-frontal electroencephalographic (EEG) signals coupled with silver wires inserted into neck muscle to record electromyographic (EMG) activity. Criteria for characterizing 'awake', 'slow wave sleep' and 'REM' sleep were derived from the EEG/EMG signals (Monassi et al., 2003).

### *1.6.5 Endocrine Changes following CCI*

While no differences were found pre-injury between the behaviourally different groups of rats, changes in endocrine function have also been demonstrated following CCI in our laboratory. Testosterone levels were altered in the PD group only (Russo et al., 2005). Changes in HPA axis activity were shown in ND and PD rats; however there were differences between the groups. While increased levels of corticosterone were demonstrated in both groups, the PD group had decreased ACTH levels when compared to the ND group. Moreover they also show increased ACTH receptor expression in the adrenal cortex, indicating an increased adrenocortical sensitivity. Moreover, relative to ND and control rats, the PD group showed evidence of altered hypothalamic regulation, with decreased numbers of both glucocorticoid receptor (GR) and corticotrophin-releasing factor immunoreactive cells in the paraventricular nucleus of the hypothalamus (Russo et al., 2005) (Sosa, 2008).

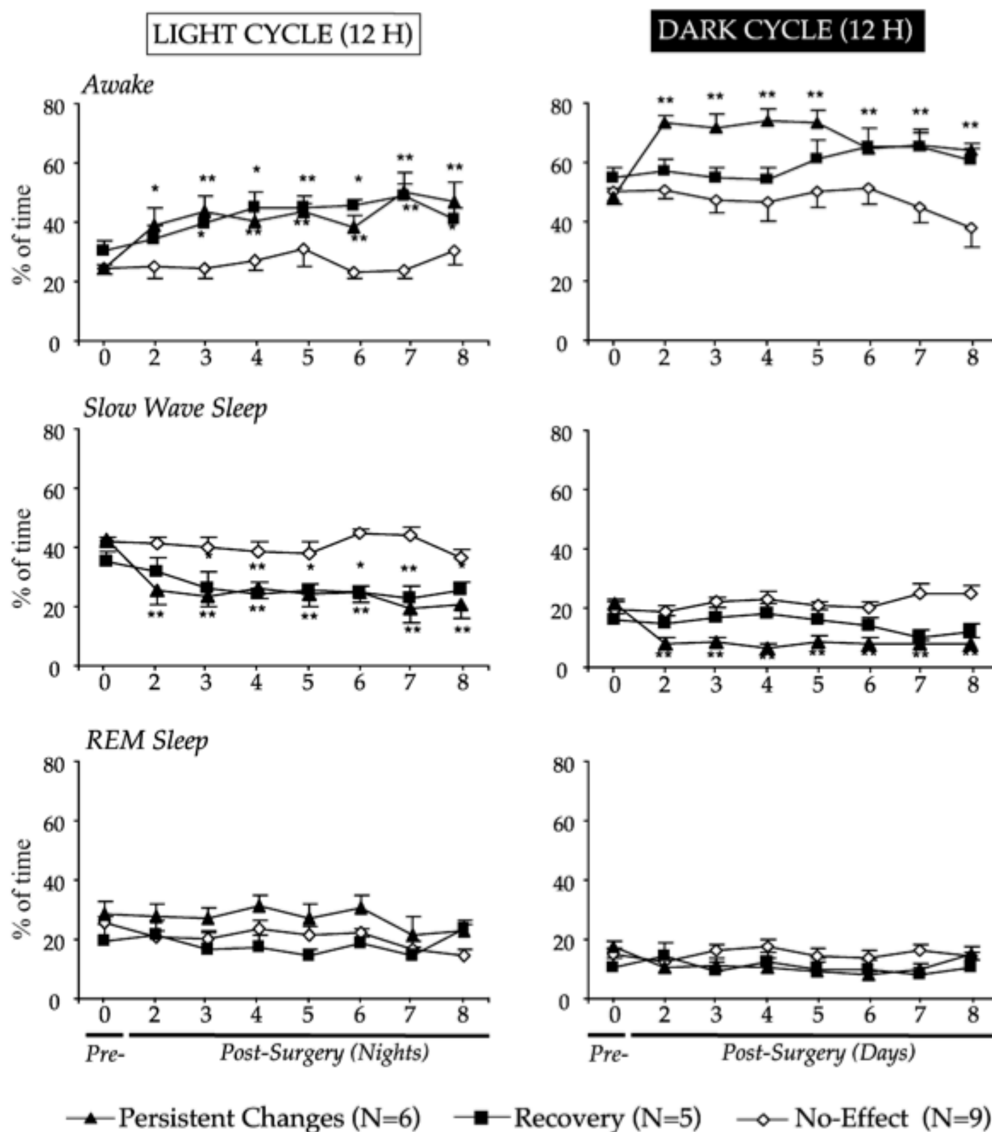



**Figure 1.6.4:** Graphs comparing the mean duration (± SEM) of wakefulness, slow-wave sleep and REM sleep during either the light or dark phase of the circadian cycle for each day. Data are shown for Persistent Change/Persistent Disability, Recovery/Transient Disability and No Effect/No Disability groups of animals. *P<0.05, **P<0.01 with respect to Pre-CCI levels (adapted from Monassi *et al.,* (2003).

### *1.6.6 Sciatic Nerves post mortem*

The changes documented in the group of rats that change behaviour could not be attributed to differences in the effectiveness of the CCI in producing inflammatory and mechanical stress. Post mortem examination of the nerves revealed that there was no difference between rats in terms of the degree of inflammatory response and all nerves displayed gross morphological changes identical to those reported using the same surgical injury (Monassi et al., 2003). Subsequently, it has been reported that the inflammatory changes seen post-CCI are in some respects greater than for more destructive lesions such as transection or ligation of the sciatic nerve (Hu et al., 2007). The authors conclude that the inflammatory changes seen post CCI are representative of an adaptive immune response and that this response is still present seven days after injury.

### *1.6.7 Summary*

CCI injury in the rat was developed as a model to further our understanding of chronic pain. All animals show the same degree of pain, as determined by the presence of allodynia, hyperalgesia and spontaneous pain behaviours. Thus the CCI is equally effective in producing the sensory changes characteristic of neuropathic pain, and examination of the nerves post mortem reveals no differences between rats. While no differences are evident pre-injury, a sub-group of the rats shows changes in social behaviour post-injury. Dominance towards an intruder into the home cage is characteristic behaviour of male rats; decreased time spent in this activity is a symptom of social disability. The same sub-group exhibits changes in their sleep-wake cycle and hormonal changes. Hence this sub-group displays similar changes to those reported by a significant sub-group of patients following nerve injury as discussed earlier. Thus, as in humans, the degree of disability does not correlate with the pain intensity. The demonstration of behavioural change in a sub-group makes this animal model more representative of the human condition of neuropathic pain and possibly of chronic inflammatory conditions in general.

## 1.7 The Question

A syndrome of decreased peripheral thyroid hormones with altered central regulation occurs in acute and chronic critical illness in humans. Experimental models of acute and chronic illness in rodents have demonstrated altered hypothalamic regulation and mechanisms have been proposed that explain this dysregulation.

Low thyroid hormones are associated with changed behavioural states and also with chronic disease states and syndromes in humans. In the CCI rat model of neuropathic pain, a sub-population show changed social behaviours analogous to those reported by a sub-group of patients. These changes include altered social behaviours, disturbed sleep, fatigue, altered appetite, reduced interest in the environment and very often depression (section 1.1.3).

In the sub-population that is characterized by behavioral change, are the thyroid hormones altered by the injury and is there evidence that these changes are due to altered central regulation?

## 1.8 Thyroid Hormone and the Brain

The critical need for adequate levels of thyroid hormone to ensure development of the nervous system in the human foetus and during the first years of life is well documented and many supportive studies in animals have confirmed the devastating effects of perinatal hypothyroidism (Bernal et al., 2003) (Morreale de Escobar et al., 1985) (Stoica et al., 2007) (Kobayashi et al., 2005) (Anderson, 2001) (Bernal, 2009). Bernal (2009) details the many studies in foetal and neonatal animals that have demonstrated abnormalities of neural development when TH is not present at critical periods. Thyroid hormone controls such diverse functions as cell migration in the cortex, hippocampus and cerebellum; myelination of neurons; neuronal differentiation and morphology; numbers of interneurons in the cortex and mitochondrial morphology and function (Bernal, 2009).

As well, an ongoing role for thyroid hormone in normal brain function throughout life is now well recognised by many investigators (Konig and Neto, 2002) (Courtin et al., 2005) (Bernal, 2009). However this concept is still viewed with caution by some and deficits in cognitive or emotional function, attributed to thyroid dysfunction, are viewed as possibly indirect effects or due to other causes (Anderson, 2001) (Samuels and Samuels, 2008). As previously discussed in section 1.2, based on patient symptoms and their reversal with correction of thyroid abnormalities, psychiatrists questioned the validity of the belief that thyroid hormone had no role to play in the adult brain as early as the 1970's (Whybrow and Hurwitz, 1976). Joffee *et al.,* in questioning the continued lack of acknowledgement of the sensitivity of the adult brain to thyroid hormones, believed that these ideas originate from early studies by Barker and Klitgaard (1952). These investigators showed "that oxygen consumption by brain slices obtained from adult rats was not affected by their thyroid status, in contrast with that of many other tissues" (Joffee et al., 1994). The demonstration that when peripheral thyroid hormone levels are manipulated, hormone levels within the brain remain within narrow limits, due to compensatory altered metabolism (auto regulation), assists in the prolonging of such beliefs (Dratman et al., 1983) (Broedel et al., 2003). Over the last two decades, however, there is increasing

evidence that normal levels of thyroid hormone are integral to normal brain function. That the mood disorders, dementia, confusion, and personality changes seen in dysfunction are reversible with proper treatment, indicate that thyroid hormone alterations of adult onset do not leave permanent structural defects (Bernal, 2009).

### *1.8.1 Thyroid hormones in the brain*

As in peripheral tissues, T3 is the iodothyronine found bound to thyroid receptors. Using a double radiolabelling techniques it has been demonstrated that 90% of TRs bind T3 in the euthyroid rat cerebral cortex and that 65-80% of the T3 bound to TRs was derived from T4. Similarly, 50-65% of T3 in cerebellar tissue was derived from local conversion of T4 (Crantz et al., 1982) (van Doorn et al., 1983). Thus local production of T3 is greater in brain than other tissues (Crantz et al., 1982). Deiodinase 2 is responsible for the conversion of T4 to T3 in the brain; Dio3 is responsible for metabolism of both T3 and T4. The local regulation of these deiodinase enzymes is responsible for local hormonal levels in brain tissue as in other tissues (Kohrle, 2000).

### *1.8.2 Thyroid hormone homeostasis in the brain*

Auto regulation of T3 levels appears to be a protective mechanism for the brain when peripheral levels of thyroid hormones fluctuate. In chronic thyroid hormone deficiency or excess in rats, levels of T4 and T3 in the brain remained within a narrow range as a result of central and peripheral changes. Thus under conditions of decreased peripheral T4, conversion to T3 was decreased in liver tissue and markedly increased in the brain; the reverse occurred in hyperthyroid rats (Dratman et al., 1983). Thyroidectomised rats infused with increasing levels of either T4 or T3 demonstrated the control of peripheral thyroid hormones levels on deiodinase enzymes in the brain. Thyroidectomy resulted in elevated Dio2 in cortical tissue that fell with progressive levels of infused T4 until levels of plasma T4 and Dio2 were similar to those of control animals. Further falls in Dio2 followed further increases in infused T4. Infusion of T3 did not alter cortical levels of Dio2 even when levels of T3 were very high, but at hyperthyroid levels, T3 infusion did cause increased levels of cortical Dio3 (Escobar-Morreale et al., 1997). Similar experiments examining regional differences in deiodinase activity with change in plasma hormone levels demonstrated that not all brain regions were equally protected from such fluctuations. At moderately increased levels of

plasma T4, causing increased T4 levels in multiple brain regions, T3 levels remained at control levels or lower. However, at higher T4 levels, while T3 levels remained normal in cortical areas, the hippocampus and amygdala, they were elevated in the hypothalamus and regions where D3 activity is low (cerebellum and medulla) (Broedel et al., 2003). In the hypothyroid animals, in which peripheral hormones were undetectable, increased levels of Dio2 were found in all brain regions and decreased levels of Dio3 were found in cortical areas, hypothalamus and hippocampus. T3 and T4 levels throughout the brain were, however, extremely low relative to controls as would be expected (Broedel et al., 2003). The effect of hypothyroidism with less extreme levels of thyroid hormone deficiency can be inferred from the study of increasing infusions of T4 on Dio2 activity. Below control levels of T4, there is an inverse relationship between Dio2 activity and T4 but no relationship between Dio3 and T4. In this study only cortical areas and pituitary were examined; however the activity of these enzymes in response to low T4 levels provides a mechanism to protect the brain from low peripheral thyroid hormone levels within limits (Escobar-Morreale et al., 1997).

#### *1.8.2.1 Disruption of brain thyroid homeostasis*

Despite mechanisms that would appear to protect the brain from altered peripheral thyroid hormones, in pathological conditions cognitive and behavioural symptoms occur in some people with relatively mild hormonal alterations. As previously discussed, correction of peripheral hormonal levels usually results in reversal of these symptoms (Monzani et al., 1993) (Whybrow and Hurwitz, 1976) (Bernal, 2009). Other physiological mechanisms, however, are known to be altered while thyroid hormone levels in the brain are maintained: for example, cortical mitochondrial succinate dehydrogenase is decreased in hyperthyroidism induced with T4 despite T3 brain levels remaining at control levels (Broedel et al., 2003).

Disruption of the central thyroid homeostasis mechanism has been demonstrated experimentally under conditions of stress and using pharmacological manipulations. Following five different experimental stressors in rats, some quite transient, Dio2 and thyroid hormones were measured in four different brain regions: all stressors induced increases in Dio2 activity, resulting in decreased T4 and increased T3 concentrations in varied and stress-specific brain regions. No changes were seen in Dio3 brain levels or in peripheral or liver hormone levels

(Baumgartner et al., 1998). Sleep deprivation resulted in increased Dio2 activity and T3 tissue levels in all brain regions except the hypothalamus and the administration of numerous drugs (antidepressants, ethanol, lithium) resulted in changes to Dio2 activity and T3 levels that were sometimes region specific, not always easily explained, and often did not result in stable tissue levels of T3 (Eravci et al., 2000). Thus while a mechanism exists to protect the brain from fluctuating levels of peripheral thyroid hormones, as could occur naturally with change in iodine intake with the seasons for example, this mechanism is sensitive to stress. Indeed, an increase in T3 in different brain regions during stressful events may play a physiological role given the wide variety of CNS parameters that thyroid hormones are increasingly being shown to influence (Baumgartner et al., 1998).

### *1.8.3 Deiodinases in the Brain*

Since a high percentage of T3 found in the brain is derived from the intracellular conversion of T4 to T3, the importance of Dio2 in the regulation of tissue levels of T3 throughout the brain is clear (Crantz et al., 1982) (Courtin et al., 2005). The role of Dio3 in metabolizing T3 provides an additional or alternative regulatory mechanism and the relative contribution of these enzymes in differing brain regions offers an explanation for anatomically specific differences in T3 content (Baumgartner et al., 1998) (Fekete et al., 2005) (Boelen et al., 2006) (Courtin et al., 2005).

In humans, mRNA for Dio2 is widely expressed in the CNS with differing levels of expression, for example in cortex when compared to cerebellum. The pattern of distribution Dio2 gene expression is said to correlate with the relative abundance of TRs in the rat CNS (Courtin et al., 2005). Using enzyme assay techniques in the rat brain, levels of Dio2 were found to be undetectable in most brain areas in euthyroid rats except for the arcuate nucleus-median eminence (AN-ME). However in hypothyroid rats, Dio2 activity was found in multiple areas (cortex, hippocampus, amygdala, various hypothalamic regions and cerebellum) with the highest concentration being in the AN-ME and the lowest levels in the PVN (Riskind et al., 1987). These authors note that the level of activity in the euthyroid rats, even in the AN-ME, was at the level of sensitivity of the assay, but their finding that sub-regions of the hypothalamus differ in the activity of this

enzyme has been supported by more recent studies. Using mRNA *in situ* hybridization techniques it was confirmed that the most abundant site of Dio2 synthesis was located in the tanycytes lining the floor and walls of the 3rd ventricle in the MBH and that there was no evidence of Dio2 gene expression in the PVN or in ependymal cells in the dorsal 3rd ventricle (Tu et al., 1997) (Fekete et al., 2004) (Lechan and Fekete, 2005). Light and electron microscopy studies using immunohistochemical techniques confirmed localisation of enzyme in tanycytes and astrocytes with a much greater immunoreactivity in the MBH and less in the PVN concurring with the distribution of mRNA for Dio2 (Diano et al., 2003). Moreover, multiple studies, in vitro and in vivo, have demonstrated that Dio2 activity is expressed in astrocytes throughout the brain, is associated with proliferating astrocytes but not neurons, and is released by astrocytes in culture. Thus glial cells are implicated in the local deiodination of T4 and regulation of tissue T3 for neurons (Courtin et al., 2005) (Anderson, 2001) (Lechan and Fekete, 2005).

In the adult rat, Dio3 activity is seen in several brain regions including cerebral cortex, hippocampus, limbic forebrain as well as hypothalamus (Courtin et al., 2005) (Lechan and Fekete, 2005). Gene expression of Dio3 is low in the euthyroid brain, in keeping with the finding that Dio3 is elevated when T3 levels are above normal (Escobar-Morreale et al., 1997). Dio3 mRNA is expressed in astrocytes and oligodendroglia and has also been shown to be localised in neurons (Tu et al., 1999) (Courtin et al., 2005) (Lechan and Fekete, 2005). Using in situ hybridization techniques, mRNA for Dio3 was shown to vary with thyroid hormone levels: hypothyroidism reduced mRNA for Dio3 whereas T3 infusion resulted in up regulation of mRNA for Dio3. It was noted that Dio3 mRNA distribution closely followed that of the known distribution of TRs (Tu et al., 1999).

### *1.8.4 Thyroid Hormone Receptors in the brain*

As discussed in section 1.3.4, there are multiple TRs. In humans, TRα1 and TRβ1 are thought to be the dominant isoforms, with varying tissue distributions, although both are found in the CNS. In the rat, TRα1 and TRβ2 as well as TRβ1 have been identified in wide distribution in the brain (Lazar, 1993). The three isoforms are found in the TRH neurons of the PVN and thus have a probable role

in the control of TSH. Immunohistochemical techniques demonstrated the co-localisation ppTRH and the TRα1,TRβ1 and TRβ2 isoforms in the same cells (Lechan et al., 1994). The ability of the ppTRH secreting cells to respond to both hypothyroidism and infused T3 was lost in TRβ2 knockout mice, confirming the central role of this isoform in regulation of TRH (Abel et al., 2001). TRα1,TRβ1 and TRβ2 have also been identified in oligodendroglia but not astrocytes in rat brain using immunohistochemical techniques (Carlson et al., 1994). However, in a more recent review it was noted that this is an area of controversy, probably due to differing techniques and the presence of TRs has been reported in astrocytes (Trentin, 2006).

### *1.8.5 Thyrotrophin Releasing Hormone in the brain*

As discussed in section 1.3.5, ppTRH is synthesised by neurons in the parvocellular division of the PVN. The hypophysiotrophic TRH neurons, the neurons that control the activity of the TSH-producing thyrotrophs, are said to be specific to the medial parvocellular (PaMP) and periventricular (PeV) subdivisions (Fekete and Lechan, 2007). The description of the boundaries of the respective subdivisions of the PVN however varies and others include the ventral parvocellular (PaV) subdivision when describing the distribution of the TRH neurons (Espinosa et al., 2007). Figure 1.8.1, adapted from Fekete and Lechan (2007), illustrates the distribution of the TRH neurons and together with the schematic diagrams shows that their localisation is confined to a very specific region of the PVN, identifiable as rostrocaudal sections approximately -1.7 to -1.9 mm to bregma (Paxinos and Watson, 2005) (Geerling et al., 2010). While the authors used the illustration, as here, for the purpose of demonstrating the localisation of the TRH neurons, they gave no details as to the technology employed or the physiological state of the rat. The technology appears to be immunohistochemical staining, but there is little support in the literature for this degree of staining in the euthyroid rat. There are many reports demonstrating the hypophysiotrophic TRH neurons in rats rendered hypothyroid (treated with propylthiouracil (PTU) or thyroidectomised) and compared to euthyroid rats. While hypothyroidism results in significant staining of cell bodies, control animals show many fewer or no immunoreactive cells (Nishiyama et al., 1985) (Kreider et al., 1985) (Espinosa et al., 2007). Alternatively, in order to demonstrate TRH neurons, rats are treated with colchicine prior to euthanasia.

Colchicine prevents secretion of the cellular product, increasing the concentration of TRH in the soma of the cells and enhancing visualisation (Nishiyama et al., 1985) (Cintra et al., 1990) (Kreider et al., 1985).

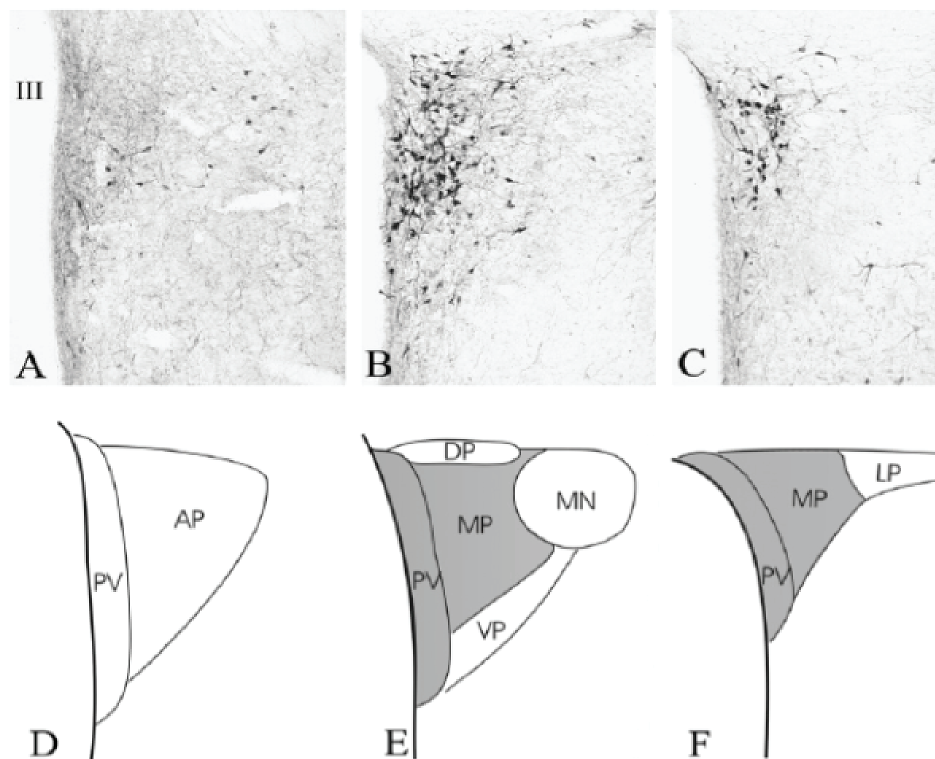



**Figure 1.8.1:** Distribution of TRH-synthesising neurons in the PVN. Micrographs showing immunohistochemical staining (A-C) and corresponding schematic diagrams (D-F) illustrate the TRH neurons at three rostrocaudal levels of the PVN. The TRH neurons are concentrated in the medial parvocellular (MP), periventricular (PV) subdivisions at rostrocaudal levels that approximate to -1.9 to – 1.7 mm to bregma. Other authors include the ventral parvocellular (VP) subdivision. Lateral parvocellular (LP), dorsal parvocellular (DP), anterior parvocellular (AP) and magnocelluar (MN) subdivisions of the PVN. Adapted from Fekete and Lechan (2007).

As well as in the PVN, TRH immunoreactive protein has been demonstrated in multiple regions of the rat CNS. These include, hypothalamic nuclei other than the PVN, brainstem nuclei, olfactory bulb, preoptic nuclei, spinal cord and hippocampus (Chung et al., 1989) (Kreider et al., 1985) (Nishiyama et al., 1985) (Cintra et al., 1990) (Fekete and Lechan, 2007). However, only the TRH neurons of the medial parvocellular PVN, which project to the ME, have been shown to regulate TSH (Nishiyama et al., 1985) (Lechan et al., 1994).

### *1.8.6 Transport of thyroid hormones in the CNS*

As discussed in section 1.3.7, the movement of T4 and T3 to their intracellular sites of action appears to be dependent on a number of specific transport molecules. Thus, it appears that the property of lipid solubility alone is not sufficient for intracellular localisation of these molecules in various tissues but more specifically in the brain. For example, one anion transporter, OATP1C1, is expressed in capillaries throughout the brain, suggesting it is critical for transport of T4 over the blood-brain barrier (Jansen et al., 2005). MCT8 is highly expressed in brain tissue and mutations of the gene for this transporter in humans result in severe psychomotor retardation and abnormal TH plasma levels (Visser et al., 2007). Mice lacking this transporter also exhibit abnormal hormone levels (abnormally low T4 with high T3) and diminished uptake of T3 into the brain. Deiodinase levels within the brain were indicative of hypothyroid tissue (elevated Dio2 and suppressed Dio3) although CNS function overall was not as impaired as that reported in humans with mutant genes (Trajkovic et al., 2007). Genetic variability in such molecules affords yet more opportunity for individual variation in thyroid hormone metabolism in the brain.

### *1.8.7 The role of Glial Cells*

As previously discussed, Dio2 gene expression in the hypothalamus is primarily found in the MBH and ME, the tanycytes lining the third ventricle and in astrocytes and there is little expression of Dio2 mRNA or activity in the PVN. These findings have lead to a construct of the relationship between the glial cells and TRH neurons; this relationship provides the framework for how T3 levels in the PVN are controlled and thus the set point for the TRH neurons established (Courtin et al., 2005) (Fliers et al., 2006b) (Lechan and Fekete, 2005). Astrocytes

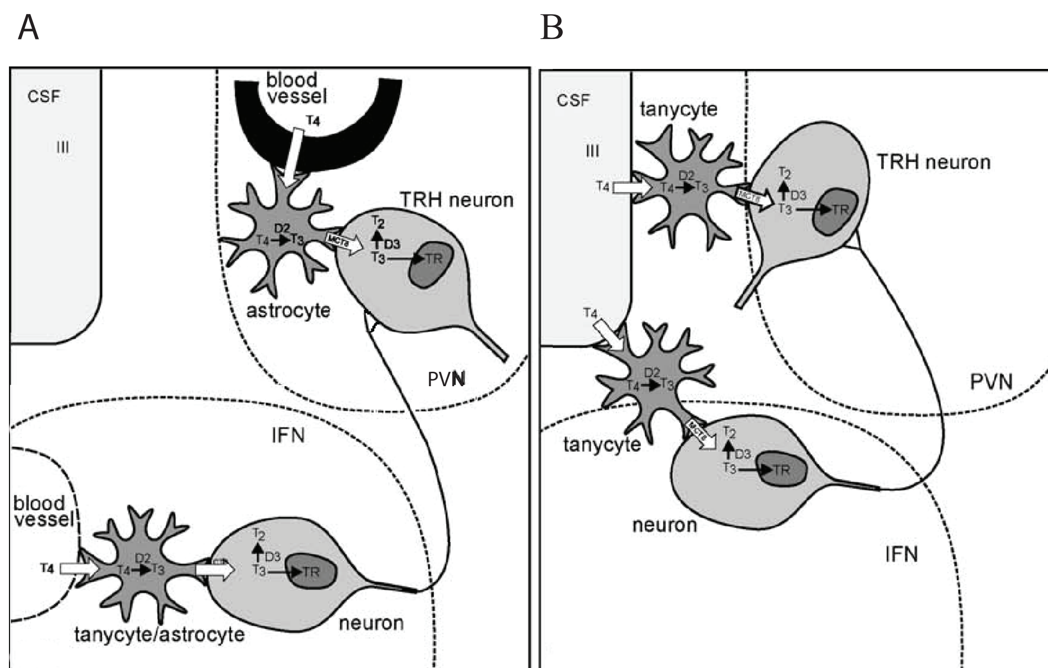



**Fig.1.8.2:** Schema of proposed routes for thyroid hormone feedback on TRH neurons in the human PVN.

(A) T4 is taken up locally from the vascular compartment by astrocytes or tanycytes expressing Dio2: this can occur in the PVN or in the infundibular nucleus/median eminence (IFN/ME) in which the blood brain barrier is absent. These cells convert T4 to T3 which is transported either directly to TRH neurons by MCT8 or alternatively to IFN neurons. In the TRH neuron, T3 may bind to the TR and exert a negative feedback on TRH gene expression. Dio3 present in the TRH neuron can inactivate T3. In the IFN neuron, T3 may bind locally to the TR, altering gene expression of IFN neurons, or may be inactivated by Dio3. T3 may thus result in altered firing pattern of IFN neurons projecting to TRH neurons in the PVN. Alternatively, T3 may travel to TRH neurons in the PVN through axonal transport.

(B) T4 is taken up from the CSF by tanycytes expressing Dio2. In these cells T4 is converted to T3 and subsequently transported by MCT8 to either TRH neurons of the PVN or to neurons in the IFN with possible sequealae as outlined above. Modified from Fliers *et al.,* (2006).

and tanycytes may be in direct contact with cerebrospinal fluid (CSF) and may extend processes that end on capillaries or on axon terminals in the adjacent hypothalamus or ME (Courtin et al., 2005) (Fliers et al., 2006b) (Lechan and Fekete, 2005). These glial cells express Dio2 and are responsible for converting T4 to T3 for transport into neurons which do not express this enzyme: neurons however do express Dio3 as previously discussed. Figure 1.8.2, presents a schema for regulation of TRH in the human PVN: derived from experimental data, it reflects the consensus of a number of researches in this field (Fliers et al., 2006b) (Lechan and Fekete, 2005) (Courtin et al., 2005).

This schema explains how the glial cells and the activity of the Dio2 enzyme determine the level of T3 within the PVN: it provides the mechanism whereby detection of peripheral levels of T4 (via blood and CSF) allow for TRH levels to be altered to restore peripheral hormones as per the accepted negative feedback control mechanism. It also provides a mechanism to explain the down regulation of the TRH neurons that occurs with inflammatory stress. As shown in experimental models of acute illness, cytokines alter the level of Dio2 activity within the MBH (Fekete et al., 2004) (Lechan and Fekete, 2005) (Boelen et al., 2004c). This local change in enzyme activity results in a localised thyrotoxicosis within the PVN down regulating TRH neurons resulting in the lowered peripheral thyroid hormones seen in infection (Lechan and Fekete, 2006).

### *1.8.8 Neurogenesis and Thyroid Hormones*

Recent studies have demonstrated that hypothyroid status in the adult rat affects hippocampal neurogenesis (Desouza et al., 2005) (Ambrogini et al., 2005) (Zhang et al., 2009). While thyroid deficiency resulted in abnormal hippocampal development in the young which was not reversed by replacement of thyroid hormone levels (Zhang et al., 2009), in adult rats, there was reversal of the defects when TH levels were restored (Desouza et al., 2005) (Zhang et al., 2009). Specifically, hippocampal volumes were decreased and while the number of mitotic granular cells was not influenced by hormone deficiency, the survival and neuronal differentiation of new cells was much decreased when compared to controls (Desouza et al., 2005) (Ambrogini et al., 2005) (Zhang et al., 2009). Neurogenesis in the hippocampus is a source of both new neurons and glial cells throughout an animal's lifetime allowing for continued learning. That lack of

thyroid hormone leads to the impairment of this process provides a link to the cognitive and mood disturbance described in these individuals (Zhang et al., 2009).

### *1.8.9 Thyroid Hormones and Behaviour*

As discussed in section 1.2, thyroid hormone levels outside the reference ranges, resulting from thyroid dysfunction, are associated with disturbances of cognitive and emotional functions. These disturbances are manifested through altered behaviours that reflect widespread changes in brain processes. Behavioural change such as altered sleep patterns, paucity of speech and social withdrawal observed in hypothyroidism contrasts with the emotional lability, nervousness and short attention span seen in hormone excess. A general ‘slowing’ or ‘speeding up’ of functions in general metabolic terms might explain these differences. However the evidence would support a more complex view. In a recent comprehensive review, Nunez *el al.,* (2008) stated that while recognition that TH is required for normal function of the nervous system has gained acceptance, the understanding of how this hormone elicits its functions is far from complete. There is however an emerging view that the selective cooperation of a number of genes determines the nature, time and place of TH actions in the nervous system (Nunez et al., 2008). Thus the tissue concentrations of the various hormones, the deiodinase enzymes that control these concentrations, transporter molecules and the varying expression of the thyroid hormone receptors in different regions of the brain determine function.

As previously discussed, studies in foetal and neonatal animals have documented the structural effects of lack of thyroid hormone during development of the brain resulting in profound psychomotor retardation (Bernal, 2009). The disruption of the fundamental processes documented, such as myelination and mitochondrial function, may impact on brain function when hormone deficiency occurs later in life. The specific function of thyroid hormone in regulating neurotransmitter systems, including those that regulate serotonin and norepinephrine, may underlie functional changes (Bernal, 2009).

The level of hormone required for the optimal development and subsequent function of one specific brain region may vary from another. For example, mice

deficient in the Dio2 encoding gene are deaf and exhibit some disturbances of behaviours but few deficits of neurological function when compared to hypothyroid animals. This would suggest that in some brain regions, T3 obtained from the circulation might be adequate whereas in other regions, such as the cochlea, additional T3 generated locally by type 2 deiodinase is necessary (Nunez et al., 2008). Targeted mutations for the TR isoforms have produced animals with different measurable deficiencies and behavioural abnormalities. For example, anxiety-like behaviour and learning deficiencies have been demonstrated in TRα deletions while audiogenic seizure susceptibilities and impaired 'vigilance' was found in TRβ deletions (Nunez et al., 2008).

Imaging studies in humans are increasingly demonstrating abnormalities of brain function in hypothyroidism. Glucose metabolism for example, as demonstrated by positron emission tomography, was decreased in the prefrontal cortex in hypothyroid patients as compared to controls (Bauer et al., 2003). Activity in the superior portion of the pre-frontal cortex correlated inversely with TSH levels whereas activity in the inferior portion of the prefrontal cortex was correlated with severity of depression. After treatment with thyroxine, multiple areas of the brain showed changes in glucose metabolism with improvement in symptom scores (Bauer et al., 2003). Using functional magnetic imaging and a test of working memory processing, He *et al.,* (2011) demonstrated deficiencies in hypothyroid patients compared to controls. Neural activity was decreased in bilateral prefrontal cortex and posterior cingulate; memory state and neural activity returned to control levels following treatment with thyroxine (He et al., 2011).

Despite these insights, the mechanisms underlying the behavioural changes seen in thyroid dysfunction are still to be elucidated. It is still the case that the mechanisms underlying the interactions between thyroid state and mood disorder are still as unknown (Kulikov et al., 1997).

### *1.8.10 Summary*

Although the adult brain has long been seen as insensitive to thyroid hormones the weight of evidence no longer supports this view. The concept of central thyroid homeostasis has no doubt contributed to the belief that fluctuations in thyroid hormone do not alter neural function: however numerous stresses have been

shown to alter the enzymes that control the tissue levels of T3 in various brain regions. Recent evidence that thyroid hormone deficiency inhibits the survival and development of proliferating cells in the hippocampus lends weight to the argument that even mild hypothyroidism may have significant consequences for the individual.

There are numerous molecules and thus many possible genetic variations in the factors involved in the regulation of the HPT axis. Hence, there may be individual differences in HPT axis regulation resulting in differing individual responses to various stressors.

Increasingly sophisticated investigative techniques have lead to increased understanding of the enzymes, receptors, transporters and hormones involved in the regulation of the HPT axis together with the anatomical relationships involved. This knowledge has resulted in the development of schemas which are useful in the understanding of the mechanisms involved in the regulation of the hypophysiotrophic TRH neurons. Hence, these schemas are useful in understanding the mechanisms leading to down regulation of TRH, the altering of the 'set point' that occurs in response to inflammatory stress.

## 1.9 Aims

Chronic constriction injury of the sciatic nerve has been previously shown to result in different behavioural outcomes in male Sprague-Dawley rats such that a subgroup of the population demonstrate change in complex social behaviours.

To investigate the effect of an inescapable inflammatory stressor, CCI, on the function of the hypothalamo-pituitary-thyroid axis and to correlate HPT axis function with behavioural change.

# *CHAPTER TWO*

# *Materials and Methods*

## 2.1 Ethical Considerations

All experimental procedures documented in this thesis were carried out following the guidelines of the NHMRC Code for the Care and Use of Animals in Australia and were approved by the Animal Care and Ethics Committee of the University of Sydney. The investigations were carried out as part of a larger project, supported by a NHMRC grant, entitled "Supraspinal neural applications in the transition from acute injury to chronic pain and disability" (K03/5-2008/3/4852).

### 2.1.1 Care and treatment of rats

All rats were housed in a temperature-controlled room ($22 \pm 1^{o}$C) with food and water available *ad libitum*. Whether group or individually housed, all rats were provided with environmental enrichment in the way of cardboard boxes or tubes or sections of polyethylene pipe. All animals were observed daily for general health and welfare and both the cages and the room were cleaned regularly. Experimental animals were weighed three times over the course of their procedures.

### 2.1.2 Surgical procedures and care following surgery

All surgical procedures were carried out using aseptic technique with all care given to minimising trauma and blood loss for the animal. Post-operatively, rats were observed until motor activity and exploration of environs resumed, before returning them to the home cage. Thereafter, rats were observed daily using specific criteria as to cage activity, posture, grooming, nesting activity, ano-genital cleanliness, eating, drinking and hydration. Following cathetarisation of the external jugular, catheters were cared for as detailed below. If a catheter came out of position, the rat was euthanased immediately. Rats were also monitored for signs of self-harm, particularly after Chronic Constriction Injury (CCI).

### 2.1.3 Blood Sampling

Guidelines for the removal of blood from conscious rats specify that only 10% of the rat's blood volume should be removed at any one time or 1.0% every 24 hours when repeat samples are taken (Morton et al., 1993). In rats of 250-350 g this equates to approximately 0.2 ml/day but a larger volume may be removed if cellular elements are returned to the rat (Morton et al., 1993). Sampling from an indwelling jugular catheter is considered a form of 'mild' stress for the rat (Morton et al., 1993). As the rats were to be sampled before and after injury and to maximise the number of time points post-injury while adhering to the guidelines, blood was removed in 1 ml aliquots and after removal of the plasma, cellular elements were resuspended and returned to the rat via the indwelling catheter. Seven samples were taken over the course of the procedure. Aseptic technique was followed throughout the blood removal and replacement.

### 2.1.4 Reduction in animal use

In accordance with the principles of reduction in use of experimental animals, where feasible, tissue from rats used for other experiments in the larger project was used. Specifically, for the experiment documented in Chapter 5, hypothalamic tissue was used from animals that had undergone the same experimental procedures but investigations had centred on other regions of the brain. The brain tissue not used in the initial experiments was used to develop a tissue bank. Brain tissue was stored at - 80$^{o}$C until used.

### 2.1.5 Investigations of Experimental Pain in Conscious Animals

The deliberate inflicting of an injury on a conscious animal would seem to fly in the face of guidelines related to ethical care. It is generally acknowledged however, that in order to investigate conditions such as chronic pain, animal models are necessary and indeed should resemble the human condition as closely as possible. The International Association for the Study of Pain has established ethical guidelines for such investigations (Zimmermann, 1983). The principles of those guidelines have been adhered to in all animal procedures documented in this thesis.

## 2.2 Experimental Animals

Male Sprague-Dawley rats were used in all experiments and were obtained from the Gore Hill Animal Facility, St Leonards NSW or the Animal Resource Centre, Perth, WA. Rats were obtained at 6-8 weeks of age and habituated to a 12-hour reverse light cycle (lights ON at 18.00 and lights OFF at 0600 hours) for 7-10 days. The room temperature was maintained at 21-23$^{\circ}$C.

A total of 148 rats were used in this study (64 were injured, 10 used as 'Behavioural Controls' and 74 used as 'Intruders' for behavioural testing). Rats that were subjected to the injury protocol weighed between 250-350 g. at the time of initial surgery.

### 2.2.1 Group Housing

On arrival and while habituating to the conditions, rats were housed 6 to a cage. Rats used as 'Intruder' animals remained in group housing for the duration of the study. Food and water were freely available, cages were cleaned regularly and rats were provided with environmental stimulation as discussed above.

### 2.2.2 Individual housing

After habituation to the environmental conditions, experimental animals were housed individually in Perspex cages and placed so that they were able to see and hear other rats. Food and water were freely available and rats were provided with environmental stimulation. Cleaning of individual cages was minimal to enable the animal to develop 'ownership' of the cage and will be explained more fully under the section related to Behavioural Testing.

## 2.3 Surgical Procedures

All surgical procedures were performed using aseptic technique: instruments were sterilised using 70% alcohol before and during the procedures; sterile solutions, ligatures, sutures, syringes and needles were used throughout. Fur was shaved to the skin prior to incisions being made. Riodine® Povidone-Iodine antiseptic solution (Orion Laboratories. Australia) was used to clean the skin prior to incision and after suturing; Tricin® antibiotic powder (Jurox, Australia) was applied liberally to the wound as well as quinine hemisulphate powder (Sigma-Aldrich, USA) to serve as a

deterrent to removal of sutures. Body temperature was maintained during surgery using a homeothermic blanket control unit (Hadland Photonics, Australia).

### *2.3.1 Anaesthesia*

Rats were placed in a clean Perspex box for induction of anaesthesia using 5% halothane (Rhodia® BP, Merial, Australia) in 100% oxygen at a flow rate of 2 L/min. Once an animal was unconscious it was transferred to the surgical area where anaesthesia was maintained via a facemask at 1.0-1.5% halothane in 100% oxygen at a flow rate of 1 L/min. Before commencing surgery, depth of anaesthesia was confirmed by noting absence of a blink reflex and lack of response to pinching of the foot. At this time, a subcutaneous injection of atropine sulphate (0.15 mg, Apex Laboratories, Australia) was administered. Atropine acts to decrease bronchial mucous production and thus assists respiration during the period of anaesthesia and recovery.

### *2.3.2 Catheterisation of the right external jugular vein*

#### *2.3.2.1 Preparation of catheters*

Catheters were prepared using 15cm lengths of polypropylene tubing (O.D. 0.96mm – I.D. 0.58mm) and silastic tubing (O.D. 1.19mm – I.D. 0.64mm) with a 1cm overlap and a finished length for the silastic tubing of 34mm. Catheters were filled with and immersed in 70% ethanol overnight; before use they were drained, dried and filled with a sterile solution of Saline/Heparin/Gentamicin (0.9% NaCl; 100 IU/ml Heparin; 20 mg/ml Gentamicin) (DBL® Heparin sodium BP, Hospira, Australia: Gentam®, Troy Laboratories, Australia).

#### *2.3.2.2 Isolation of the right external jugular vein*

Following confirmation of depth of anaesthesia, with the rat lying supine, a longitudinal incision was made superior to the right clavicle. Blunt dissection was used to isolate the external jugular and three silk ligatures (4-0, Ethicon©, Australia) passed under the vein; one was tied loosely just superior to the heart to prevent backflow of blood during the catheterisation procedure; a second was tied tightly approximately 12 mm proximal to this in order to occlude blood flow returning to the heart. The vein was held taut by gentle force applied to each of these ligatures.

#### 2.3.2.3 *Insertion of the catheter*

A small incision was made in the vein mid-way between the two ligatures and the silastic end of the catheter inserted and gently pushed inferiorly towards the heart until the area of overlap (silastic-polypropylene) corresponded to the point of incision. Using a syringe attached to the polypropylene end of the catheter, the patency of the catheter was confirmed by withdrawing and reinfusing a small volume of blood. The distal and the third ligature were tied tightly and separately around the vein in the area of the overlap (silastic-polypropylene) to secure the catheter. The proximal ligature was tied loosely to the catheter.

Turning the animal prone, a small incision was made in the nape of the rat's neck. Maintaining a closed system, the polypropylene end of the catheter was guided subcutaneously to the dorsal aspect of the neck and externalised via the incision. The catheter was folded and clamped using a small length of hard plastic tubing such that approximately 2 cm of the catheter protruded from the animal's neck. The catheter was trimmed approximately 1.5 cm from the fold.

The ventral incision was closed using interrupted sutures (silk suture 3-0, Ethicon©, Australia) and one suture used to close the dorsal incision and secure the externalized end of the catheter. The cathetarisation procedure was based on the method described by Thrivikraman *et al* (Thrivikraman et al., 2002).

#### 2.3.2.4 *Recovery and post-surgical monitoring*

After surgery, rats were placed in a warmed recovery box and monitored for at least 2 hours. During this time rats were observed to return to normal motor functioning. They were then transferred to individual Perspex cages. Daily monitoring continued with respect to the surgical wounds in addition to general welfare. Animals were left to recover for 3 days except for catheter care.

#### 2.3.2.5 *Post-surgical care of catheters*

The patency of catheters was maintained over the 16 days of the procedure by daily flushing. A short extension tube, with a female luer lock connector (B/Braun, Germany) and attached 1 ml syringe, was fitted to the externalised polyethylene portion of the catheter maintaining a closed system. Approximately 0.2 ml of blood

was withdrawn and discarded before 0.2 ml of a sterile solution of 20 IU/ml heparinised saline was injected followed by 0.2 ml of sterile heparinised saline with Gentamicin (0.9% NaCl; 100 IU/ml Heparin; 20 mg/ml Gentamicin). This latter volume remains in the catheter and assists with maintaining catheter patency. The rats were also habituated to handling during the flushing procedure in preparation for later blood sampling.

### *2.3.3 Chronic Constriction Injury (CCI)*

Anaesthesia was induced as described above.

#### *2.3.3.1 Isolation of the right sciatic nerve*

Following confirmation of depth of anaesthesia, with the animal prone, an incision was made in the right upper thigh parallel to the femur. Using blunt dissection, the biceps femoralis muscle was separated to expose the sciatic nerve. The section of the nerve proximal to its trifurcation was isolated.

#### *2.3.3.2 Constriction of the sciatic nerve*

The constriction injury was performed by loosely tying 4 chromic impregnated ligatures (chromic catgut 5-0, Ethicon©, Australia) around the right sciatic nerve just proximal to the trifurcation using the procedure described by Bennett and Xie (Bennett and Xie, 1988). These ligatures are tied approximately 1mm apart and so that the nerve is barely constricted and the circulation is retarded but not arrested. The muscle tissue was closed over the cavity and the skin sutured with silk suture (3-0) as before.

#### *2.3.3.3 Recovery and post-surgical monitoring*

After surgery, rats were again placed in a warmed recovery box and monitored for two hours during which motor function returned to normal except that all animals had a pronounced limp. The rats were returned to their home cages and monitored carefully. The few rats that removed sutures before adequate healing were re-anaesthetised and re-sutured.

## 2.4 Experimental Procedures

### 2.4.1 Experimental Paradigm

There were two different experimental paradigms depending on whether or not blood samples were taken from the rats. Rats that were injured using the Chronic Constriction Injury (CCI) were subjected to one of the following:

**Experimental Paradigm 1:**

- Habituation to environmental conditions – 7-10 days
- External jugular cathetarisation – individual housing
- Recovery - 3 days
- Pre-injury behavioural testing days 1–5 pre-CCI
- Pre-injury blood sampling days 4-5 pre-CCI
- CCI
- Post-injury behavioural testing days 1–6 post-CCI
- Post-injury blood sampling days 2-6 post-CCI

Or alternatively – **Experimental Paradigm 2:**

- Habituation to environmental conditions – 7-10 days
- Individual housing – 3 days
- Pre-injury behavioural testing days 1–5 pre-CCI
- CCI
- Post-injury behavioural testing days 1–6 post-CCI

### 2.4.2 Blood Sampling

In the post-catheterisation period, rats were habituated to handling during the daily procedure of flushing the catheters; they would mostly sit quietly wrapped in a towel while this process took place and this lead to a stress free experience when blood sampling commenced.

Blood samples were removed via the indwelling catheter either at the end of the dark phase (one hour prior to lights on) or at the beginning of the light phase (3-5 hours after lights on), into heparin (20 IU/ml). Both sampling times are within the nadir of plasma corticosterone levels for the rat (Kalsbeek et al., 1996) whereas the later is the time of the TSH diurnal peak (Campos-Barros et al., 1997).

Maintaining a closed system, blood was removed using the equipment described for catheter flushing in section 2.3.2.5. On each occasion, the first ~ 0.2 ml was discarded before 1.0 ml blood was withdrawn into a clean heparinised syringe. After sampling, the catheters were flushed as before. Blood was transferred to 1.5 ml Eppendorf tubes and stored on ice until centrifuged.

Plasma was separated by centrifugation and stored at - $20^0$C for later analysis. Blood cells were resuspended in sterile isotonic Plasma-Lyte 148® solution (Baxter, Australia) and returned to each rat via the indwelling catheter. Returning the cellular elements after each sampling ensured that the haematocrit remained stable. Preliminary experiments confirmed that peripheral T3, T4 and corticosterone levels were stable three days post-catheterisation and over a subsequent five day sampling period. Considering the total amount of blood that could be taken over time, blood samples were collected on the 2 days prior to CCI and on days 2-6 after CCI.

### *2.4.3 Behavioural Testing using the Resident-Intruder test of social interactions*

The animals that had jugular vein catheterisation were allowed to recover for three days before behavioural testing commenced. All animals had been individually housed for 3 days prior to commencing testing.

#### *2.4.3.1 Baseline Testing*

Baseline behavioural testing using a 'resident-intruder' paradigm was then carried out for five days. On each day, at approximately the same time during the rats' active period (2 to 4 hours after lights off), an 'intruder' partner was introduced into the home cage of the experimental rat (the 'resident'). Thus animal interactions were tested at least 6 hours prior to blood sampling. Behavioural interactions were videotaped for a period of 6 minutes for later analysis (Sony®, Japan, using

'Nightvision' low-light infra-red illumination). Although 5 days of testing pre-CCI were recorded and analysed, only days 3-5 pre-CCI are used as a measure of baseline behaviour.

#### *2.4.3.2 Post-CCI Testing*

Resident-Intruder testing was resumed the day after CCI and continued for 6 days post-CCI. As before, a 6-minute period of social interaction was recorded for later analysis. On the day of CCI surgery the cages were cleaned but only half of the bedding was renewed so that the 'home cage' environment of the 'resident' was maintained.

#### *2.4.3.3 Intruder Rats*

The 'intruder' rats were uninjured male animals that were matched for age and weight. Intruder rats were rostered such that over the eleven days of testing, a resident did not encounter the same intruder on more than two occasions and never on consecutive days. Intruders were marked on the proximal portion of the tail for identification purposes. The intruders' fur was marked (stripes) to distinguish them from the residents during interactions.

#### *2.4.3.4 Behavioural Control Rats*

A group of 10 rats used as behavioural controls for the experiment detailed in Chapter 6 were tested using the Resident-Intruder test following the same procedure as given above for the injured rats. The experimental schedule for these animals was as Experimental Paradigm B (no blood samples were taken) except that on the day of CCI they were given a period of anaesthesia for 20 minutes and then allowed to recover.

### *2.4.4 Sacrifice and Tissue Removal*

All experimental rats were euthanased on the seventh day post CCI. The method of euthanasia and subsequent tissue handling was different for each group of animals.

#### *2.4.4.1 Brain (Chapter 4)*

Rats were placed in a clean Perspex box and rendered briefly unconscious with Carbon Dioxide. While still unconscious, they were given a lethal intra-peritoneal injection of Sodium Pentobarbitone (60 mg in 1ml, Lethabarb ®, Verbac, Australia).

When the rats were deeply anaesthetised, all circulating blood was removed by transcardial perfusion using 500ml 0.1M phosphate buffer pH7.4 in 0.9% NaCl (PBS) containing 10 IU/ml heparin.

Rats were placed supine and a midline skin incision made from the abdomen to the neck region. An incision was made into the peritoneal wall which was extended bilaterally beneath the diaphragm and then superiorly, cutting through the rib cage on either side. The diaphragm was cut away and the chest wall retracted superiorly exposing the heart. After removal of the pericardium, a perfusion needle (19 G x 1.5 in) was inserted into the left ventricle of the beating heart and the right atrium was cut. The perfusion needle was connected to a motorised pump (Cole Palmer, USA).

Following the perfusion of 500 ml heparinised PBS, rats were turned prone and the brain promptly removed. The skin overlying the skull was cut away and the bone removed using rongeurs. After removal of the dura mater the brain was snap frozen in dry ice and stored at - $20^{0}$C.

#### *2.4.4.2 Brain (Chapter 5) – Preparation of Tissue for Molecular Biology - RT-qPCR*

The hypothalamic tissue used for molecular biology investigations in Chapter 5 was obtained from a tissue bank of animals that experienced the Experimental Paradigm 2. Eight rats used as control animals for the molecular biology study were euthanased in accordance with the method used in previous experiments and the tissue stored in an identical manner.

After exposure to Carbon Dioxide, and whilst unconscious, the rats were quickly decapitated by an expert. The brains were rapidly removed as above, immersed in Tri-Reagent® (Molecular Research Center, USA) on ice and transferred to storage at - $80^{o}$C as soon as possible.

#### *2.4.4.3 Brain (Chapter 6) - Preparation of Tissue for immunohistochemistry*

Rats were rendered unconscious with Carbon Dioxide as described in section 2.4.4.1. The procedure thereafter was the same for the initial transcardial perfusion of 500 ml 0.9% saline containing 10 IU/ml heparin described above. This was followed by 500 ml of 4% paraformaldehyde fixative in borate buffer pH 9.6.

After removal, the brains were post-fixed in 4% paraformaldehyde in borate buffer pH 9.6 overnight at $4^{o}C$. Brains were then transferred to 30% sucrose in 0.1PBS pH 7.4 for cryoprotection and stored at $4^{o}C$ until use.

#### *2.4.4.4 Sciatic Nerve*

The portion of the right sciatic nerve containing the ligation was removed using blunt dissection and placed in 4% paraformaldehyde in 0.1M PBS pH 7.4 and stored at $4^{o}C$. This was done for each rat used in the experiments reported in Chapters 4 and 6. The nerves were examined for gross histological appearance. Photographs were taken of the post-mortem right sciatic nerves of the animals used in Chapter 4 using a macroscope (Leica M420, Wild 400076 APOZoom 1:6, Germany) with attached digital camera (Olympus DP70, Japan).

## *2.5 Behavioural Analysis (Chapter 3)*

The videotaped interactions between the resident and the intruder partner were analysed for resident behaviours using an automated scoring device and specialised computer software (Maclab 8, Chart v3.2). This scoring device was developed specifically to facilitate the encoding and subsequent processing of visual observations of behaviours (Depaulis, 1983). An example of the output from the specialised software illustrating the scoring of the interactions according to derived criteria, as explained below, is shown in Figure 2.1.

### *2.5.1 Behavioural Categories*

Four specific resident behaviours were scored during six minutes of interactions: Dominance (D), Social (S), Non Social (NS) and Submissive (SM) behaviours. The criteria for describing these behaviours were defined by Monassi *et al.,* and are used in

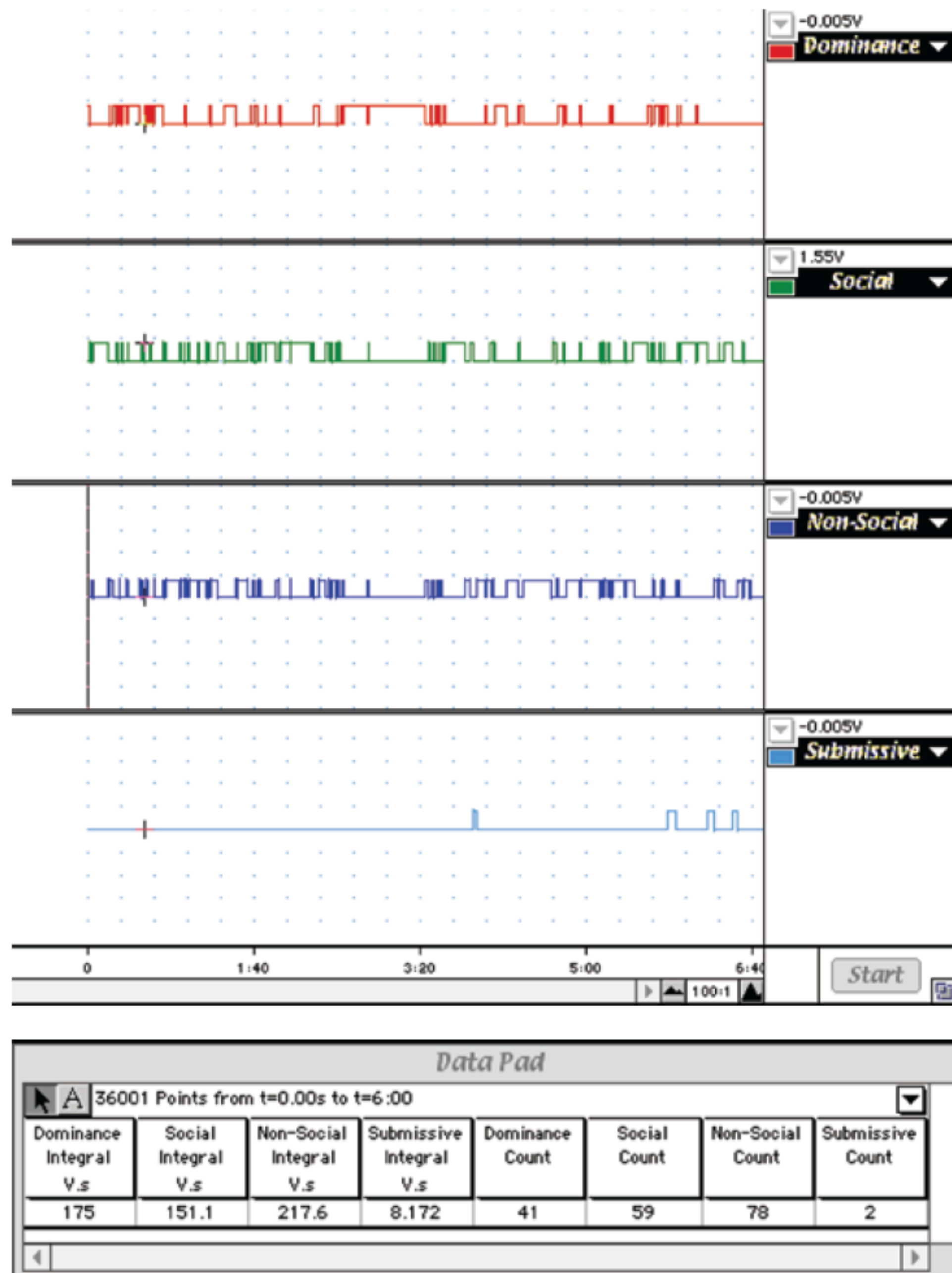



**Figure 2.1:** An example recording of observed animal interactions over six minutes. Taped videorecordings are replayed and, using derived criteria, animal interactions are scored as Dominance (D), Social (S), Non Social (NS) or Submissive (SM). Each sub-score is corrected for gain (1.55V) to give a score in seconds for each of the four behavioural categories.

all behavioural experiments in our laboratory (Monassi et al., 2003). A full description of the criteria used to describe the four behaviours was given in section 1.6.3.2.

For each rat, the score (in seconds) was calculated for each of the 4 behavioural categories for each of the 11 days of behavioural scoring (days 1-5 pre-CCI and days 1-6 post CCI). A pre-injury baseline, for each behavioural category, was derived from the mean score (in seconds) for days 3-5 pre-CCI for each rat.

### *2.5.2 Behavioural Classification*

Based on an analysis of post-injury relative to pre-injury behaviour, Monassi *et al.,* developed a behavioural classification, which is used consistently in our laboratory (Monassi et al., 2003). A description of the classification was given in section 1.6.3.3 and is repeated here with specific details relevant to these experiments. Although all 4 behavioural categories were examined, the classification is based on the duration and pattern of altered dominance (D) behaviour. A rat was defined as showing decreased dominance on any one day if there was <70% of the mean dominance pre-injury. Six days of post-CCI dominance behaviour were evaluated.

Three distinct behavioural groups were defined:

- Rats that showed reduction of dominance on 2 or less than 2 days after injury were defined as No Disability (ND).
- Rats defined as having ‘Disability’ show decreased dominance on 3 or more days.
- This group is further divided into Persistent Disability (PD) and Transient Disability (TD) groups.
- The rats are assigned to the TD group if the dominance scores on days 5 and 6 return to pre-injury levels.
- Rats that are categorised as PD all have decreased dominance on at least one of days 5 and 6.

Using the defined criteria, rats were assigned to a ‘Behavioural Group’ based on the number and pattern of days when dominance behaviour was <70% the pre-injury baseline level.

## 2.6 Peripheral Hormone Measurement (Chapter 4)

Total thyroxine (TT4), total triiodothyronine (TT3), free thyroxine (fT4), free triiodothyronine (fT3), thyroid Stimulating Hormone (TSH) and corticosterone levels were measured in rat plasma using commercially available radioimmunoassay kits.

All plasma samples were stored at - 20$^{o}$C after sampling and were thawed and refrozen no more than once. All hormone levels were determined in duplicate and all samples from any one rat were assayed using the same kit.

All the assay kits used in this study were chosen because the manufacturers included 'heparinised plasma samples' in the specifications for use of the kit. This was necessary, as heparin was used to keep the catheters patent and as an anticoagulant. There are issues related to the measurement of fT4 that are well documented related to validity of measurement and interference by drugs including heparin (Stockigt, 2001). The heparin artefact occurs when non-esterified free fatty levels are high and this can occur in stored samples of heparin-treated patients. These issues are of necessity important when clinical evaluations of thyroid status are made, however this study was concerned with relative change following the intervention. Heparinised saline was used to keep the catheters open before and after injury and in all rats. If there is a shift in the protein binding of thyroxine, it should be controlled for by the fact that each animal acted as its own control.

Where the molecule to be measured was species specific, for TSH and corticosterone, rat specific radioimmunoassay kits were used. Where the hormone to be measured was a molecule that is common across species, kits for clinical evaluations in humans were used. Arguments have been made against using kits developed for human measurements in rats (Stringer and Wynford-Thomas, 1982). Errors in absolute values can occur because of the different binding proteins (rat and human). However, pragmatically, the cost difference is considerable or the specific rat kits are not locally available. In the experimental paradigm described, each animal acts as its own control – it is change within an animal, not absolute values that are important here. Moreover the development and use of monoclonal antibodies has increased the specificity of kits now in use and decreased the importance of issues related to binding proteins.

During early trials of kits however it was realized that kits using anti-mouse antibodies had to be avoided because of cross-reactivity between mouse and rat proteins interfering with the assay.

Further choice of commercial kits for this study was based on the size of the plasma sample required and the nature of the antibody utilised in the kit. All of the kits used employed I-125 radioligands and measurements were made using a gamma counter (PerkinElmer, USA).

## 2.6.1 Radioimmunoassay

### 2.6.1.1 Total thyroxine (TT4)

Thyroxine, the primary product of the thyroid gland, is found in plasma 99.9% bound to plasma proteins. Measurement of TT4 is a measure of both protein bound and non-protein bound thyroxine. TT4 was measured using Active® Thyroxine RIA kits (Diagnostic Systems Laboratories, USA); the intra-assay error for this kit as cited by the manufacturer as 2.9 - 5.1%.

### 2.6.1.2 Total triiodothyronine (TT3)

Triiodothyronine is produced both by the thyroid gland and by peripheral deiodination of thyroxine. T3 is found in plasma 99% bound to plasma proteins. Measurement of TT3 is a measure of both protein bound and free triiodothyronine. TT3 was measured using RIA-gnost® T3 kits (CIS bio, France); the intra-assay error for this kit as cited by the manufacturer as 4.7 - 6.6%.

### 2.6.1.3 Free thyroxine (fT4)

The small percentage of thyroxine that is not protein bound is measured using a variation of the traditional RIA in which free thyroxine competes for binding sites on radiolabelled monoclonal anti-thyroxine antibody. Free T4 was measured using FT4 RIA kits (Immunotech, Czech Republic); the intra-assay error for this kit as cited by the manufacturer as < 6.7%.

#### *2.6.1.4 Free triiodothyronine (fT3)*

The non-protein bound T3 is measured by competition for binding sites with a T3 antibody. Free T3 was measured using RIA-gnost® FT3 kits (CIS bio, France); the intra-assay error for this kit as cited by the manufacturer as 3.3 - 6.8%.

#### *2.6.1.5 Thyroid stimulating hormone (TSH)*

Rat TSH was measured using species-specific kits, Rat TSH IRMA® (Biocode, Belgium): the intra-assay error for this kit as cited by the manufacturer as 5.3 - 6.1%.

#### *2.6.1.6 Corticosterone*

Corticosterone was measured using rat-specific kits, Rat Corticosterone RIA, (Diagnostic Systems Laboratories, USA); the intra-assay error for this kit as cited by the manufacturer as 2 - 4.2%.

#### *2.6.1.7 Hormone measurement analysis*

Duplicate hormone determinations were averaged: as each rat served as its own control, hormone levels were analysed for change post injury relative to pre-injury levels.

Criteria for individual rat data to be included in the analysis were: normal interactions between resident and intruder pre-CCI; corticosterone levels returned to baseline pre-CCI; stable haematocrit; no injuries during post-CCI interactions.

## *2.7 Reverse Transcription quantitative real-time polymerase chain reaction (RT-qPCR) (Chapter 5)*

Using RT-qPCR techniques, relative expression of mRNA for a number of molecules of interest was determined in hypothalamic tissue from injured rats and from two groups of control rats. The polymerase chain reaction (PCR) is one of the most powerful technologies available in molecular biology. Specific sequences of DNA or cDNA (produced by reverse transcription from RNA) can be copied (or amplified) exponentially allowing for the detection of sequences that are found in very small concentrations. Theoretically, PCR amplifies the DNA by doubling the number of molecules present with each amplification cycle. RT-qPCR allows for the reaction to be monitored such that the PCR product can be measured at each cycle due to the use

of florescent markers attached to specific gene sequence probes that are incorporated into the PCR product. The increase in fluorescent signal is directly proportional to the number of amplified molecules and is detected by the RT-PCR instrument that generates amplification plots (fluorescence against cycle number); these plots represent the accumulation of specific product over the duration of the reaction. Sequences of DNA/RNA that are more abundant in the sample will be amplified at an earlier cycle compared to those of lower abundance which appear at a later cycle. Relative abundance of a specific RNA sequence is obtained by examining the threshold cycle (Ct) at which amplification is first observed (Reischl and Kochanowski, 1995) (Bustin et al., 2009). This technology is however completely dependent on the identification of appropriate reference genes. Gene expression can only be determined relative to a reference gene that remains stable in expression across the experimental paradigm (Wan et al., 2010).

### *2.7.1 Preparation of hypothalamic tissue*

Rat brains, previously stored in Tri-Reagent® (Molecular Research Centre, USA) at - 80$^{o}$C, were blocked while still frozen and the hypothalamus from each rat isolated, by an experienced individual, by micro-dissection according to strict anatomical points of reference. The hypothalamic block was defined as follows: rostral border - the optic chiasm: caudal border – the decussation of the posterior commissure; superior border – the mid-point of the internal capsule fibres; lateral border – the margin of the cerebral cortex. Once isolated, the hypothalamus of each rat was homogenised in 1 ml Tri-Reagent® using RNase free pestles (Astral Scientific, Australia) and RNase free Eppendorf tubes (Astral Scientific, Australia). All steps in the following preparation and handling of hypothalamic samples were carried out in an RNase free environment (RNase Away®, Molecular Bioproducts, USA) using RNase free reagents and equipment (Bioshere® filter tips, Starstedt, Germany).

### *2.7.2 Extraction of RNA*

After homogenisation, total RNA was extracted with chloroform (Ajax Finechem, Australia). The aqueous phase was separated by centrifugation at 4$^{o}$C at 13,000 rpm for 15 minutes and transferred to a fresh tube with care taken not to include any DNA. Total RNA was precipitated with 100% 2-propanol (Sigma-Aldrich, USA) (13,000 rpm at 4$^{o}$C) and the pellet washed with 75% ethanol (Sigma-Aldrich, USA). After

removal of the supernatant, the RNA pellet was allowed to partially air-dry to remove remaining ethanol and then resuspended in 60 μl RNase free water. A small aliquot was removed for quality assurance testing and the remainder of each sample stored in multiple aliquots at - 80$^{o}$ C.

### *2.7.3 Quantitative analysis of RNA*

The total RNA concentration of each sample was determined using spectrophotometry (ND-1000 Spectrophotometer, Nanodrop$^{TM}$, Thermo Scientific, USA). For each sample a concentration is given in ng/μl based on measurement at wavelength 260nm. Additional assessments of the quality of the preparation are given by ratios of absorption at 260/280 and 260/230. A value of approximately 2 indicates acceptable quality with respect to DNA contamination or contamination with organic solvents respectively.

### *2.7.4 Re-precipitation of RNA*

Samples where the 260/230 ratios were unacceptable (< 1.5 indicating organic contamination) were re-precipitated as follows. Glycogen (RNase free, 1μl of 20mg/ml, Roche, Germany) was added to RNA on ice and then times 2 volumes of 100% ethanol added. Mixtures were incubated overnight at -20$^{o}$C and then total RNA precipitated, washed and resuspended as before. Samples where the 260/230 remained below 1.5 were evaluated for inclusion in final data analysis.

### *2.7.5 Qualitative analysis of RNA*

The quality of each sample was assessed using a 2100 Bioanalyser$^{TM}$ (Agilent Technologies, Germany) and the program Eukaryote Total RNA Nano Series II (Copyright © 2003-2006 Agilent Technologies, Germany). A measure of quality is given by the RNA Integrity Number (RIN), which uses a scale of 0-10 units. This parameter assesses total RNA integrity based on the ratio of 28S/18S ribosomal peaks. Samples where the RIN values were below 6.5 were evaluated for inclusion in final data analysis. Samples with RIN below 7.0 are usable provided that the RIN does not correlate with the Ct number obtained using RT-qPCR assays (Bustin et al., 2009).

### 2.7.6 cDNA synthesis by reverse transcription

Using RNA sample concentrations obtained using the Nanodrop™ spectrophotometer, equivalent amounts of total RNA from each preparation were used to synthesise double stranded cDNA using the Superscript™ Reverse Transcriptase kit (Invitrogen, Australia), following the manufacturer's protocol. For each sample, RNA (3 μg), 1 μl random hexamers (Invitrogen), 4 μl 2.5 mM dNTP mix (Bioline, Australia) and RNase free water to a volume of 14 μl, were heated for 5 minutes at 65°C. Samples were cooled and centrifuged.

Reverse transcriptase (Superscript™ III RT, 1 μl of 200 U/μl), 4 μl buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$)), 1 μl dithiothreitol (DTT, 0.1M) were then added to each sample (total reaction volume of 20 μl) prior to a sequence of heating steps; 5 minutes at 25°C, 50 minutes at 50°C, 15 minutes at 70°C and cooling at 4°C. Control reactions, with RNase free water replacing reverse transcriptase, for each RNA sample were prepared and included in the same synthesis run (RT minus). Post synthesis, the cDNA (RT plus) samples were diluted to 80 μl and stored in multiple aliquots at -20°C until use. RT minus samples were stored undiluted.

### 2.7.7 Real time polymerase chain reaction (RT-PCR) using Taqman® Gene Expression Assays

The relative expressions of mRNA for molecules of interest were measured using real-time Taqman® quantitative reverse transcription polymerase chain reaction (RT-qPCR) assays utilising Taqman® Universal PCR Master Mix, No Ampase® UNG and Taqman® probes (Applied Biosystems, USA). The LightCycler 480 ™ Real-Time PCR Instrument (Roche, Germany) was used for reactions. Assays were performed using either a 96 (reaction volume 20 μl) or a 384 well plate (10 μl reaction volume). When a 384 well plate was used, automated pipetting was performed using a Tecan® Freedom EVOware instrument, supported by Freedom EVOware® Standard 2.3 software (Switzerland).

Prior to each relative gene expression assay, standard curves were run to determine the efficiency of each probe and when two probes were to be run in the same assay,

optimisation of probe concentrations was performed. No template controls were included in each assay and possible genomic DNA contamination was controlled for using no reverse transcriptase (RT minus) samples. All sample measurements were performed in triplicate and all samples measured in the same assay where comparisons were to be made. The RT-qPCR reaction conditions were; 95°C for 10 minutes; 45 cycles of 95°C for 15 seconds followed by 60°C for 1 minute; 37°C for 10 minutes.

For the respective mRNA assays, the following FAM™ dye-labelled Taqman® probes (Applied Biosystems, USA) were used:

- Rn00564880_m1 - thyrotrophin releasing hormone (TRH)
- Rn00581867_m1 - deiodinase, iodothyronine type II (Dio2)
- Rn00568002_s1 – deiodinase, iodothyronine type III (Dio3)
- Rn00562044_m1 – thyroid hormone receptor beta (TR β)

- Rn01462137_m1 – corticotrophin releasing hormone (CRH)
- Rn00566449_m1 – arginine vasopressin (AV)
- Rn00561369_m1 – glucocorticoid receptor (GR)

- Rn00580432_m1 – interleukin-1β (IL-1β)
- Rn01525859_g1 – tumour necrosis factor (TNF)

Two reference genes were used:

- Rn99999916_s1 – glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (VIC®/MGB Probe, Applied Biosystems, USA) was used as a within assay reference gene with all of the above probes.
- Hs99999901_s1 – Eukaryotic 18S rRNA (18S) a FAM™ dye-labelled probe, was run as an independent reference gene.

The threshold cycle (Ct) for each sample was determined using the Fit-Points analysis function of the Roche LightCycler 480 ™ software (Germany).

## 2.8 Immunohistochemistry (Chapter 6)

Immunohistochemistry techniques were used to determine the tissue distribution of a number of molecules of interest in hypothalamic tissue from injured rats and from a group of behavioural control rats. Immunohistochemistry employs the use of specific antibodies, produced to a specific protein (antigen), for direct localisation of proteins *in situ* in tissue (Cuello, 1993). Using amplification techniques and reaction with a chromagen, protein so localised can be visualised using the light microscope (Cuello, 1993). In theory, this technology allows for confirmation of translational change in protein expression identified using technologies that determine gene expression. However, the technology is entirely dependent on the antibody used being both specific for the protein in question and sensitive in the experimental conditions of the assay (Saper, 2009). When protein localisation is attempted in post-mortem tissue, issues related to fixation techniques can result in loss of antigenicity due to the formation of cross-linkages between proteins and conformational change (D'Amico et al., 2009). Techniques can be employed to restore antigenicity lost during tissue fixation.

### 2.8.1 Preparation of brain tissue

Rats were euthanased and perfused as detailed in sections 2.4.4.1 and 2.4.4.3. After the perfusion of paraformaldehyde, brains were removed as detailed, post fixed, cryoprotected in 30% sucrose and stored at 4$^{o}$C until use (section 2.4.4.3).

### 2.8.2 Sectioning of brain tissue

For each brain, a block of tissue was cut: rostral margin - the optic chiasm (0.0 mm to bregma); caudal margin - the anterior margin of the Pons (- 6.0 mm to bregma). The right side of each rat brain was marked using a gauge #21 needle. The block of tissue containing the hypothalamus was mounted onto the freezing stage (- 15$^{o}$C) of a cryostat (Leica, Germany) using a tissue-mounting medium (Tissue-Tek®, ProSciTech, Australia). Coronal sections 40 μm thick were cut in a 1 in 5 series: four series were collected into sterile plastic jars containing 0.1 M phosphate buffered saline pH 7.4 (PBS); one series was collected sequentially into separate wells containing PBS.

### 2.8.3 Nissl staining

For each rat brain, the sequentially collected sections were stained with Nissl stain to enable anatomical landmarks to be identified and to aid in sequencing the specific antibody labelled brain sections. The sections were mounted onto glass slides (Superwhite, ProSciTech, Australia), which had been coated with gelatine impregnated with chrome alum as an antifungal agent. The slides were air dried for at least a week and then immersed in chloroform: ether (50:50) for 48 hours before staining. The mounted sections were passed through a descending ethanol series (100%, 90%, 70% and 50%) for one minute each followed by one minute in acetic acid: ethanol (1:4). Slides were then immersed in 1% thionin for 15-20 seconds before distilled water for one minute and then an ascending ethanol series. The stained sections were cleared in histolene (Fronine, Australia) for 24 hours before cover slipping.

### 2.8.4 Antigen labeling in the hypothalamus

The following antibodies were utilised in immunohistochemical protocols to assess the distribution of specific proteins in the PVN and in particular in sections close to -1.8 mm to bregma where the hypophysiotrophic ppTRH secreting neurones are primarily located (Fekete and Lechan, 2007).

- Rabbit polyclonal antibody to Thyroid Hormone Receptor β (TRβ) (ab5622, abcam®, UK); the manufacturer states that the antibody reacts with rat TRβ but does not detect TRα1 or TRα2: this antibody has been used in human heart muscle and rat liver (Modesti et al., 2008) (Sun et al., 2007).
- Rabbit polyclonal antibody to synthetic Thyrotrophin Releasing Hormone (TRH) (ab87764, abcam®, UK); the manufacturer states that the antibody reacts with human TRH; there are to date no published articles citing the use of this specific antibody.
- Goat polyclonal antibody to Deiodinase 2 enzyme (Dio2) (ab77481, abcam®, UK); the manufacturer states that the antibody reacts with mouse Dio2 protein and is predicted to react with rat Dio2 protein; this product has been used to identify Dio2 in mouse heart muscle and characterised using western blot (Wang et al., 2010).

- Rabbit polyclonal antibody to Deiodinase 3 enzyme (Dio3) (ab82041, abcam®, UK); the manufacturer states that the antibody reacts with rat Dio3 protein and was produced using a synthetic peptide to a region within internal residues 250-300 Rat Dio3; using western blot a band is identified at 31 kDa which is the predicted band size; this product has been used to identify Dio3 in rat retina and characterised using western blot (Bedolla and Torre, 2011).

#### *2.8.4.1 Preparation of sections*

Following sectioning, the free-floating sections were washed x3 in PBS to remove traces of sucrose and fixative. Washing procedures were performed in plastic jars at room temperature (RT) using a orbital rotor set at 100 rpm to ensure movement of brain sections with incubation times of approximately 10 minutes per wash.

#### *2.8.4.2 Preliminary studies*

Initial attempts to optimise immunohistochemical protocols resulted in poor or no specific staining while confirming the lack of specific staining in the absence of primary antibodies. Subsequently, various means of antigen retrieval were employed to attempt to enhance the antigenicity of the proteins. Sodium borohydride had been successfully used to enhance antigenicity for TRH (Chung et al., 1989) (Rinaman et al., 1989); heat and acidic pH are well-documented methods for breaking intra and intermolecular cross-links (D'Amico et al., 2009). Further optimisation of protocols using these techniques resulted in specific staining.

#### *2.8.4.3 Antigen retrieval techniques*

The following Antigen retrieval techniques were employed.

- Dio3 and TRβ: sections were incubated in 1% sodium borohydride (Fluka, UK) at RT for 30 minutes with shaking, followed by washing x3 in PBS.
- TRH: sections incubated in 0.5% borohydride at RT for 30 minutes and washed as above.
- Dio2: sections were incubated in 100 ml 0.01 M citrate buffer pH 4.5 in the microwave oven for 3 minutes (heated to boiling point) followed by standing a further 10 minutes before washing x3 in PBS at RT.

#### *2.8.4.4 Quenching of endogenous peroxidase and blocking of non-specific binding sites*

Following antigen retrieval protocols, as detailed above, sections were washed in 50% ethanol for 30 minutes and then incubated in 3% hydrogen peroxide in 50% ethanol for 15 minutes at RT to remove endogenous peroxidase activity. After further washing x3 in PBS, the sections were incubated in PBS containing 2% horse serum and 0.1% bovine serum albumin (phosphate buffered horse serum, PBHS) to reduce non-specific binding of antibodies.

#### *2.8.4.5 Incubation of sections with primary antibodies*

The free-floating sections were incubated with the primary antibodies diluted in fresh PBHS as follows:

- Dio3: incubated at $4^{o}$C for 40 hours at 1:2000.
- TRβ: incubated at $4^{o}$C for 40 hours at 1:1000
- TRH: incubated at $4^{o}$C for 68 hours at 1:500
- Dio2: incubated at $4^{o}$C for 68 hours at 1:500

Sections were then washed x3 with PBS at RT.

#### *2.8.4.6 Incubation of sections with secondary antibodies*

After thorough washing, the sections were incubated with the appropriate secondary antibody diluted 1:500 in fresh PBHS: for TRβ, TRH and Dio3, with biotin-SP-conjugated AffiniPure donkey anti-rabbit IgG (Jackson Immunoresearch, USA) and for Dio2, biotin-SP-conjugated AffiniPure donkey anti-goat antibody IgG (Jackson Immunoresearch, USA). After further washing (x3 in PBS) all sections were incubated for 2 hours in ExtrAvidin® Peroxidase (Sigma-Aldrich, USA) at a 1:1000 dilution in PHS at RT. Sections were washed as before after this incubation.

#### *2.8.4.7 Chromagenic reaction with 3-3'-diaminobenzidine (DAB)*

Amplified antigen-antibody complexes were visualised by reaction with the chromagen DAB (Sigma-Aldrich, USA). Sections were incubated in a 20 ml solution of PBS containing 10 mg DAB, 200 μl 0.4% $NH_4Cl$, 200 μl 20% D-glucose and 125 μl $Ni_2SO_4$ for 20 minutes on ice. The reaction for each sample was started (on ice) by adding 20 μl glucose oxidase (Sigma-Aldrich, USA). The reaction was allowed to

continue for the same reaction time for each sample of a specific antibody: TRβ - 12 minutes; TRH – 14 minutes; Dio2 – 24 minutes; Dio 3 – 12 minutes. The chromagenic reaction was stopped in each case by washing with PBS X3 for 10 mins at RT; the samples were placed in the freezer for 10 minutes and washed again with PBS.

### *2.8.5 Mounting, dehydration and cover-slipping*

DAB reacted sections were mounted on gelatinised slides and left to air-dry for six days. The mounted sections were dehydrated by passing through an ascending ethanol series (30%, 50%, 70%, 90% and 100%) for three minutes each followed by three minutes in ethanol: histolene (50:50). The sections were cleared in histolene (Fronine, Australia) for 24 hours before cover slipping (Deckglaser, ProSciTech, Australia) using DPX Mountant (Sigma-Aldrich, USA). When the DPX was dry, excess was removed with a razor blade and the slides cleaned with ethanol: acetone (50:50).

## *2.9 Data Analysis and Statistics*

### *2.9.1 Analysis of Rat Behaviour (Chapter 3)*

Scores (in seconds) for each behavioural category for each rat for each testing day were determined as described in section 2.5.1. Mean pre-CCI behavioural scores (days 3-5 pre-CCI) were determined for each rat. Post-CCI scores: mean score days 1-6 and mean score on days 1-3 and days 4-6 post-CCI were determined for each rat.

#### *2.9.1.1 Behavioural Scores by Disability Group*

Once rats were classified behaviourally into the three behavioural groups (ND, TD and PD) using the criteria given in section 2.5.2, mean behavioural scores for each group (±SEM) were calculated. Percentage of mean pre-CCI behaviour on days 1-3 and 4-6 post-CCI for each behavioural group was also calculated. Change in dominance (D) was determined from mean (D) post-CCI relative to mean (D) pre-CCI for each behavioural group.

#### *2.9.1.2 Statistics*

Mean group behavioural scores were examined for difference to the ND group in each case using the Independent Samples T-test. Levene's Test for equality of variances was used to determine whether equal variance could be assumed and thus the appropriate 2-tailed significance obtained in each case (Pallant, 2007). Levels of significance were taken at $p<0.05$.

### *2.9.2 Analysis of Hormone Levels (Chapter 4)*

#### *2.9.2.1 Hormone concentrations*

Hormone concentrations for each sample were determined using the relevant with-in assay standard curve and duplicates averaged. Mean hormone concentrations (± SEM) for each of the hormones measured were determined for each group pre-CCI and post-CCI (days 1-6). Change in mean hormone concentrations relative to pre-CCI levels was determined for each behavioural group.

#### *2.9.2.2 Time course*

Hormone levels relative to the individual rat's baseline concentrations were calculated for days 2, 3, 4, 5 and 6 post-CCI for the thyroid hormones and TSH. Mean behavioural group levels (±SEM) were calculated and presented as a function of time after injury.

#### *2.9.2.3 Statistics*

All data are expressed as means ± standard error of the mean. T-test (equal variances assumed or not assumed as determined by Levene's Test) was used to determine differences between 2 sets of data (Pallant, 2007). Testing for statistical significance between 3 groups was performed using one-way ANOVA. When an overall significant effect was reported ($p<0.05$), *post hoc* analyses were done using Dunnett t-tests to determine where the difference lay. Correlation coefficients were determined using Pearsons Test for Correlation. Levels of significance were taken at $p<0.05$.

## *2.9.3 Analysis of RT-qPCR measurement of gene*

### *2.9.3.1 Reference genes*

Mean Ct values (±SEM) for each of the reference genes were determined for each behavioural group. The Mann-Whitney U test was used to examine whether there were differences between different experimental groups for raw gene expression and hence evaluate the appropriateness of each reference gene for use in the experimental paradigm.

### *2.9.3.2 Relative gene expression*

For each sample, the expression for the gene of interest was determined relative to the reference gene and then normalised to the relevant BC group. Mean relative mRNA expression (± SEM) for each behavioural group of rats was determined.

### *2.9.3.3 Statistics*

Mean relative mRNA expression (±SEM) for each behavioural group was examined for difference to the BC group using the Mann-Whitney U test for evaluating differences between small groups where equal variances and normal distributions cannot be assumed (Pallant, 2007). The Mann-Whitney U test compares group medians (Md). Levels of significance were taken at $p<0.05$.

## *2.9.4 Analysis of TRH, THβ, Deiodinase 2 and Deiodinase 3 expression in the Hypothalamus*

### *2.9.4.1 Identification of relevant sections*

The Nissl-stained series of sections for each rat were used to identify four consecutive sections of the hypothalamus for each of the four antibody series. The sections identified were at approximately -2.1, -1.9, -1.7 and -1.5 mm from bregma; these hypothalamic sections include the PVN and more specifically the medial (PaMP) and ventral (PaV) parvocellular subdivisions of the PVN identified as the location of the ppTRH hypophysiotrophic neurons (Geerling et al., 2010) (Espinosa et al., 2007). The respective Nissl-stained sections were used as a template in drawing regions of interest for each antibody stained section.

#### *2.9.4.2 Identification of immunoreactive profiles*

Staining that was identifiably twice background in intensity and of a discrete shape was identified as an immunoreactive profile; streaks of staining were not included. There was a range of size of profiles identified and an arbitrary division was made between those >10 μm and those <10 μm. Where there was staining in the ependymal layer, these profiles were not included; only profiles fully within the PVN were identified and included in the quantifying of profiles.

#### *2.9.4.3 Quantitative analysis*

Images of each of the identified sections of the hypothalamus (-2.1, -1.9, -1.7 and -1.5 mm to bregma) for each antibody were captured under standard illumination conditions using a light microscope (Olympus BX51) with a Digital camera attachment (Olympus DP70) at x 200 magnification. Each image was then processed in Photoshop CS3® (Adobe®, USA) using standard parameters: for evaluation of staining for each different antibody, identical contrast, illumination and magnification parameters were used. Profiles were counted as either, >10 μm or < 10 μm, as defined by consistent application of a grid overlaid on the images used for counting.

Regions of interest were defined to approximate to those shown in Figure 2.2. Immunoreactive profiles were counted in the areas defined. That is, on section -2.1 mm to bregma, profiles within the medial PVN (PaMP) only were counted: on section -1.9 mm to bregma, profiles in the PaMP, in the ventral PVN (PaV) and in the dorsal PVN (PaDP) were counted: similarly on section -1.7 mm to bregma, profiles in the PaMP, PaV and PaDP were counted; on section -1.5 mm to bregma, profiles were counted in the PaMP and PaV only. Each of the three regions was counted separately and right and left sides of each section were counted separately.

#### *2.9.4.4 Photography*

Digital images of representative sections of the PVN and other relevant regions of the rat CNS were taken using a light microscope (Olympus BX51) with a Digital camera attachment (Olympus DP70). The magnification used in each case is specified on the specific image.

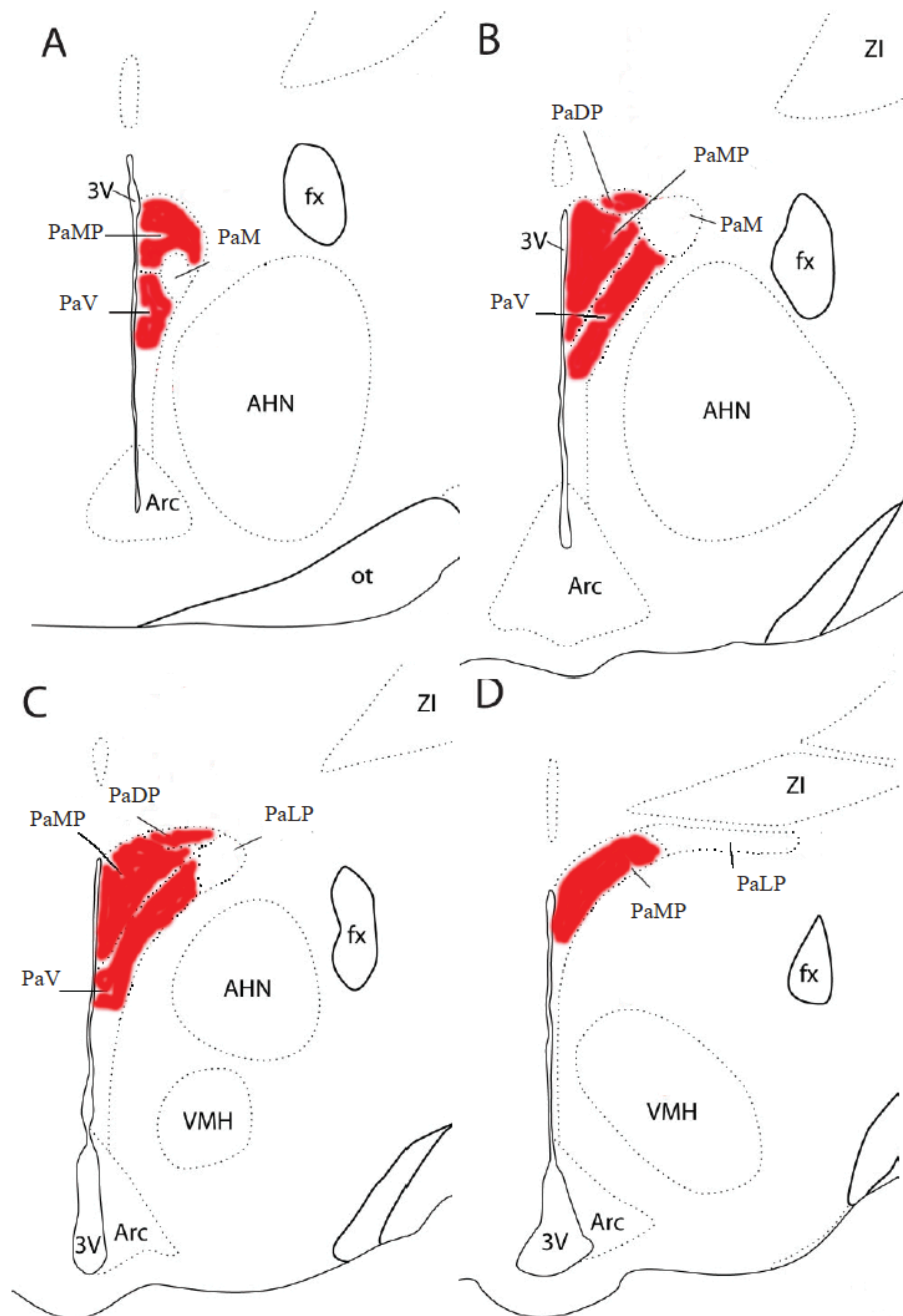



**Figure 2.2:** Schematic diagrams indicating the hypothalamic sections and the regions on each section included in quantifying immonoreactive profiles. A) -1.5 mm to bregma B) -1.7 mm to bregma C) -1.9 mm to bregma and D) -2.1 mm to bregma. Subdivisions of the PVN included were: medial parvocellular (PaMP), ventral parvocellular (PaV) and dorsal parvocellular (PaDP). The magnocellular (PaM) and lateral parvocellular (PaLP) regions of the Paraventricular Nucleus were not included. (Third Ventricle (3V), fornix (fx), Anterior Hypothalamic Nuclus (AHN), Ventromedial Hypothalamic Nucleus (VMH), Zona Incerta (ZI)). Adapted from Geerling et al (2010) and Paxinos and Watson (2005).

### *2.9.4.1 Statistics*

Mean numbers of immunoreactive profiles (±SEM) for the two groups of injured rats were examined for difference to the BC group using the Mann-Whitney U test for evaluating differences between small groups where equal variances and normal distributions cannot be assumed (Pallant, 2007). Levels of significance were taken at $p<0.05$.

# *CHAPTER THREE*

## *Behavioural Responses Following Injury*

### *3.1 Introduction*

Disability or 'sickness behaviour' is experienced by a significant sub-group of patients with ongoing pain following healing of a nerve injury. They report behavioural changes, which include changes in social interactions, family relationships, sleep-wake cycle and depression, said to be more debilitating than the pain itself (Fredheim et al., 2008) (Meyer-Rosberg et al., 2001). Similar behavioural change is documented in other chronic conditions in a proportion of patients as discussed in section 1.2. A common feature of many of these conditions is ongoing inflammatory stress.

As detailed in section 1.6, using the Chronic Constriction Injury (CCI) model of nerve injury, Monassi *et al.,* have demonstrated that a sub-group of rats display persistent behavioral change following constriction of the sciatic nerve – an injury which both inflicts mechanical stress and provokes an inflammatory response. Importantly, this behavioural change occurs while pain thresholds are demonstrated to be no different to rats where behaviour is unaltered (Monassi et al., 2003).

### *3.2 Aim*

To assess the effect of Chronic Constriction Injury, using the resident-intruder test as a measure of complex social behaviour in the rat.

### *3.3 Materials and Methods*

A total of 64 male Sprague-Dawley rats, used in the experiments reported in Chapters 4 and 6 (Group A and Group B repectively), were tested for behavioural response to an age and sex-matched intruder to the home cage. The experimental paradigm for these rats is described in section 2.4.1: all these rats were subjected to Experimental Paradigm 1, that is, all rats in Groups A and B were catheterised.

In addition, 10 rats used as behavioural controls in the experiment reported in Chapter 6 were tested using the same resident-intruder instrument. A description of these rats is given in section 2.4.3.4.

The method used for resident-intruder testing is described in section 2.4.3. Briefly, for 5 days pre-CCI and 6 days post-CCI, interactions between the rats were video-recorded for analysis of behavioural responses of the resident to the intruder.

Using the derived criteria for behaviour classification given in section 1.6.3.2, resident behaviour was scored as detailed in section 2.5.1. Thus, for each rat, a baseline (pre-injury) score and daily post-injury scores for all behaviours Dominance (D), Social (S), Non-social (NS) and Submissive (SM)) were determined.

Experimental rats were placed in a behavioural category using the criteria detailed in section 2.5.2. Thus, based on the pattern of dominance post-injury relative to the pre-injury baseline, rats were classified as No Disability (ND), Transient Disability (TD) or Persistent Disability (PD).

As described in section 2.4.4.4, the right sciatic nerves of the injured rats were examined post mortem.

## *3.4 Results*

### *3.4.1 General observations*

After both the jugular catheterisation and CCI procedures all rats included in the analysis exhibited normal grooming, nesting and exploratory behaviour. All rats ate and drank freely and gained weight over the course of the procedure. A total of 5 rats were excluded from analysis due to incomplete or unstable pre-CCI data, injury while fighting or unstable haematocrit. Results are given for the remaining 59 rats. Where appropriate, the results for the total group (n=59) will be given as well as for the two sub-groups (Group A; n=31 and Group B; n=28).

### *3.4.2 Resident behaviour pre-CCI*

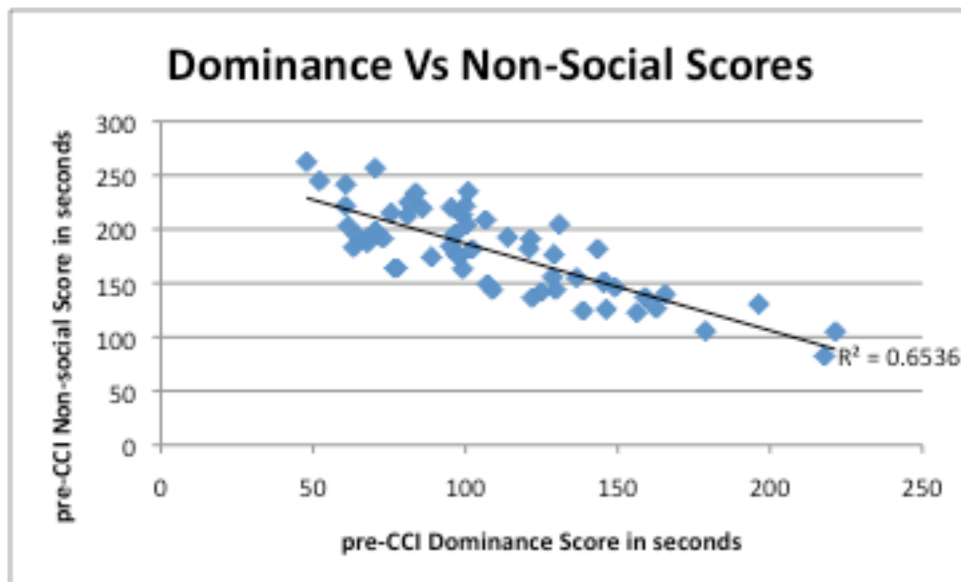
As previously discussed, the resident-intruder paradigm is predicated on the resident animal 'learning' the paradigm. For this reason the first two days of interaction, while recorded and analysed, were used only to confirm that each animal had indeed 'learned'. Consistent with previous findings, over these first 2 days the rats spent more time engaged in social (sniffing of the intruder partner) and non-social activities (self-grooming, cage exploration). Typically the amount of dominance behaviour (aggressive grooming of the intruder, chasing, fighting) exhibited by the resident animals increased over the first 3 days. By day 3, dominance had generally peaked and through days 3-5 pre-CCI the dominance behaviour remained stable. This pattern of development of dominance was consistent with previously reported studies (Monassi et al., 2003) (Sosa, 2008).

#### *3.4.2.1 Behaviour scores pre-CCI*

Resident behavioural response to an intruder varied markedly between animals. Pre-injury Dominance scores ranged from 48-221 seconds (mean 109.7 ± 5.2) in a 360 second testing period. Thus there is a wide spectrum of pre-injury Dominance behaviour evident. Similarly, Non-social pre-injury scores ranged from 82-262 seconds (mean 179.0 ± 5.2) and there was a direct relationship between these two measurements. As shown in Figure 3.1, rats with high pre-injury Dominance had low pre-injury Non-social scores (Pearson Correlation Coefficient 0.808, $p<0.001$). The range in Social behaviour exhibited pre-injury was 21-114 seconds (mean 64.9 ± 3.1) and for Submissive behaviour 0-32 seconds (mean 6.0 ± 0.9). There was no relationship between these behaviours and pre-injury dominance.

The mean pre-injury D scores did not vary between the experimental groups nor did these scores vary from that of the total group: the mean Dominance score for group A was 107.1±7.5 seconds and for group B 112.5±7.4 seconds ($p>0.05$). There was more than two years between the behavioural testing of the two experimental groups. There were differences between the two groups for mean pre-injury NS scores: the mean score for group A was 167.4±6.7 seconds

**a)**




**b)**

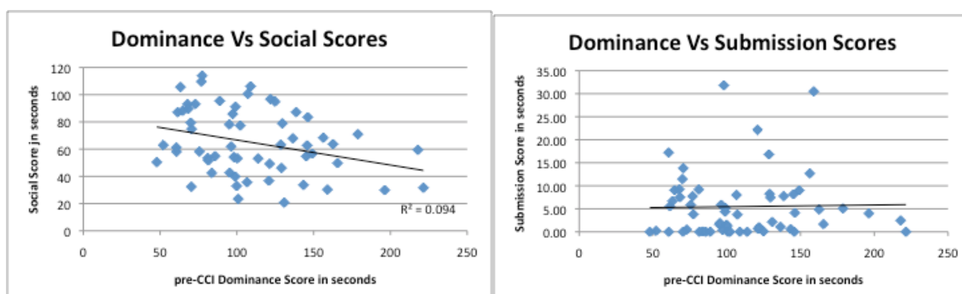



**Figure 3.1:** a) Relationship between Dominance (D) and Non Social (NS) scored behaviours pre-CCI in 59 rats. Pearson Correlation Coefficient 0.808 $p<0.001$.
b) There is no relationship between D and Social (S) scores or between D and Submissive scores (n=59).

compared to 191.8±7.6 seconds for group B ($p<0.05$), however neither group differed in mean pre-injury NS scores from the total group. A possible recent shift, in the population of rats, to higher pre-injury Non Social behaviour will be discussed in section 3.4.8.

#### *3.4.2.2 Intruder behaviour*

There was evident difference in the behaviour of intruder animals and the response of any one resident animal varied depending on the behaviour of the intruder. While many intruders submitted to the dominance behaviours of the resident, others responded with defensive behaviours leading to fighting. The behaviour of the residents with lower dominance was of interest when faced with a more reactive intruder. Residents clearly change strategies of dominating behaviours depending on the response of the intruder but the drive to dominate the stranger remains. When both rats are dominant animals there can be a great deal of fighting behaviour, with some wins going to the intruder and hence submissive scores can also be quite high. When the intruder is an overly passive animal, dominance scores may be lower because there is no fighting. Despite this variability, D scores for any one rat remained remarkably stable in the three days pre-CCI. Rotation of intruder animals assured that residents were exposed to a range of intruder dominance.

### *3.4.3 Resident Behaviour Post-CCI*

#### *3.4.3.1 Spontaneous Pain Behaviours*

Following CCI all rats exhibited behaviours which are typical of the spontaneous pain behaviours described by Monassi *et al.,* and by Bennett and Xie in the original description of the CCI injury as a model for neuropathic pain (Monassi et al., 2003) (Bennett and Xie, 1988). All rats had a pronounced limp, weight bearing minimally on the right hind leg when moving around the cage. While standing or sitting, the leg was held off the ground in a 'guarded' position and rats could be observed shaking and licking the leg and interrupting other behaviours to focus attention to the leg even after obvious healing of the wound. These behaviours were still evident 6 days post-CCI. There was no observable difference between rats in these spontaneous pain behaviours. Numerous studies from our laboratory have now confirmed the finding that, post-CCI, all rats demonstrate

evidence of allodynia and hyperalgesia in response to sensory testing and cannot be distinguished by using sensory testing alone (Monassi et al., 2003) (Sosa, 2008,) (Mor et al., 2010) (Austin et al., 2010). Thus, while sensory testing was not part of the protocol for these rats, based on spontaneous pain behaviours post-CCI it is reasonable to assume that these rats show evidence of ‘pain’ following CCI.

#### *3.4.3.2 Social Behaviours*

When resident-intruder testing was continued on the day after CCI, rats resumed social interactions, including vigorous dominating behaviours, despite obvious limping. While most rats show some decrease in D on the first days post-CCI, most return to pre-injury levels of D by the third day post-injury. However, a percentage of rats continue to show decreased D (and increased NS) behaviour and the duration and pattern of this decrease in D behaviour is the basis of the behavioural classification previously described.

#### *3.4.3.3 Behavioural classification of resident animals*

Behavioural scores for each rat were determined for each of the four behaviours for each day post-CCI. Based on the derived criteria outlined in section 2.5.2, rats were behaviourally categorised. The results of this classification for all the resident rats and for each of the two experimental groups are given in the following table.

| | No Disability ND | Transient Disability TD | Persistent Disability PD |
|---|---|---|---|
| Total Rats (n=59) | N=41 69.5% | N=6 10.2% | N=12 20.3% |
| Group A (Chapter 4) (n=31) | N=21 67.7% | N=4 12.9% | N=6 19.4% |
| Group B (Chapter 6) (n=28) | N=20 71.4% | N=2 7.1% | N=6 21.4% |

**Table 3.1:** Percentage of rats classified as No Disability (ND), Transient Disability (TD) and Persistent Disability (PD) in the total experimental rat group and in each of the sub-groups A and B.

The percentage in each group was consistent with those previously reported although the percentage of rats with disability was lower than previously reported (PD, 30-32%; TD 17-25%) (Monassi et al., 2003) (Sosa, 2008) (Mor et al., 2010).

### *3.4.4 Pre-CCI scores for Behavioural Groups*

The pre-injury behavioural scores for the groups defined by post-CCI behavioural response were determined. For the total experimental group (n=59), the mean time scored (±SEM) for each of the four behaviours (D, S, N S and SM) for the rats subsequently classified as No Disability, Transient Disability or Persistent Disability are shown in Figure 3.2(a) (ND, n=41, TD, n=6: PD, n=12). There is a significant difference between the ND group and the PD group for both Dominance (p=0.014) and for Non Social (p=0.019) behaviours but no difference for Social or Submissive scores. There is no difference between the ND and TD groups for any behavioural scores.

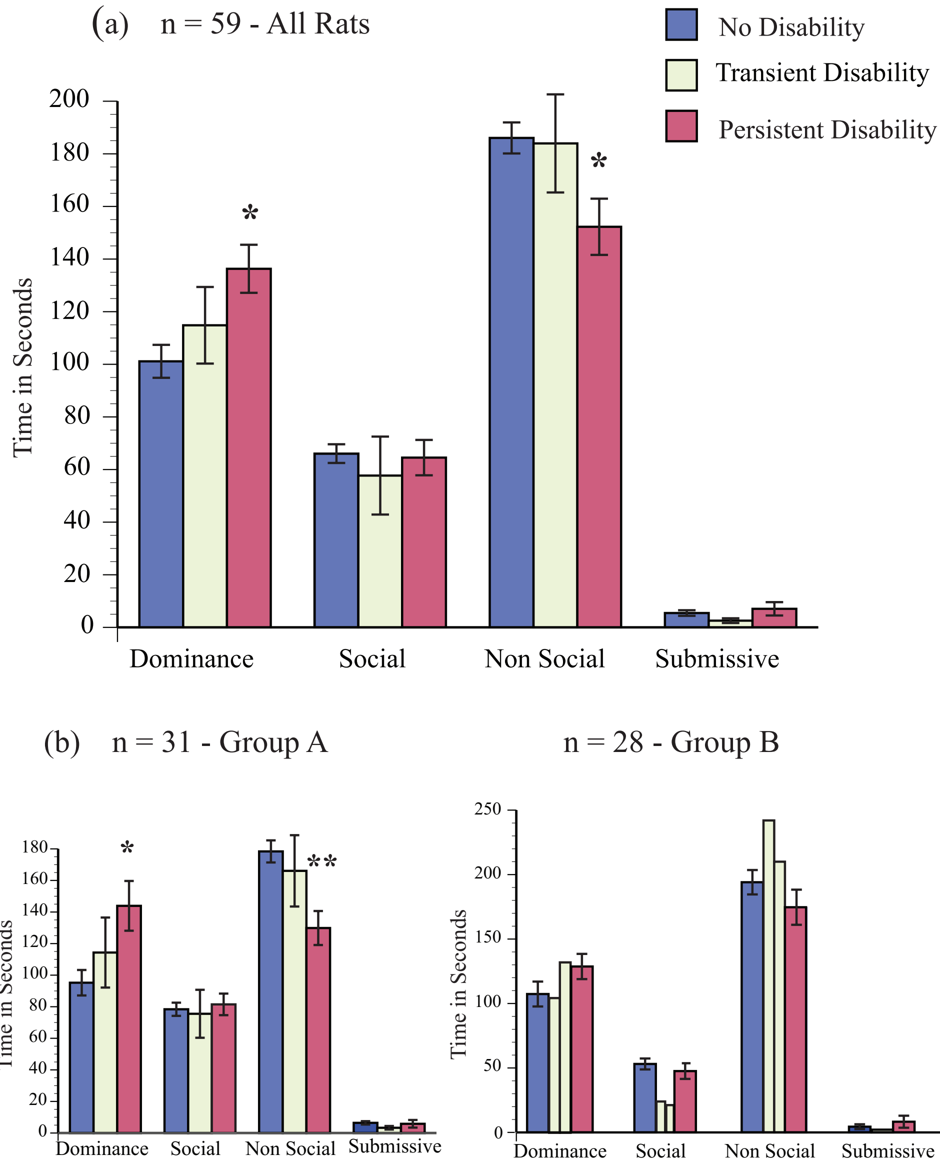



**Figure 3.2:** Histograms illustrating mean pre-injury levels (± SEM) of behaviour (Dominance, Social, Non Social and Submissive) in animals classified as No Disability (ND), Transient Disability (TD) and Persistent Disability (PD). (a) Pre-injury levels of behaviour for all experimental rats (n=59) and (b) for the two experimental groups n=31(Group A) and n=28 (Group B) respectively. Changes that are significantly different to the ND group are indicated: **p<0.01 and *p<0.05.

The pre-injury behavioural scores for each of the two experimental groups of rats are shown in Figure 3.2 (b). In Group A (n=31: ND=21, TD=4, PD=6) significant differences between the ND and PD groups for D (p=0.020) and NS (p=0.007) behaviours were also found. For Group B (n=28: ND=20, TD=2, PD=6) there were no significant differences found between the ND and PD groups for any behaviour pre-CCI. The differences found for pre-CCI D and NS behaviours between the ND and PD group are not consistent with previously reported results (Monassi et al., 2003) (Sosa, 2008) (Mor et al., 2010) (Austin et al., 2010).

The differences found in the total group of rats are due to the differences found in Group A (n=31). One PD rat (n=6) had a D score of 219 seconds (mean D for all rats in this sub-group was 107.1±7.5). When this rat was removed from the analysis, the PD group was not significantly different from the ND group for D scores (p=0.125) but still significantly different for NS scores (p=0.04). With this rat removed from the analysis, there were no differences between behavioural groups for any of the pre-injury behaviours for the total group of rats (n=59). Another possible explanation for the higher D scores (and therefore lower NS scores) in the PD rats in Group A, relates to the blood sampling procedure. Some rats had samples taken during the light phase (3 hours after lights 'on'). The mean D score for the rats sampled during the light was 122.9±12.7 seconds (n=12) and in the dark 97.1±18.7 seconds (n=19): the corresponding NS mean scores were 154.0±10.3 seconds and 175.9±8.3 seconds. The differences between the light and dark phase sampling groups are not significant but as the light phase group contributed 4/6 animals to the PD group but only 7/21 to the ND group, this factor may have contributed to the differences seen.

### *3.4.5 Post-CCI scores for behavioural groups*

#### *3.4.5.1 Dominance Behaviour*

The mean post-CCI behavioural scores were grouped for days 1-3 and 4-6 post-injury and the mean raw scores (±SEM) for the total rats (n=59) are shown in Figure 3.3(a). The D score for days 1-3 post-CCI for the PD group fell to 50% of the pre-CCI score but was not significantly different to the ND group whereas the mean D score for days 4-6 for the PD rats is significantly different to the ND

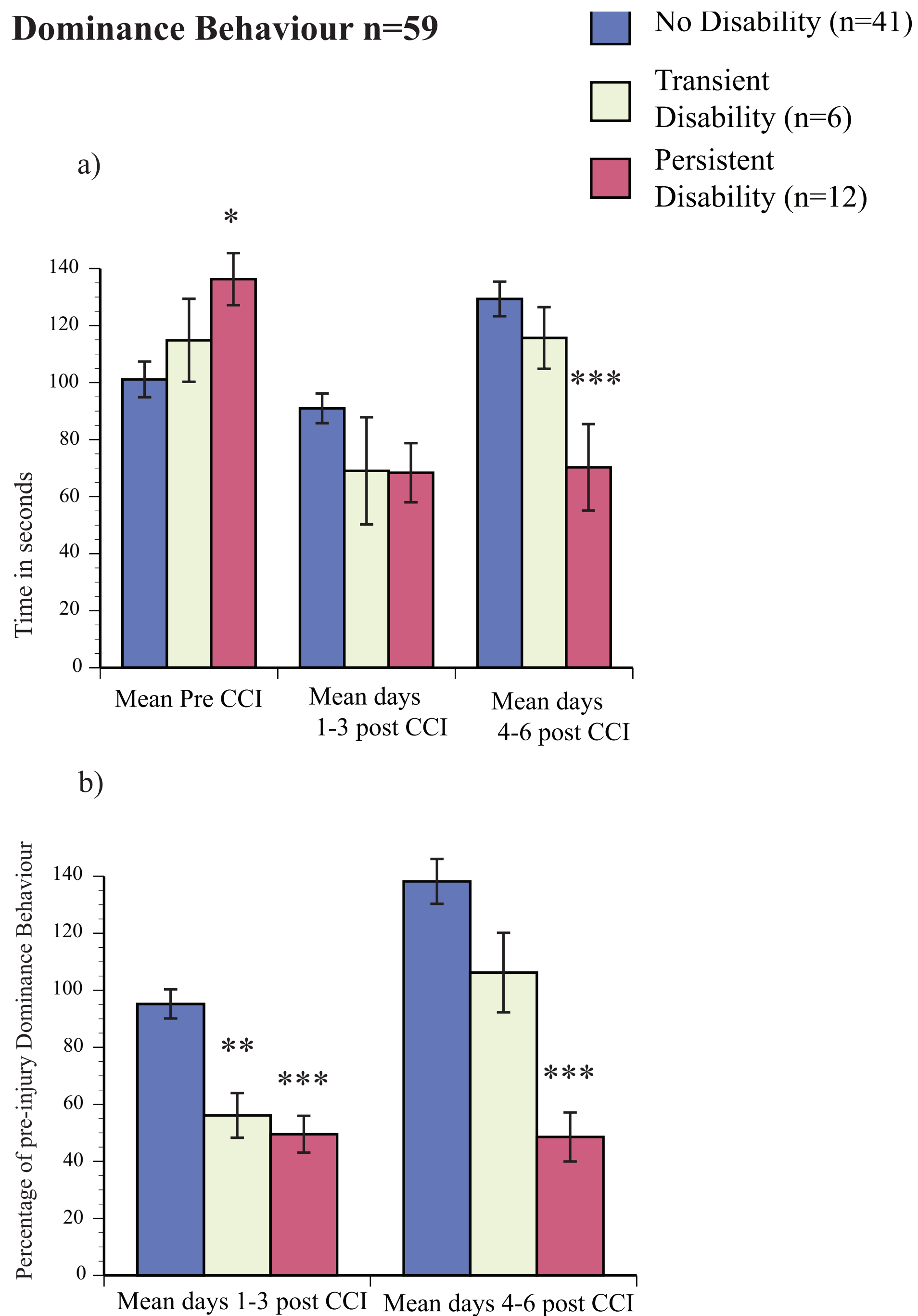



**Figure 3.3:** Histograms illustrating mean levels of Dominance (D) in experimental rats (n=59) classified as No Disability (ND), Transient Disability (TD) and Persistent Disability (PD). a) Mean pre-injury scores in seconds compared to mean scores days 1-3 and 4-6 post injury. b) The mean change in Dominance behaviour relative to pre-injury levels is shown for days 1-3 and 4-6 post-CCI. Changes that are significantly different to the ND group are indicated: ***p<0.001, **p<0.01 and * p<0.05 (Independent t-test).

group ($p<0.001$). Similarly, the TD group does not differ from the ND group on days 1-3 or days 4-6 post-CCI using mean D scores.

Figure 3.3(b) presents the post-injury Dominance behaviour relative to pre-injury levels (n=59). Compared to rats with ND, PD rats show a reduction in Dominance behaviour relative to their pre-injury levels on days 1-3 ($p<0.001$) and on days 4-6 ($p<0.001$). Thus the change in behaviour persists. In contrast, the TD rats, while showing a fall in Dominance on days 1-3 ($p=0.008$), show recovery and a similar pattern to the ND rats on days 4-6.

When the post-injury D scores relative to the pre-injury scores are examined for the two sub-groups, differences consistent with the total population are seen. Compared to ND rats, PD rats show a reduction in D on days 1-3 post-CCI ($p<0.001$ and $p=0.017$ for groups A and B respectively) and on days 4-6 post-CCI ($p=0.003$ and $p<0.001$ respectively).

#### *3.4.5.2 Non Social Behaviour*

Since there is a correlation between time spent in Dominance behaviour and Non Social behaviour, Non Social scores were also examined. Figure 3.4 (a) shows the mean scores for days 1-3 and 4-6 post CCI compared to pre-CCI scores. For the PD rats, the mean NS score increased but was not different to the ND group on days 1-3 but was different on days 4-6 post-CCI ($p<0.001$). The mean NS scores for the TD group differed from the ND group on days 1-3 post-CCI ($p=0.017$) but not on days 4-6 post-CCI.

Figure 3.4(b) presents the relative change in NS behaviour after injury (n=59); PD rats show significant increase relative to ND rats on days 1-3 ($p<0.001$) and 4-6 ($p<0.001$) post-injury while TD rats show an increase relative to ND rats on days 1-3 only ($p=0.026$). The same pattern of increase in NS behaviour in PD rats when compared to ND rats is shown in the two experimental groups (1-3 days post-injury $p<0.001$, group A; days 4-6 post-injury $p<0.001$ and $p<0.001$ groups A and B respectively).

Thus the rats with decreased Dominance show an increase in Non Social behaviour following injury. This pattern of shift from time engaged in Dominance to Non Social behaviour in the group identified as PD, is consistent

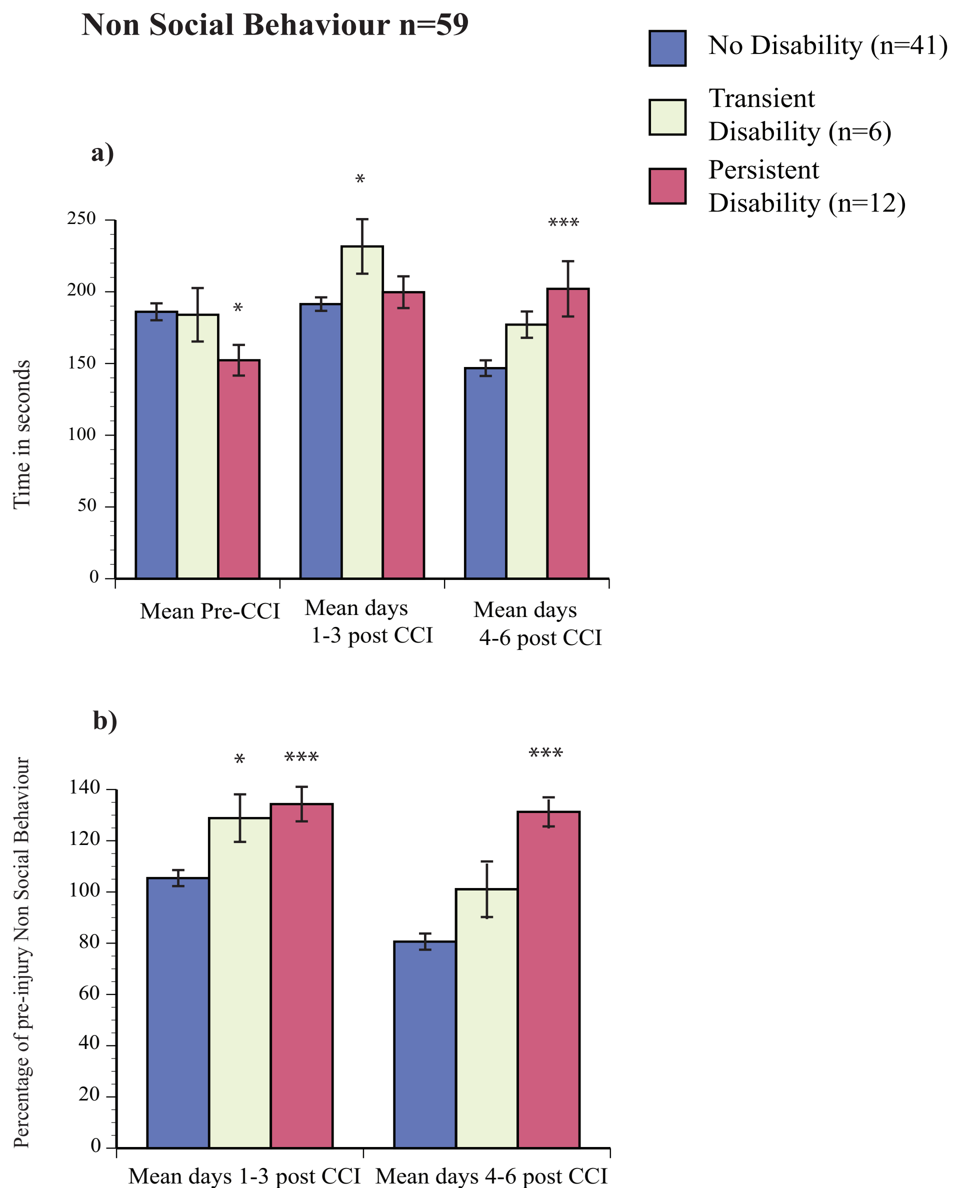



**Figure 3.4:** Histograms illustrating mean levels of Non Social (NS) behaviour in experimental rats (n=59) classified as No Disability (ND), Transient Disability (TD), and Persistent Disability (PD). a) Mean pre-injury scores in seconds compared to mean scores days 1-3 and 4-6 post-injury. b) The mean change in Non Social behaviour relative to pre-injury levels is shown for days 1-3 and 4-6 post-CCI. Changes that are significantly different to the ND group are indicated: ***p<0.001 and *p<0.05 (Independent t-test).

with previously reported results (Monassi et al., 2003) (Austin et al., 2010) (Mor et al., 2010).

##### *3.4.5.3 Other social behaviours*

There were no differences between the behavioural groups for pre and post-CCI Social and Submissive behaviours or for post-injury relative to pre-injury scores for S and SM behaviours.

### *3.4.6 Sciatic Nerve Injury Site*

The right sciatic nerves of all injured rats were examined post-mortem. In all rats the four chromic ligatures were confirmed to be still in place. All nerves showed evidence of inflammatory change; the nerve in the area of ligation was swollen to at least twice the diameter of the nerve proximal to the injury. The area of ligation and distal portions of the nerves, including the area of trifurcation, was encapsulated in a mass of connective tissue. The appearance of the nerves was consistent with the description given by Bennett and Xie (Bennett and Xie, 1988). Figure 3.5 shows some examples of nerves taken from rats from the three behavioural groups. There were no gross anatomical differences between nerves from rats in the different behavioural groups.

### *3.4.7 Behavioural control rats*

The 10 rats were behaviourally tested as per the experimental rats; on the day after establishing baseline behaviours they experienced a period of anaesthesia in lieu of sciatic nerve injury as described in section 2.4.3.4.

Development of Dominance behaviour occurred over the first three days of testing and in days 3-5 pre-intervention the D behaviour stabilised as for the experimental animals. The mean baseline behavioural scores for these rats were similar to those for the experimental rats reported in section 3.4.2.1. Mean D scores were 101.3±10.7 seconds, S scores 39.3±3.2 seconds, NS scores 194.3±13.4 seconds and SM scores 23.5±6.5 seconds.

Post-intervention, behavioural scores remained stable across days 1-3 and days 4-6 post-intervention as shown in Figure 3.6.

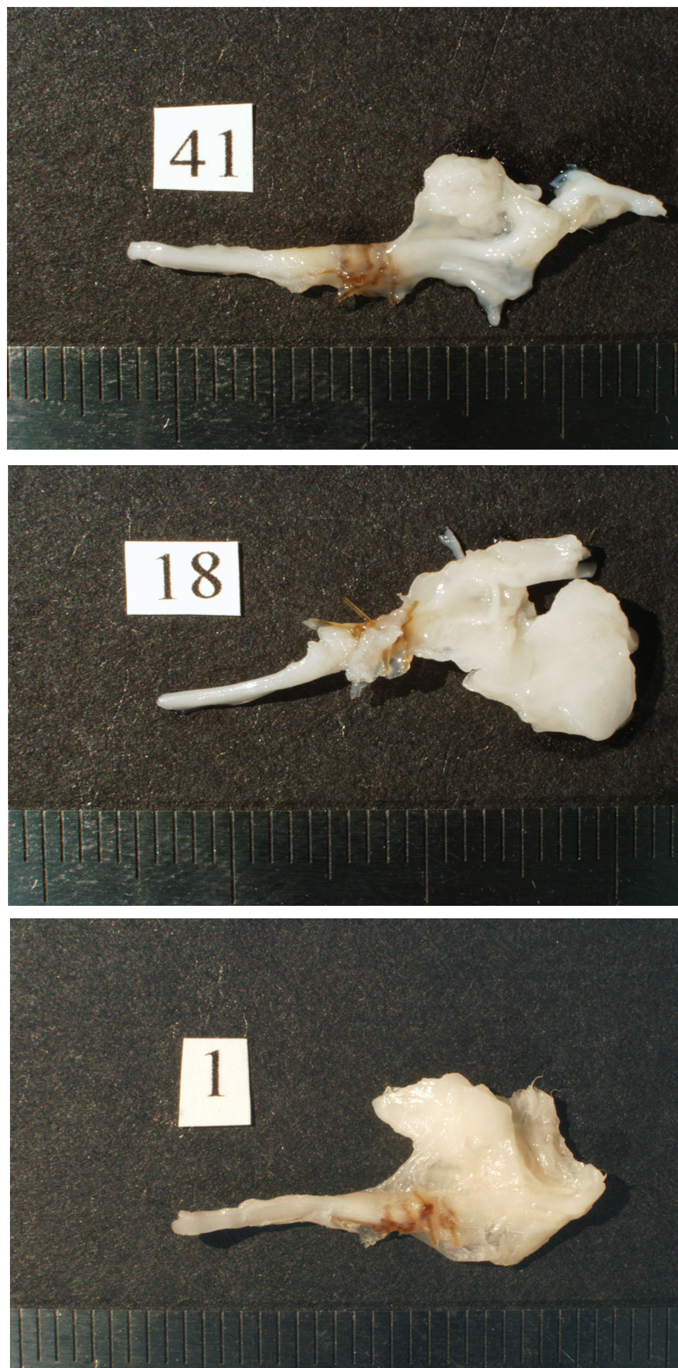



**Figure 3.5:** Representative examples of right sciatic nerves removed post-mortem from experimental rats. In each image, the portion of the nerve proximal to the injury (on the left), the area of the ligation with chromic gut ligatures in position and the inflammed tissue mass involving the distal porrtion of the nerve is seen, Nerves from a rats cassified as No Disability rat (No, 41), Transient Disability (No. 18) and Persistent Disability (No,1).

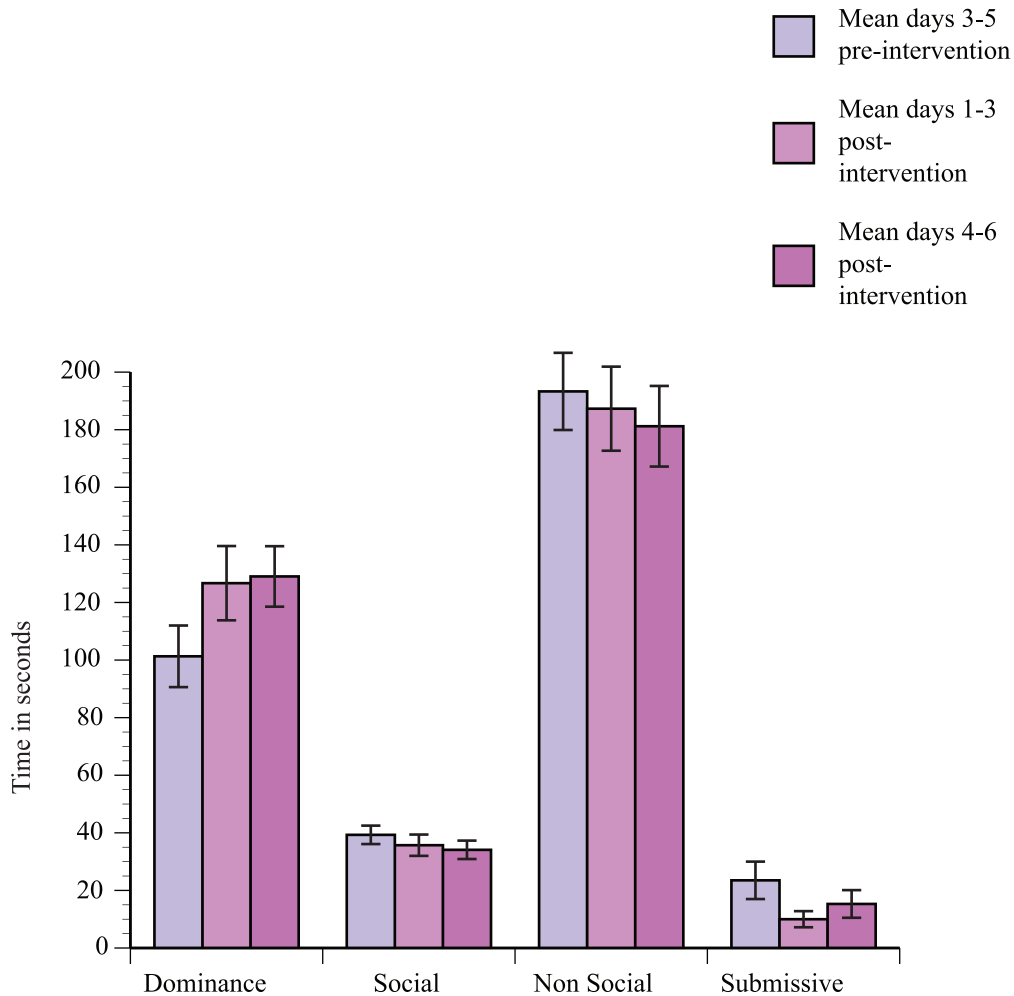



**Figure 3.6:** Histogram illustrating the mean scores in seconds (+/-SEM) for each behavioural category (Dominance, Social, Non Social and Submissive) in 10 rats used as Behavioural Controls for the immunohistochemical studies (Chapter 6). Mean pre-intervention (anaesthesia) as well as post-intervention scores on days 1-3 and 4-6 are shown. There are no differences between pre and post levels for any of the behaviours. ($p>0.05$) Independent samples t-test).

### 3.4.8 *Behavioural Scores over years*

The behavioural scores reported in this Chapter were obtained over a number of years. In results to be presented in Chapter 5, brain tissue was stored for varying periods of time. An examination of baseline behavioural scores for all the groups of animals is therefore of interest since the passage of time could introduce additional variables.

**Mean Pre-injury/intervention Scores in Seconds (±SEM)**

| | Year Tested | D Score | S score | NS score | SM score |
|---|---|---|---|---|---|
| Gp. A (n=31) (Chapter 4) | 2006 - 2007 | 107.1 (±7.5) | 78.6 (±3.5) | 167.4 (±6.7) | 5.9 (±0.9) |
| Gp. B (n=28) (Chapter 6) | 2009 | 112.5 (±7.4) | 49.7 (±3.6) | 191.8 (±7.6) | 5.0 (±2.5) |
| BC (n=10) (Chapter 6) | 2010 | 101.3 (±10.7) | 39.3 (±3.2) | 194.3 (±13.4) | 23.47 (±6.5) |
| BC (n=12) (Chapter 5) | 2009 | 99.9 (±12.6) | 52.6 (±5.5) | 203.7 (±14.1) | 3.2 (±1.8) |
| Gp. C (n=24) (Chapter 5) | 2004 - 2005 | 136.5 (±6.7) | 88.0 (±6.1) | 128.4 (±6.5) | 6.2 (±1.0) |

**Table 3.2:** Mean baseline behavioural scores (in seconds) for all groups of experimental (Group A, B and C) and behavioural control rats (BC) for which data is presented in this thesis. There is a trend over time for deceasing Dominance (D) and Social (S) scores and increasing Non Social (NS) scores.

The mean baseline scores for rats tested using the resident-intruder paradigm from 2004 to 2010 (as shown in Table 3.2) show evidence of a shift in scores over this time. There has been a decrease in D and S scores and an increase in NS scores. The scores for the rats reported in Section 3.4.7, used as behavioural controls for the animals tested in 2009 (Group B, Chapter 6), do not differ in mean baseline scores from this group. However, this is not true for the rats used as control animals for Chapter 5. These behavioural controls (tested in 2009) are markedly different in pre-injury/intervention behavioural scores from the 2004-2005 rats (Group C, Chapter 5). The significance of this finding for the results documented in Chapter 5 will be addressed in that chapter.

## 3.5 Discussion

Using resident-intruder testing as a measure of complex social behaviour in the rat, this study confirmed previous findings that, following a mechanical and inflammatory stress, chronic constriction injury of the sciatic nerve, a sub-group of animals exhibits evidence of social disability. That is, the well documented drive to exhibit dominance over a stranger introduced to the living space is diminished as evidenced by a decreased amount of time engaged in the behaviours that characterize a resident rat (Blanchard et al., 1975) (Blanchard et al., 1977). As the behaviours of each animal are compared to its own established pre-injury baselines, they are not subject to the normal variations in dominance found between male rats. Blanchard *et al.,* have documented the dominant behaviours toward a stranger of both an alpha male and a subordinate cage-mate: both animals exhibit all the characteristic behaviours of over-the–top, lateral attack, chasing and biting. The difference lies only in the total amount of time each rat spends engaged in these interactions and the mix of the dominating activities. Thus alpha males spend more total time interacting with the stranger and relatively more time in chasing, biting and attacking behaviours than the subordinate cage-mate (Blanchard et al., 1975). The wide variation in D scores shown in this study and the variation in strategies displayed by the resident rats are consistent with these findings.

Moreover, in each of the sub groups, to be used in separate experiments, the identified socially disabled rats are similar to the total group in terms of changed behaviours and percentage of effected animals.

### *3.5.1 Baseline behavioural scores*

Pre-injury dominance was not a predictor for the development of social disability. There was in fact a difference found between the pre-injury D (and NS) scores for these experimental animals with the animals that developed disability having higher pre-injury dominance. This finding was not consistent with the results of previous studies from our laboratory, which found no difference in pre-injury scores for the different behavioural groups (Monassi et al., 2003) (Sosa, 2008) (Austin et al., 2010) (Mor et al., 2010). Data related to possible reasons for this difference were presented. Removal of one animal from the PD group resulted in this difference disappearing. This animal however was not an outlier, as there were other rats, included in the ND group, with similar D scores. The small number of PD rats (n=6) compared to the number of ND rats (n=21) allows for one individual in the group to distort the mean values. Time of blood sampling, again because of the small size of the PD group, may also have contributed to this finding. There is no statistical difference between D scores for rats sampled in the light compared to those sampled in the dark, however the scores tend to be higher for those sampled in the light. There were relatively more rats sampled in the light in the PD group, although any attempt to explain why time of sampling would influence D scores would be purely speculative.

However, the pre-CCI difference in D in the PD rats was in the opposite direction from the effect. That is, the animals subsequently classified as having a decrease in dominance and social disability had higher pre-injury dominance scores. The pre-injury differences found in fact do not detract from the finding that the rats identified using the derived behavioural classification, changed their social behaviour.

### *3.5.2 Post-injury behaviour*

What is most surprising about the post-injury behaviour in the CCI model is the continued dominant behaviour of most of the rats. Despite obvious limping and

other spontaneous pain behaviours they continue to vigorously exert dominance over the stranger in the cage.

What is distinctive in the behaviour of the identified sub-group is that they appear to accept defeat, that is, they decrease their attempts to dominate as well as the actual time spent in dominance type behaviour. The shift to time spent in non-social activities is consistent with the correlation between these two types of behaviour across the spectrum of pre-injury dominance. They become less dominant animals. This is contrary to what has been shown, that the dominance behaviours of rats, once established, remain constant over a long period of time (Blanchard and Blanchard, 1990) (Monassi et al., 2003).

This decrease in dominance is not related to the behaviour of the intruder animals. Intruder animals do not display the dominance behaviours characteristic of resident rats. Any fighting behaviours are defensive in nature. The behaviour of intruder rats was not a focus of this study but unless provoked, usually by bites from the resident, intruder rats spend the majority of their time in non-social activity. This does not change when the resident is a less dominant animal (Blanchard et al., 1978) (Blanchard and Blanchard, 1990).

### *3.5.3 Reproducibility of CCI model*

In most respects, the results obtained with the rats tested for these studies were consistent with behavioural results reported by others in our laboratory using the same methodology (Monassi et al., 2003) (Mor et al., 2010) (Austin et al., 2010). Other than the difference in pre-injury behaviour discussed above, the results concurred with others except in the percentage of animals identified as Persistent Disability animals. Although in the same range, there were only 20% of PD rat in this study compared with 30-32% as reported by others from our laboratory. While the behavioural methodology was the same, these rats experienced an additional surgical procedure, catheterisation of the external jugular vein. This procedure of itself carries some risk for the rat. Additionally, if at any time during the two weeks of the procedure the catheter is displaced, the rat must be euthanased. It is possible that these factors disproportionally affected the rats susceptible to behavioural disability.

The rats in this study displayed the same spontaneous pain behaviours reported by others and gross pathology of the sciatic nerves post-mortem did not differ from other reports. Sensory testing in numerous studies has produced the same finding, that the behavioural groups exhibit the same degree of 'pain' (Monassi et al., 2003) (Sosa, 2008) (Austin et al., 2010) (Mor et al., 2010). It is therefore reasonable to assume that the sub-group identified as PD rats would likely display the other aspects of disability previously identified; change in sleep-wake patterns and endocrine disturbances (Monassi et al.,2003) (Keay et al., 2004) (Sosa, 2008).

### *3.5.4 Other models of inflammatory stress*

There are not many examples of measurements of behavioural change in animal models following inflammatory injury. In most of the models that have examined changes in thyroid hormones and their regulation, the rodents have been extremely sick and die within days (Fekete et al., 2004) (Boelen et al., 2004) or behavioural aspects, other than altered gait indicating discomfort, are not considered in the study (Boelen et al., 2006). To date, there are no other animal models reporting differential behaviour post-injury, that is, identification of a sub-group that responds differently to injury/inflammatory stress.

### *3.5.5 Variation in the rat population over time*

Despite the wide range of dominance scores in resident rats, the mean D scores for the experimental group (n=59) and the two sub-groups A and B are remarkably consistent. However, as shown in Table 3.2, there appears to have been a change in baseline behaviour when mean scores over 6 years are examined. In particular there has been a decrease in D and S scores and an increase in NS scores. While most of the rats were sourced from WA, when there was a problem with supply, rats were obtained from UTS. This, of itself, does not explain the trend. It is possible that there has been a change in the population over time due to out-breeding of the colonies. If so, this variable is outside the researcher's control. However it does not detract from the primary finding that some animals, having established baseline dominance behaviour, show a decrease in this dominance after injury. The majority of rats maintain their pre-injury dominance despite injury.

## *3.6 Summary*

Following injury to the sciatic nerve, a sub-group of animals was identified in which there was a change in complex social behaviours when compared to the majority of rats. The group identified is similar to that identified by others using this model in which it has been shown that the pain levels do not differ between groups identified by behavioural change This model provides an opportunity to examine the activity of the HPT axis under conditions of uncontrollable stress where a sub-group demonstrates changes consistent with disability.

# CHAPTER FOUR

# Peripheral Thyroid and Corticosterone Hormones Following Injury

## 4.1 Introduction

Altered peripheral thyroid hormones have been shown in response to uncontrollable physical stressors in animal models as previously discussed (Boelen et al., 2004) (Helmreich et al., 2006). The decrease in peripheral thyroid hormones without a compensatory increase in their regulatory factor, TSH, suggests strongly a re-setting of the accepted negative feedback relationship between thyroid hormones and TSH.

Elevated corticosteroids have been implicated in the altered hypothalamo-pituitary-thyroid (HPT) axis activity seen in various stress models but this is not uniformly reported and may be dependent on the specific stress model (Helmreich et al., 2005) (Kondo et al., 1997).

Following Chronic Constriction Injury (CCI) of the sciatic nerve a subgroup of rats are unable to cope with the uncontrollable and inescapable stress of peripheral nerve injury, and display changes in social behaviours or disability. Individuals with behavioural and affective changes have been shown to have disturbed HPT axis function while those with disturbed thyroid indices are known to exhibit mood and behavioural change (Gold et al., 1981) (Esposito et al., 1997) (Davis et al., 2003).

## 4.2 Aim

The aim of these experiments was to determine the effects of an uncontrollable physical stressor, an inflammatory mechanical nerve injury, on peripheral thyroid hormones and to correlate these measures with the expression of behavioural disability following nerve injury. In addition, the effect of this stressor on peripheral corticosterone levels was determined.

## 4.3 Materials and Methods

Thirty-four rats were subjected to Experimental Paradigm 1 as described in section 2.4.1. In brief, after external jugular vein catheterisation and recovery, pre-injury behaviour for 5 days was determined as outlined in section 2.4.3. Plasma was collected as described in section 2.4.2 on days 4 and 5 pre-injury. CCI was performed on all rats. The day following CCI, behavioural testing recommenced and continued for 6 days: blood sampling resumed on day 2 after surgery and continued daily till day 6. Plasma was collected from in-dwelling jugular catheters as described in section 2.4.2 and stored at - 20$^{o}$C until assayed.

The behaviour of the rats was categorised and rats were behaviourally classified as described in sections 2.51 and 2.52. Three rats were excluded from the analysis based on criteria for inclusion of data (section 2.6). Of the remaining 31 rats, the peripheral hormones were measured in 21; all the animals categorised as Persistent Disability (PD) (n=6) or Transient Disability (TD) (n=4) were tested together with 11 No Disability (ND) rats chosen to include rats from both sampling times (that is, 1 hour prior to and 3-5 hours after lights ON).

The TT4, TT3, fT4, fT3, TSH and corticosterone hormone levels were determined using radioimmunoassay: the specific the kits used are detailed in section 2.6. In brief: all assay kits were suitable for use with heparinised samples; all samples were measured in duplicate and all samples from any one rat were determined within the one assay.

## 4.4 Results

The following experimental results have been published in the journal article cited below. A copy of this article can be found in the Appendix:

Kilburn-Watt E, Banati R and Keay KA, Altered Thyroid Hormones and Behavioural Change in a Sub-population of Rats following Chronic Constriction Injury, *Journal of Neuroendocrinology,* 2010; **22**(8): 960-70.

### *4.4.1 Behaviour*

The results of behavioural analysis for the 21 rats used for hormonal analysis are shown in Figure 4.1. Figure 4.1(a) illustrates the mean time scored for each behaviour (Dominance, Social, Non Social and Submissive) for the 3 groups of rats pre-injury (ND, n = 11; TD, n = 4; PD, n = 6). The results show no difference between groups prior to CCI in D, S and SM behaviours and a difference for NS behaviour between ND and PD groups. Figure 4.1(b) shows the change in Dominance for the three groups relative to pre-CCI levels. Compared to rats with ND, PD rats show a reduction in Dominance behaviour relative to their pre-injury levels on days 1-3 (p=0.001) and on days 4-6 (p=0.007). Thus the change in behaviour persists. In contrast, the TD rats, while showing a fall in Dominance on days 1-3, show recovery and a similar pattern to the ND rats on days 4-6. Further, the PD rats have a significant increase (compared to the ND group) in Non Social behaviour relative to pre injury levels on days 1-3 (p=0.001) and 4-6 (p=0.006) (not shown). This pattern of shift from time engaged in Dominance to Non Social behaviour in the group identified as PD, is consistent with our previously reported results (Monassi et al., 2003).

### *4.4.2 Thyroid Hormones: Pre-CCI*

There was a difference in the pre-CCI mean TSH levels between rats sampled in the "dark" (1 hour before lights on (11 rats)) and those sampled in the "light" (3-5 hours after lights on (10 rats)) (3.11+/- 0.27 versus 5.07+/- 0.66 ng/ml, p=0.018). This was expected since the diurnal TSH peak in Sprague-Dawley rats has been shown to occur at 4-6 hours after lights on (Campos-Barros et al., 1997). However, there were no differences between the two sampling times in the pre-CCI mean levels for T4, fT4, T3 and fT3 (p>0.05). As each rat was sampled at the same time each day and acted as its own control, the rats were analysed as one group.

### *4.4.3 Thyroid Hormones: Post-CCI:*

Pre-CCI and post-CCI mean thyroid hormone levels were calculated for each rat and each behavioural group. The mean changes in hormone levels from pre to

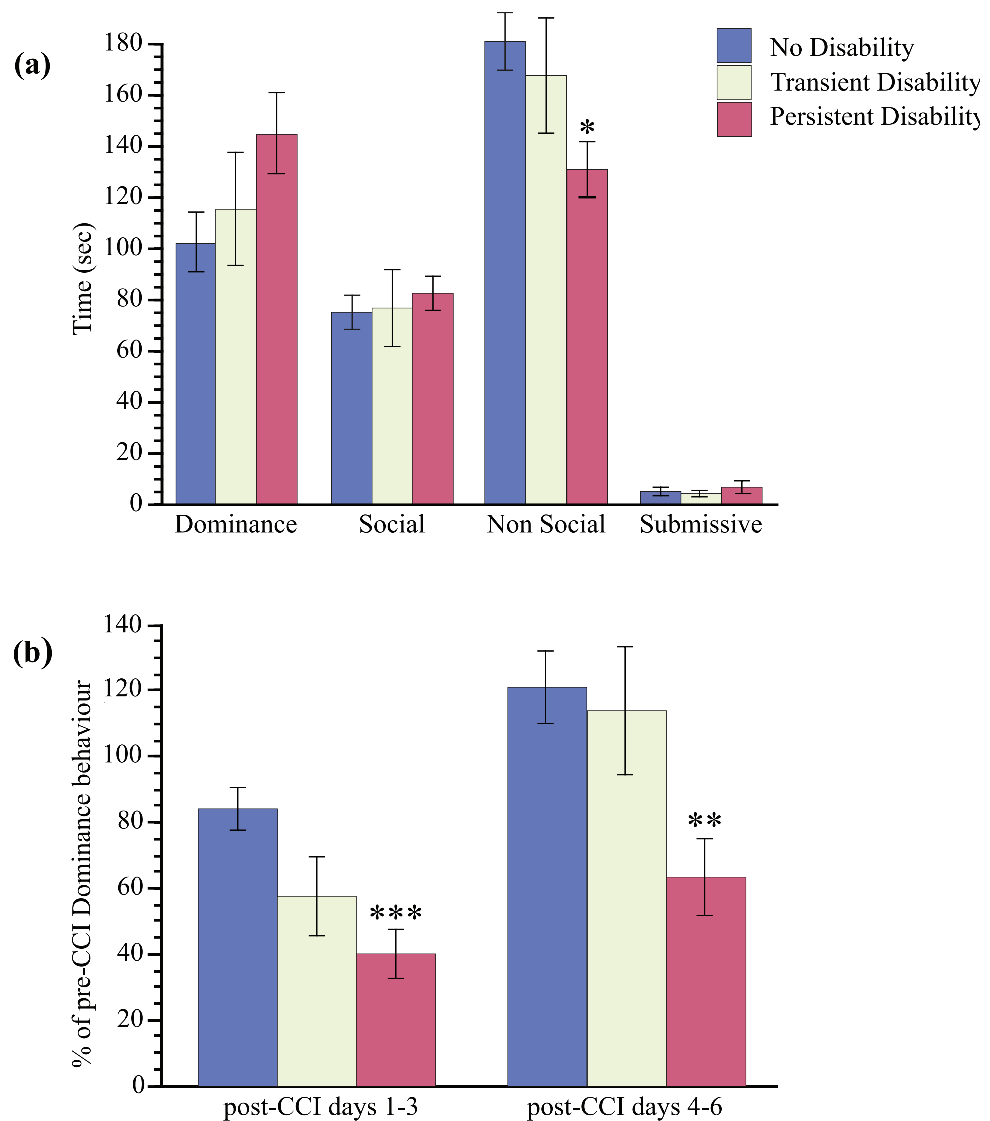



**Figure 4.1:** (a) Bar graphs illustrating mean pre-Chronic Constriction Injury (CCI) levels (+/- SEM) of behaviour (Dominance, Social, Non Social and Submissive) in animals classified as No Disability (ND), Transient Disability (TD) and Persistent Disability (PD) for 21 rats where hormone levels were measured. Difference between groups was found for Non Social behaviour only.
(b) The mean change in Dominance behaviour relative to pre-CCI levels (+/- SEM) is shown for days 1-3 and 4-6 post-CCI for 21 rats. One-way ANOVA followed by Dunnett t-tests shows the decrease in Dominance for the PD group to be different to the ND group on both days 1-3 and 4-6 whereas the TD group differed from the ND group on days 1-3 only. Values that are significantly different to the ND group are indicated: ***p=0.001, **p<0.01.and *p<0.05.

post-CCI were compared. A graphical representation of the mean changes in hormones for the three behavioural groups is shown in Figure 4.2.

In PD rats, T4 fell significantly following CCI compared to both ND and TD rats (PD *vs*. ND $p<0.001$; PD *vs*. TD $p= 0.001$; one-way ANOVA, post hoc comparison using the Dunnett t-tests). The mean fall in T4 for the PD rats was 8.5±1.12 ng/ml (~20% from pre-CCI levels). However, T4 levels in both ND and TD rats were unchanged following CCI and did not differ from each other (Figure 4.2a).

In PD rats, fT4 also fell significantly compared to the ND and TD rats (PD *vs.* ND $p=0.002$; PD *vs.* TD $p=0.01$). The mean fall in fT4 for the PD rats was 2.4±0.40 pg/ml or 10% from pre-CCI levels. The fT4 levels did not change for the ND and TD rats and did not differ from each other (Figure 4.2b).

The mean fall in T3 for the PD rats was 10.6±0.94 ng/dl or 14%. The mean change was again significantly different to that in ND and TD rats (PD *vs.* ND $p=0.001$; PD *vs*. TD $p= 0.001$) whereas the ND and TD groups did not differ from each other (Figure 4.2c).

The fall in mean fT3 for the PD rats was greater than for the ND rats (0.38± 0.10 *vs*. 0.13± 0.07 pg/ml)) but the differences were not significant.

There was no difference between the change in mean TSH for the ND and PD rats (0.38±0.33 *vs.* 0.11±0.45 ng/ml) ($p>0.05$). Figure 4.2d illustrates the change in TSH for the three groups of rats.

### *4.4.4 Post CCI hormones relative to baseline*

The daily levels of hormones relative to pre-injury baselines were determined and shown in Figure 4.3. Hormone levels relative to pre-CCI levels (+/- SEM) are shown for 10 ND rats in Figure 4.3a. The relative levels for TSH are shown for the total group as well as for the different sampling times. Rat # 20 (clearly shown as an outlier for T4 in Figure 4.2a), was identified as a ND rat in the initial analyses based on the behavioural criteria (i.e., decreased Dominance on 2 or

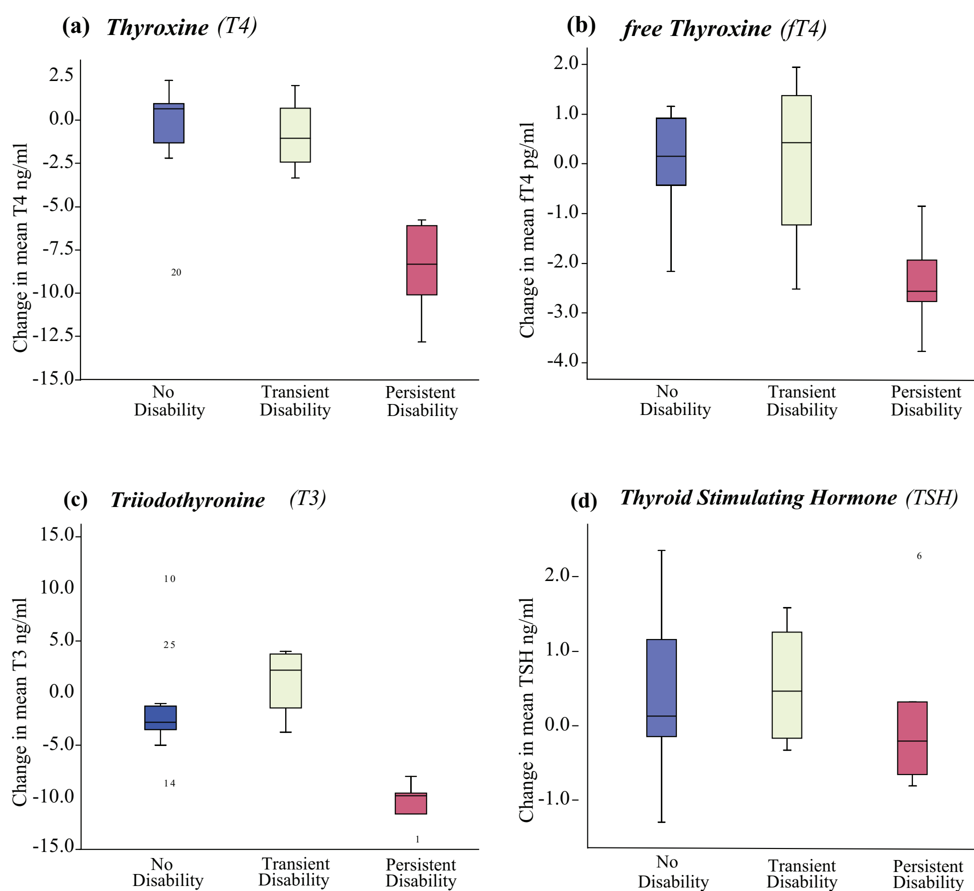



**Figure 4.2:** Graphical representation of changes in mean thyroid hormones following Chronic Constriction Injury (CCI) for the three behavioural groups a) thyroxine (T4), b) free thyroxine (fT4), c) triiodothyronine (T3) and d) TSH. One way ANOVA followed by Dunnett t-tests show the changes in T4, fT4 and T3 for the Persistent Disability group to be different to the No Disability group $p<0.001$, $p=0.002$ and $p=0.001$ respectively. There is no difference between the groups for change in TSH. Statistical outliers are shown using animal identification numbers.

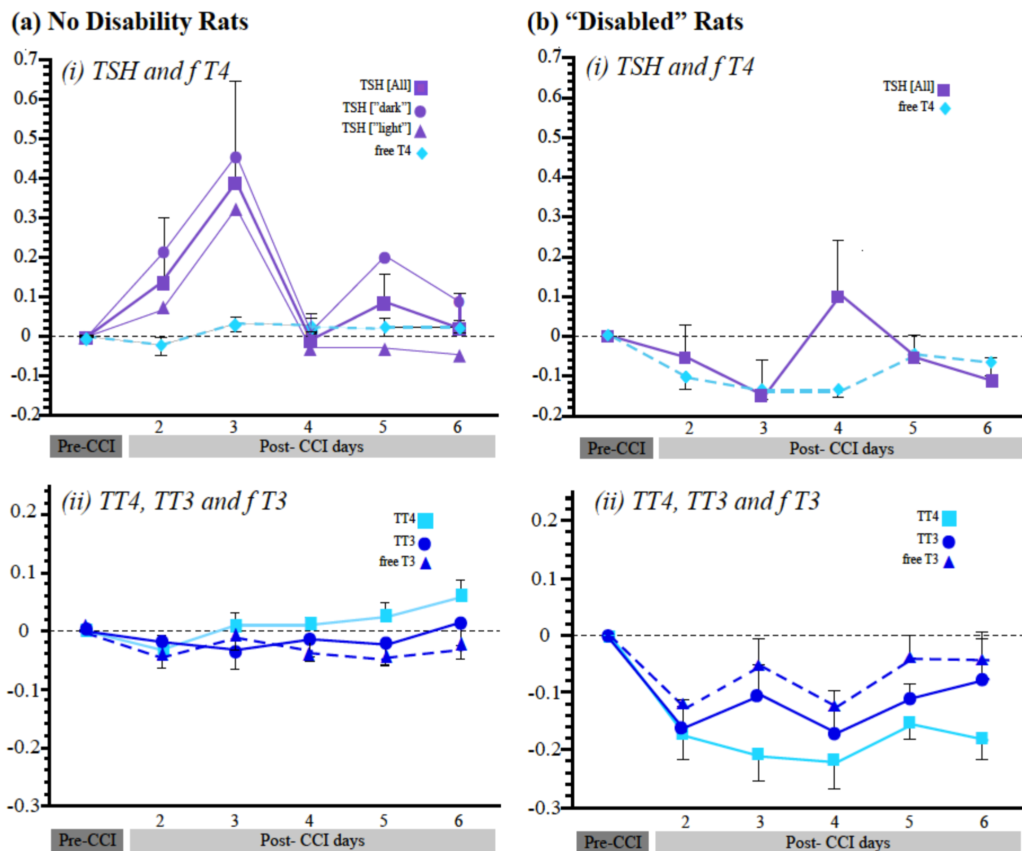



**Figure 4.3:** (a) Mean hormone levels (+/– SEM) relative to baseline on days 2, 3, 4, 5 and 6 post-Chronic Constriction Injury (CCI) in No Disability rats: (i) TSH and free thyroxine (fT4) and (ii) thyroxine (T4), triiodothyronine (T3) and free triiodothyronine (fT3). TSH values for all animals (10) and the two different sampling times (5 in each group) are shown (animal #20 was excluded).
(b) Mean hormone levels (+/– SEM) relative to baseline on days 2, 3, 4, 5 and 6 post-Chronic Constriction Injury (CCI) in Persistent Disability rats (excluding animal #6): (i) TSH and free thyroxine (fT4) and (ii) thyroxine (T4), triiodothyronine (T3) and free triiodothyronine (fT3).

fewer days relative to pre-CCI levels). The behaviour of this rat was however unusual in that the decreased dominance occurred on days 5 and 6 post-CCI. This rat was removed from the ND group for this and further analyses.

Hormone levels relative to pre-CCI levels (+/- SEM) are shown for 5 PD rats in Figure 4.3b. The data for rat #6 was excluded here, as the change in TSH was more than 4 SD outside the mean for the rest of the group.

### *4.4.5 Thyroid Hormones and Behaviour*

Correlation coefficients were determined to examine the relationship between the change in mean Dominance behaviour and the changes in mean hormone levels for the rats classified as ND and PD. Significant relationships were found for T4 and fT4. The Pearson Correlation for change in mean Dominance and mean T4 was 0.616 (p=0.009) and for mean fT4 was 0.710 (p=0.001). There was no significant correlation between change in Dominance behaviour and changes in T3, fT3 or TSH. Graphical representations of the relationship between change in Dominance and change in T4 and fT4 are shown in Figure 4.4.

### *4.4.6 Corticosterone*

There were no differences in the pre-CCI mean corticosterone levels between rats sampled in the “dark” (1 hour before lights on (11 rats)) and those sampled in the “light” (3-5 hours after lights on (10 rats)). Both sampling times lie within the nadir of plasma corticosterone levels (Kalsbeek et al., 1996). The mean corticosterone levels for each behavioural group are shown in Figure 4.5. Post-CCI, all groups of rats had increased corticosterone levels, which peaked between days 2 and 3 post-injury. (Note, some animals also had a second peak on day 6). In TD rats the corticosterone peak levels were significantly higher when compared to both ND and PD rats (TD *vs.* ND p=0.039; TD *vs*. PD p= 0.032; one-way ANOVA, post hoc comparison using the Dunnett t-tests). The mean peak value for the TD rats was 162.3± 32.6 ng/ml, for the ND rats 59.6±25.6 ng/ml and for the PD rats 45.7± 6.0 ng/ml. The increase in mean corticosterone post-CCI for the TD rats was 60.7±14.3 ng/ml, for the ND rats 21.7±12.0 ng/ml and for the PD rats 14.0± 2.5 ng/ml, however these differences were not significant. The

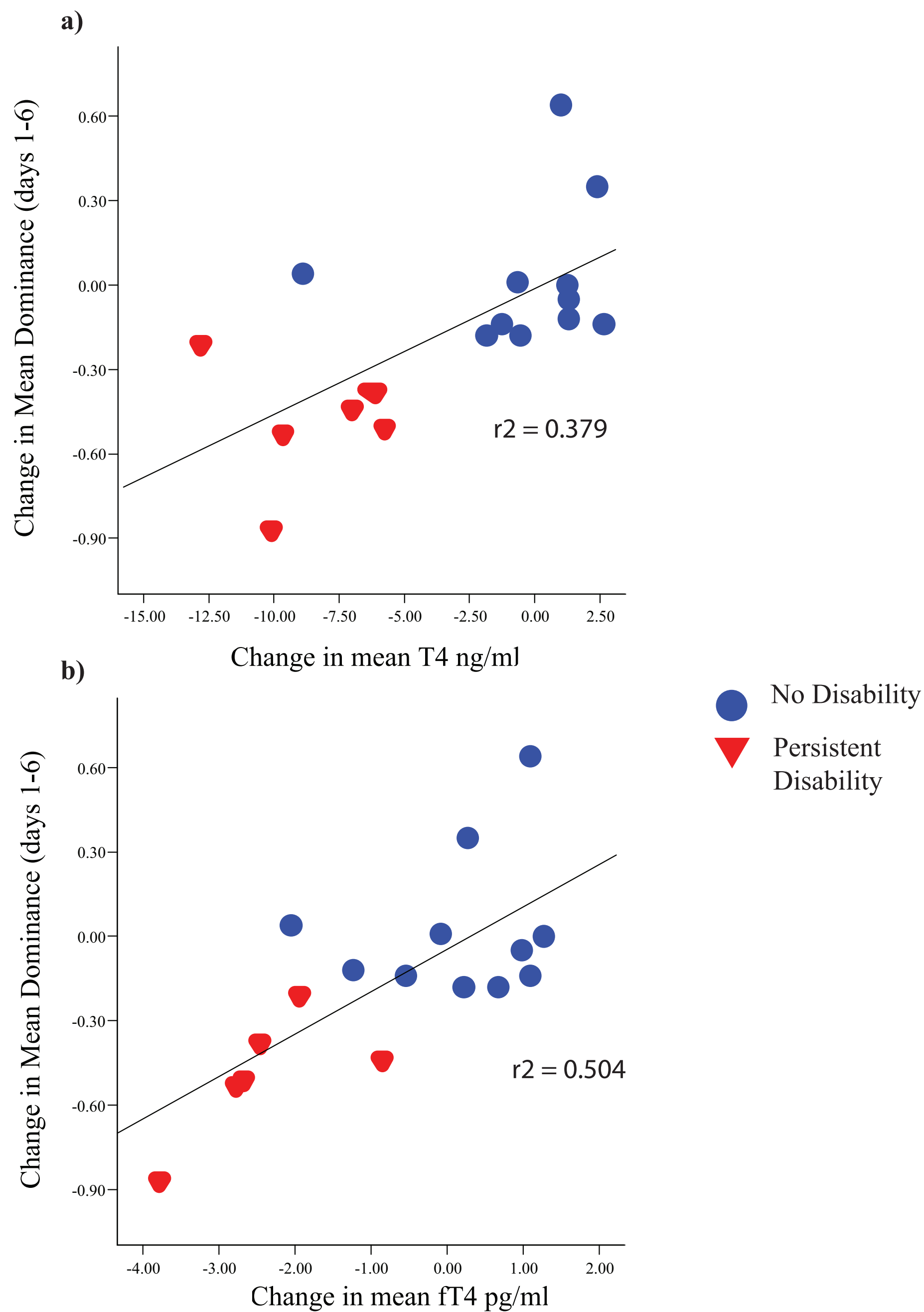



**Figure 4.4:** Correlation between change in mean Dominance (D) post-Chronic Constriction Injury (CCI) and change in a) mean thyroxine (T4) and b) mean free thyroxine (fT4) in No Disability (n=11) and Persistent Disability (n=6) rats. Pearson Correlation Coefficients were a) 0.616, p=0.009 and b) 0.710, p=0.001.

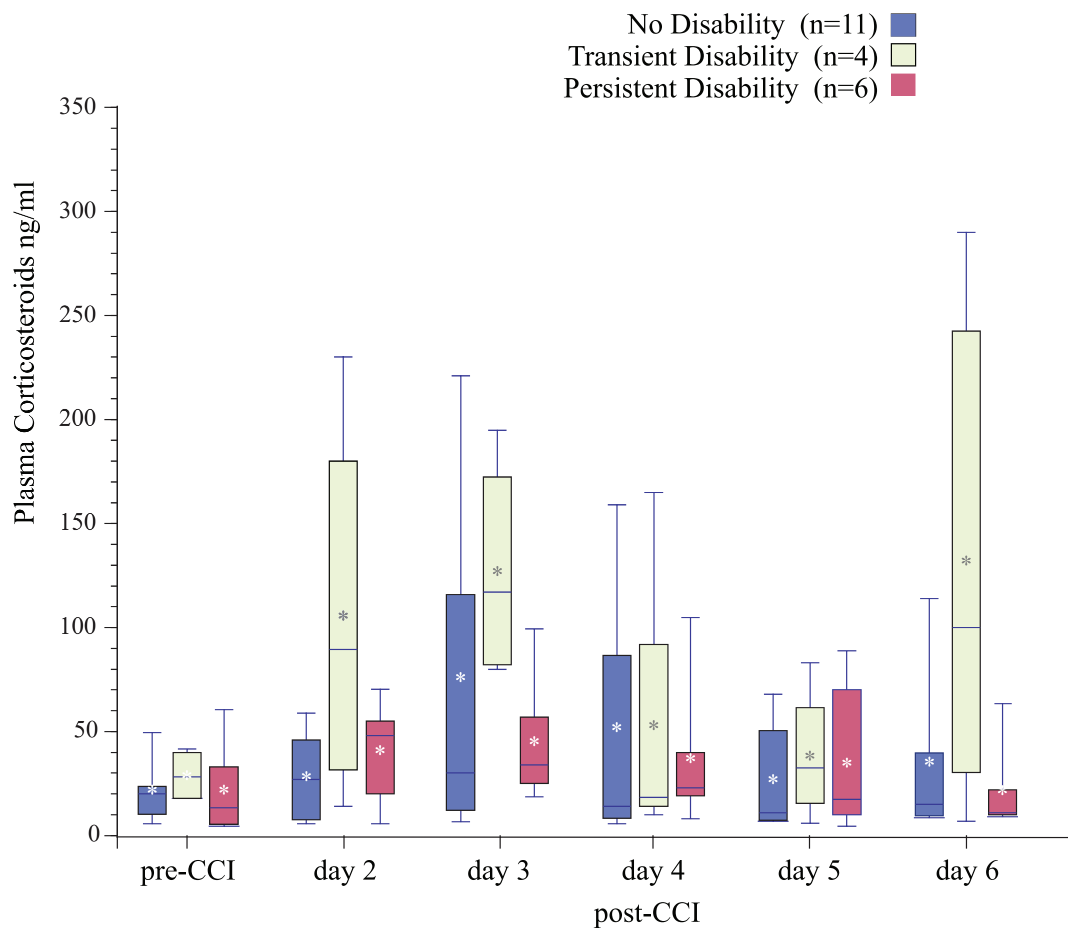



**Figure 4.5:** Box-plot showing mean corticosterone levels (asterisks) relative to baseline on post-Chronic Constriction Injury (CCI) days 2 to 6 in rats with No Disability (ND) (n=11), Transient Disability(TD) (n=4) and Persistent Disability (PD) (n=6). There were no differences between groups for increase in mean corticosterone post-CCI; the peak corticosterone levels post-CCI for the TD group were significantly higher than the ND group (p=0.039) and the PD group (p=0.032) (One-way ANOVA, post-hoc comparison using the Dunnett's t-test).

increases in plasma corticosterone did not correlate with changes in plasma TSH. (Pearsons correlation $p<0.05$).

## *4.5 Discussion*

The aim of this study was to investigate thyroid hormones in a subgroup of rats that exhibit behavioural change following chronic constriction injury of the sciatic nerve. This peripheral nerve injury, through both mechanical and inflammatory mechanisms, provokes uncontrollable and inescapable stress for the rats. The findings were that while there was no difference between rats prior to injury, in the six days following CCI, the subgroup with persistent behavioural change showed a significant decrease in T4, fT4 and T3 when compared to the group with unchanged behaviour. TSH did not rise in these rats as would be expected and there was a significant correlation between changed Dominance behaviour and decreases in both T4 and fT4. In addition, the TD rats showed a greater and more prolonged corticosterone response following CCI. The animals selected for hormone measurement did not differ in behavioural profiles from the total group of rats (see Figures 3.2, 3.3 and 3.4).

### *4.5.1 Changes to the HPT equilibrium following nerve injury*

A role for T3 in tissue repair has been suggested on the basis of a general increase in metabolism and the induction of specific proteins involved in the repair process (Anderson et al., 1998) (Safer et al., 2005). Following injury to the sciatic nerve in the rat, increased deiodinase 2 and deiodinase 3 mRNA expression and activity, as well as induced expression of thyroid hormone receptors have been reported (Li et al., 2001a) (Li et al., 2001b). Type II deiodinase is responsible for the conversion of T4 to T3 in the central nervous system, but is not normally present in adult rat peripheral nerves, whereas type III deiodinase is responsible for the metabolism of T3 and T4 and hence important in control of tissue T3 levels (Courtin et al., 2005) (Kohrle, 2002).

Expression of these enzymes following injury would suggest an increased tissue demand for T3. Increased utilisation of T3 should initiate mechanisms, which would result in the maintenance of plasma T3 levels. There should be a shift in

the equilibrium of protein bound and freeT3 and an increased conversion of T4 to T3. Also, if T3, fT4 and T4 levels all fall, increased release of TSH from the pituitary is expected in turn, leading to stimulation of the thyroid gland, restoration of hormone levels and maintenance of these levels during the period of increased metabolic need.

There are differences in secretory patterns between the ND rats and the rats in which thyroid hormones decreased as shown in Figure 4.3. Although there was no change in mean hormones for the ND group (Figure 4.2), the pattern of secretion following CCI reflects what might be expected if, as suggested, there is an increased tissue requirement for T3 after the nerve injury. While free and bound thyroid hormones remain steady, there is a TSH response, peaking on day 2 or 3 post-injury suggesting that there has been a disturbance in the equilibrium (Figure 4.3).

Restoration of thyroid hormone equilibrium did not occur in the rats where behaviour changed. In 5 PD rats with decreased thyroid hormones, the falls in hormone levels were greater before a TSH response was initiated, when compared to ND rats (Figure 4.3). The response occurred later and the amplitude of the response was lower. The peripheral hormone levels were not restored to pre-injury levels and after six days, the mean TSH level in these rats was below pre-injury level (Figure 4.3).

The variance in TSH values for the PD rats, is accounted for by a single outlier (rat #6). This rat showed the expected increase in TSH following a fall in peripheral hormones but fT4, T4, fT3 and T3 levels all remained depressed relative to pre-injury levels. The lack of response suggests primary thyroid failure in this rat; the decrease in fT4 was the largest of all rats with the falls in T4 and T3 among the largest; the change in behaviour was profound with post injury dominance decreased to 13% of pre injury levels.

As discussed in section 2.6, the measurement of fT4 in heparinised samples is of concern in some clinical situations (Stockigt, 2001). However, the concern is that, because heparin can cause interference with T4 binding to TBG, higher than

warranted fT4 results are obtained. It was hypothesised that as heparinised saline was used to keep the catheters open before and after injury and in all rats, a shift in the protein binding of thyroxine should be controlled for by the fact that each animal was its own control. The results do not show any increase over the course of the study in fT4 levels in the ND rats, moreover there was a significant decrease in fT4 is the PD rats. Any change in fT4 levels due to heparin have either not occurred, as suggested, or change in TBG-T4 binding occurred prior to the pre-CCI sampling. It seems therefore that the heparin has not influenced the fT4 results here in that there was no change for the ND rat while the fT4 levels for the PD rats fell significantly.

### *4.5.2 Altered hypothalamic regulation of TSH in the PD rats*

The inadequate and blunted TSH response in the sub-group of rats with changed behaviour would suggest a change in the central regulation of TSH, a change in the 'thyrostat', resulting in a relative hypothyroid state existing post-injury. While it could be argued that these hormone levels would still be within wide 'normal' limits, it has been shown in humans that intra-individual fluctuations in thyroid hormones are small (Andersen et al., 2002). In view of this, it is argued that a fall in thyroid hormone levels within the normal range could well be significantly abnormal for the individual and hence have systemic and neural effects (Danzi et al., 2005). A fuller discussion of possible mechanisms that could be involved in altered hypothalamic regulation in the PD rats will be given in Chapter 7.

### *4.5.3 Decreased T4 and changed Behaviour*

The majority of the T3 produced in the cerebral cortex of the rat is derived from local conversion of T4 to T3 due to type II deiodinase activity (Crantz et al., 1982) (van Doorn et al., 1983). Estimates of T4 contribution to T3 found in the cortex by these authors, using double radioisotope labelling techniques, varied between 60 and 75% with only 20-25% contributed by plasma T3. Thus the level of T3 in the cortex depends primarily on the circulating levels of T4 and to a lesser extent T3. In humans, a correlation between psychological well-being and fT4, but not fT3, has been demonstrated in patients on thyroid hormone replacement (Saravanan et al., 2006).

The PD rats, with peripheral decreases in T3 as well as fT4 and T4 could well have decreased delivery of both thyroid hormones to the brain which in turn could alter neural function and offer an explanation for altered behaviour. However, despite the clinical evidence for the effect of decreased thyroid hormones on behaviour, the mechanisms that result in this dysfunction are unclear. The tissue levels of T3 in different regions of the rat brain have been shown to be maintained despite changes in peripheral hormones. This is thought due to an auto-regulatory mechanism whereby peripheral T4 regulates the activity of the deiodinase 2 responsible for converting T4 to T3 (Broedel et al., 2003). Disruption of this mechanism however has been shown to occur under various situations of stress, in sleep deprivation and calorie restriction as well as following pharmaceuticals which inhibit norepinephrine and serotonin reuptake mechanisms (Baumgartner et al., 1998) (Eravci et al., 2000). Broedel *et al.,* suggest the possible role of immunological factors, as occur in Graves' disease, in disturbing the fundamental auto-regulatory mechanism, which is clearly protective of the brain under conditions of altered peripheral thyroid hormone levels (Broedel et al., 2003).

### *4.5.4 The role of Corticosterone*

Suppression of peripheral thyroid hormones has been documented in many instances of elevated corticosteroid; both exogenous and endogenous (Visser and Lamberts, 1981) (Coiro et al., 2002) (Alkemade et al., 2005) (Greenspan and Gardner, 2001). It seemed possible therefore that there could be a relationship between decreased TSH levels and increased corticosterone levels in the PD rats. However the results do not support this view. The corticosterone response in the PD rats is not different to the ND rats and there is no correlation between decreased TSH and increased corticosterone. While exogenous and pathological levels of corticosteroids are associated with suppressed TSH, Samuels concluded that the "specific nature of the interactions between cortisol and TSH within the physiological range remains to be fully elucidated"(Samuels, 2000). Moreover, Kondo and colleagues demonstrated that the suppression of TRH and TSH mRNA levels following LPS in rats was independent of the LPS induced increase in corticosteroid (Kondo et al., 1997).

While increased levels of corticosterone were demonstrated in both ND and PD groups, the response was in fact the lowest in the PD group although levels were not significantly lower in the PD rats when compared to the ND rats. It is perhaps a limitation however that corticosterone levels were not measured on the day immediately following CCI. These results support the previous finding by Sosa (2008) that, corticosteroid levels in the two groups were not significantly different. However Sosa (2008) also demonstrated that the PD group had decreased ACTH levels when compared to the ND group. Moreover, relative to ND and control rats, the PD group showed evidence of altered hypothalamic regulation, with decreased numbers of both glucocorticoid receptor (GR) and corticotrophin-releasing hormone (CRH) factor immunoreactive cells in the paraventricular nucleus of the hypothalamus (Sosa, 2008).

## *4.6 Summary*

Compared to rats that coped with the stress of CCI, the sub-group of rats characterized by changed social behaviour had decreased peripheral thyroid hormones post-injury. The decreased thyroid hormones were associated with a blunted TSH response, which suggests altered central regulation of these hormones. There was a significant correlation between changed Dominance behaviour and decreases in both T4 and fT4 for the ND and PD rats.

Increased corticosterone levels post injury were greatest in the rats with Transient Disability and do not explain the suppressed TSH levels in the PD rats.
Since decrease in fT4 should stimulate TSH release, suppression of the hypothalamus as shown in other animal models of inflammatory stress seems more likely (Boelen et el., 2004) (Fekete et al., 2004).

# *CHAPTER FIVE*

# *Evidence for Altered Hypothalamic Regulation – Relative Gene Expression of Components of the HPT and HPA Axes Following Injury*

## *5.1 Introduction*

When peripheral thyroid hormones fall, in particular free thyroxine, the expected homeostatic response is an increase in TSH secretion to stimulate the thyroid gland and restore and maintain hormone levels. This negative feedback relationship is known to directly involve the pituitary gland but the hypothalamus is now recognised as the regulator which establishes the set point of TSH secretion (Aron et al., 2001) (Chiamolera and Wondisford, 2009).

As presented in Chapter 4, following injury, the pattern of thyroid hormone levels in the rats classified as ND suggests a change in thyroid homeostasis and the expected response to such change. The response in the rats classified as PD however suggests that, in this sub-group, the set point for TSH has been down regulated. In addition, changes in corticosterone secretion following injury implicate the HPA axis in the post-injury response.

As previously discussed, animal models of stress have demonstrated changes in gene and/or protein expression in molecules involved in regulation of both the HPA and HPT axes. In models of inflammatory stress, changes in the enzymes responsible for metabolising thyroid hormones as well as inflammatory cytokines have been reported (Boelen et al., 2004) (Fekete et al., 2004) (Boelen et al., 2005) (Helmreich et al., 2005) (Mebis et al., 2009).

RT-PCR techniques can be used to measure the relative gene expression of a number of molecules involved in hypothalamic regulation. Given the different

peripheral hormone responses in the three behavioural groups of animals, relative mRNA expression could provide supporting evidence for altered hypothalamic regulation of the HPT axis in the PD rats.

## *5.2 Aim*

To determine relative hypothalamic mRNA expression of a number of molecules known to be involved in regulation of the HPT and HPA axes or shown to be implicated in the response to uncontrollable or inflammatory stress. To correlate the relative gene expression of these hypothalamic factors with the behavioural responses demonstrated by rats following chronic constriction injury of the sciatic nerve.

## *5.3 Materials and Methods*

### *5.3.1 Experimental animals*

Animals used in this experiment experienced Experimental Paradigm 2 (section 2.4.1); that is, there was no jugular vein cathetarisation prior to commencing behavioural testing. In brief, rats were acclimatized to the reverse light/dark cycle for one week prior to commencement of behavioural testing. Resident-intruder behavioural testing was carried out as described (section 2.4.3) for 5 days; CCI surgery was performed and resident-intruder testing continued for six days post surgery. On day 7, the rats were decapitated, the brains removed and frozen in Tri-Reagent® at -80$^{o}$C (section 2.4.4.2).

As previously explained in section 2.4.4.2, these rats were subjects in different experiments conducted by other members of the laboratory; the behavioural testing of these rats was conducted by other individuals; euthanasia of these rats and removal and storage of brain tissue were also performed by other individuals. The tissue was obtained from a tissue bank; the development of the tissue bank has been possible because of the very robust nature of the behavioural classification. Inter-rater reliability for scoring of behaviour is < 10%.

Eight rats, acclimatised to the same light/dark cycle and group housed were used as a naïve control group for the experiment. These animals were euthanased and

brain tissue obtained using the identical procedures as used with the experimental rats (section 2.4.4.2).

The behavioural classification of injured rats was used as the basis for selecting tissue for RT-PCR measurements. Thus eight rats classified as belonging to each of the behavioural groups (ND, TD and PD) were randomly selected. In addition, stored tissue from 12 animals used as Behavioural Control rats in a previous experiment, (same experimental paradigm as reported in section 2.4.3.4), were included. Together with the eight control rats described above, there were a total of 44 rat brains used for relative gene expression measurements.

### *5.3.2 Measurement of relative gene expression*

The hypothalamus was removed from each of the 44 rats by micro-dissection as described in section 2.7.1. Total RNA was extracted and quality assurance procedures performed as given in sections 2.7.2-5. First-strand cDNA was synthesised using each RNA preparation as described (section 2.7.6). Relative gene expression was measured for a number of molecules of interest using Taqman® quantitative reverse transcription polymerase chain reaction (RT-qPCR) assays and two different reference genes as detailed in section 2.7.7.

## *5.4 Results*

### *5.4.1 Behavioural analysis*

The pre-injury (or pre-anaesthetic) behaviours for the 41 rats for which PCR data were included are shown in Figure 5.1a. There was no difference in pre-injury levels in the four different scored behaviours for the three groups of injured animals ($p>0.05$, independent samples t-test). There are however differences between the BC group and the injured animals. Compared to the ND group, the BC rats displayed less dominance, although this difference was not significant ($p>0.05$). However the BC rats displayed significantly less social ($p<0.01$) and more non-social behaviours ($p<0.001$) than the ND group. Figure 5.1b illustrates the change in mean dominance behaviour following intervention for the four groups of animals. Compared to the ND rats, the TD rats show a temporary decrease in dominance on days 1-3 only ($p<0.01$) whereas the PD group show decreased D on days 1-3 and days 4-6 post-injury ($p<0.01$ and $p<0.001$

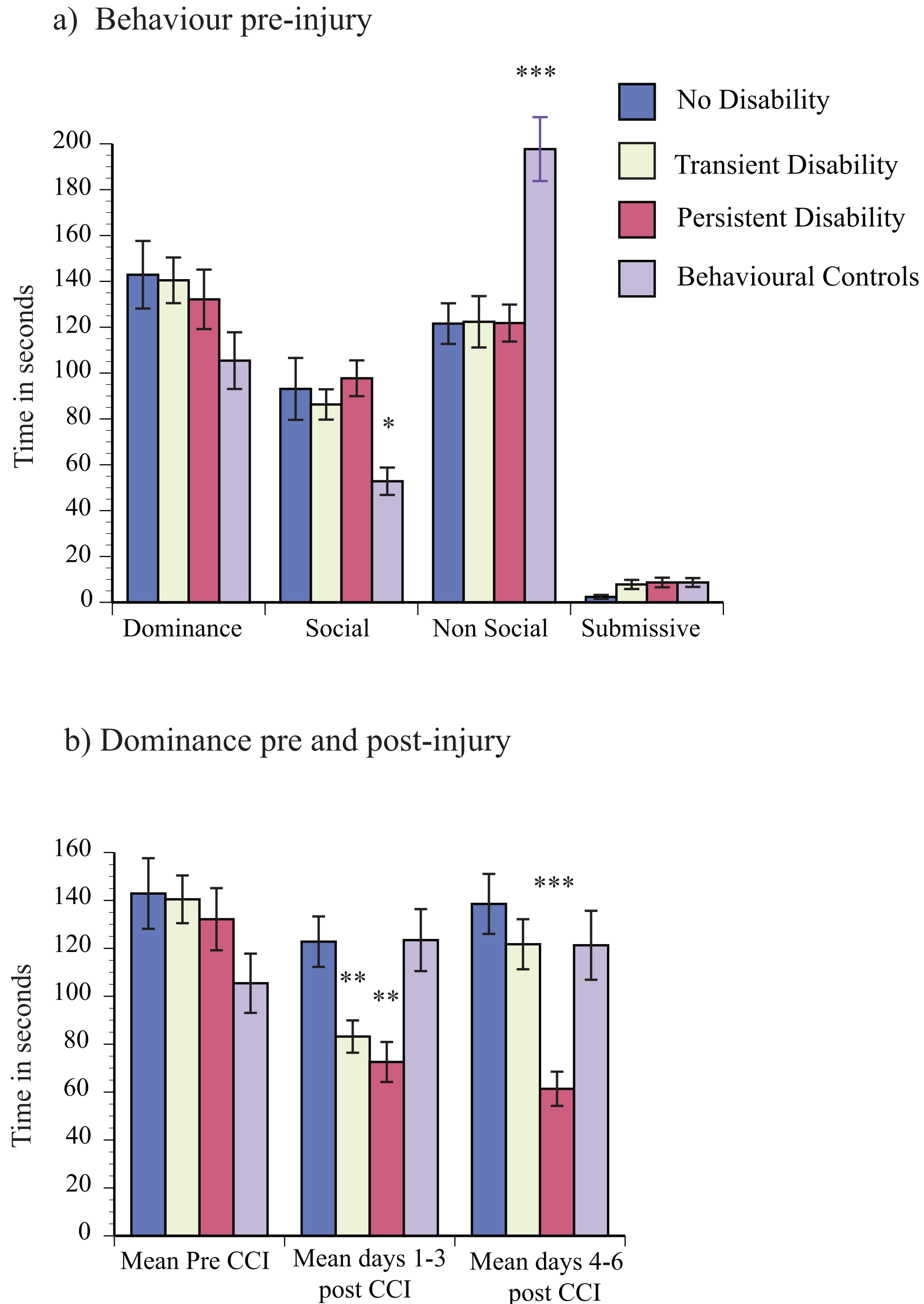



**Figure 5.1:** (a) Histogram illustrating mean pre-injury levels (± SEM) of behaviour (Dominance, Social, Non Social and Submissive) in animals classified as No Disability (ND, n=8), Transient Disability (TD, n=7), Persistent Disability (PD, n=7) and Behavioural Controls (BC, n=11). (b) Post-injury levels of Dominance behaviour for the four groups of rats. Changes that are significantly different to the ND group are indicated: *p<0.05,**p<0.01 and***p<0.001 (independent samples t-test).

respectively). The BC group however, like the ND animals, maintains dominance behaviour over the six days following the injury/anaesthetic.

### *5.4.2 RNA analysis*

The mean RIN (RNA Integrity Number: a measure of RNA quality) for the 44 RNA samples was 7.3± 0.07 with a range of 6.2 - 8.1. The mean for the two control groups was 7.5 (range 6.9 – 8.1) and for the 3 injured groups 7.1 (range 6.2 – 7.8). There was no significant difference between the mean RIN for the control and injured animals although the brains of the injured rats had been stored for several years whereas those for the control groups were stored for weeks to months only (Independent samples t-test, $p>0.05$). For the two samples with RIN below 6.5 (RIN 6.2 and 6.3 from a PD and TD animal respectively) as there was no evidence that gene expression correlated with RIN for these samples they were not excluded from the relative gene expression analyses.

There were no samples excluded due to poor 260/280 ratios; there was no significant DNA contamination in the samples.

There were five samples where the 260/230 values were below the acceptable level of 1.5. For four of these samples the 260/230 ratios were above 1.4. As there was no consistent difference in relative gene expression between these samples and the mean relative expression for the respective behavioural groups, they were included in the analyses. One sample, with a 260/230 of 1.24, was excluded. Relative gene expression for this sample was consistently an outlier relative to the mean values for the group; it was possible that organic contamination in the sample interfered with the RT-qPCR assay results (Bustin et al., 2009).

### *5.4.3 Reference genes*

#### *5.4.3.1 GAPDH*

GAPDH was initially used as a within assay reference gene for the genes of interest. GAPDH was reliably amplified over serial dilutions giving an equal cycle threshold. Reaction efficiencies for GAPDH were all within acceptable levels (>1.8).

Examination of Ct values for GAPDH, determined in the same assay as TRH, TRβ, Dio2 and Dio3 respectively, revealed that GAPDH was significantly down regulated in the PD group relative to the other four groups of animals in each case. For example, when measured with the TRH probe, the mean GAPDH Ct value for the PD rats (17.724±0.07) was different to those of the other groups as follows; ND rats, 17.075±0.16; TD rats, 17.007±0.13; BC rats, 17.202±0.14; C rats, 17.188±0.20. A Mann-Whitney U test showed a significant difference in Ct values for GAPDH for the PD rats (Md=17.769) and the other rats (Md=17.124), p=0.005. The mean Ct values for GAPDH, determined in assays with each of the HPT axis gene probes, are shown in Figure 5.2a illustrating that the values obtained in the PD animals are different to the other groups in each case. There were no differences between the mean GAPDH Ct values for any of the other groups (p>0.05). It was concluded that GAPDH was not a suitable reference gene to use to investigate alteration to relative gene expression involving the HPT axis, as the fundamental criterion, that the reference gene be stable across the experimental paradigm, was not true with respect to hypothalamic tissue.

GAPDH was also measured in assays with CRH and Vasopressin. As shown in Figure 5.2b, the Ct values for GAPDH for the PD group were again different to those of the other experimental groups. For example, when measured with the Vasopressin probe, the mean GAPDH Ct value for the PD rats (18.571±0.09) was different to those of the other experimental groups as follows; ND rats, 17.812±0.15; TD rats, 17.853±0.13; BC rats, 17.935±0.12. A Mann-Whitney-U test showed a significant difference in Ct values for GAPDH for the PD rats (Md=18.559) and the other rats (Md=17.882), p=0.004. However the mean Ct value for GAPDH for the control group of animals was not different to the PD group (p=0.487). While the decision to use a different reference gene had already been made, this finding raised the issue of the appropriateness of this control group especially with reference to the HPA axis probes.

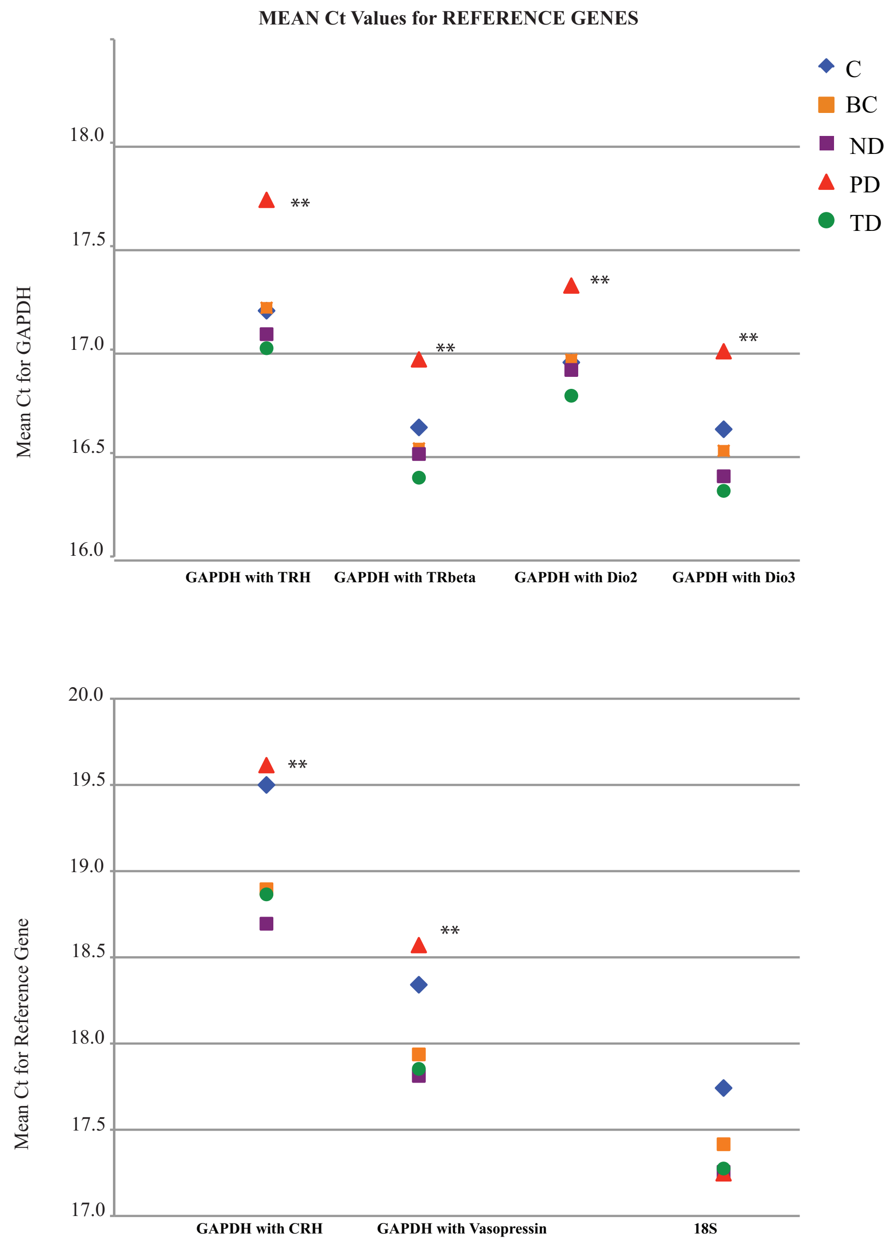



**Figure 5.2:** Mean Ct values for reference genes for each group of rats, Control (C), Behavioural Control (BC), No Disability (ND), Persistant Disability (PD) and Transient Disability (TD) groups. Ct values for GAPDH were measured in the presence of the other gene probes as indicated; Ct values for 18S were the mean of two assays (all samples assayed in triplicate). The mean Ct values for the PD group were different to the other four groups of rats, (p<0.01 (Mann-Whitney U test).

#### *5.4.3.2 rRNA -18S*

Serial dilutions using the other reference gene used, 18S, also demonstrated reliable amplification and a reaction efficiency of 1.9.

Ct values obtained measuring gene expression in the rat samples were similarly examined. Two samples (one each from the PD and TD groups) were excluded from all further analysis since the Ct values were clear outliers (> 3 SD outside the mean for the respective group of values). With these samples removed, there was no correlation between RIN and Ct values for 18S expression for all samples ($r=0.142:p=0.376$).

There was no difference between the mean Ct value of the PD group and the other rats ($p>0.05$). Mean Ct value for C rats, 17.742±0.34; ND rats 17.256±0.16: PD rats, 17.247±0.05; TD rats, 17.275±0.13 and BC rats 17.416±0.09. There was no difference between the mean Ct value for the C group and the other rats ($p>0.05$) although the variance within this group was much greater than for the other groups. Mean Ct values for 18S for the five groups of animals is shown in Figure 5.2b. It was concluded that 18S was a suitable reference gene to use to determine relative gene expression, as the expression of this gene was stable across the experimental paradigm. However, the use of the C group as the control group was again questioned as the mean Ct values for18S indicate that this gene may be down regulated in this group relative to the experimental groups and the behavioural control group.

### *5.4.4 Relative mRNA expression*

Using 18S as the reference gene, relative mRNA expression was determined for all of the genes of interest as detailed above (section 2.7.7). The data for the following animals were included:

- Control (C): N = 8
- Behavioural Control (BC): N = 11
- No Disability (ND): N = 8
- Permanent Disability (PD): N = 7
- Transient Disability (TD): N=7

### *5.4.4.1 Control Groups*

Initially, relative mRNA expression was determined and normalised to the control group (C). It was apparent however, that there were differences between the two control groups. The relative expression for the genes measured for the two control groups of animals is shown in Figure 5.3 normalised to the BC group.

The mean relative mRNA expression was up regulated in the C group relative to the BC group for all the genes measured but there were no significant differences between the groups for relative mRNA expression for TRH, TRβ, deiodinase 2 or deiodinase 3 (Mann-Whitney-U test, $p>0.05$).

There was no difference between the control groups in relative mRNA expression for GR. Relative mRNA expression for CRH for the C group (Md=1.780) was significantly different to the BC group (MD=0.965), $p=0.014$ and relative mRNA for Vasopressin for the C group (Md=2.410) significantly different to the BC group (Md=0.930), $p=0.039$. Similarly, mRNA for IL-1β for the C group (Md=2.380) was significantly up regulated compared to the BC group (Md=1.015), $p=0.041$.

In view of these differences and considering the closer environmental (housing) and experimental (anaesthetic and behavioural testing) experiences of the BC group relative to the experimental animals, all of the following results are presented normalised to the BC group of control animals.

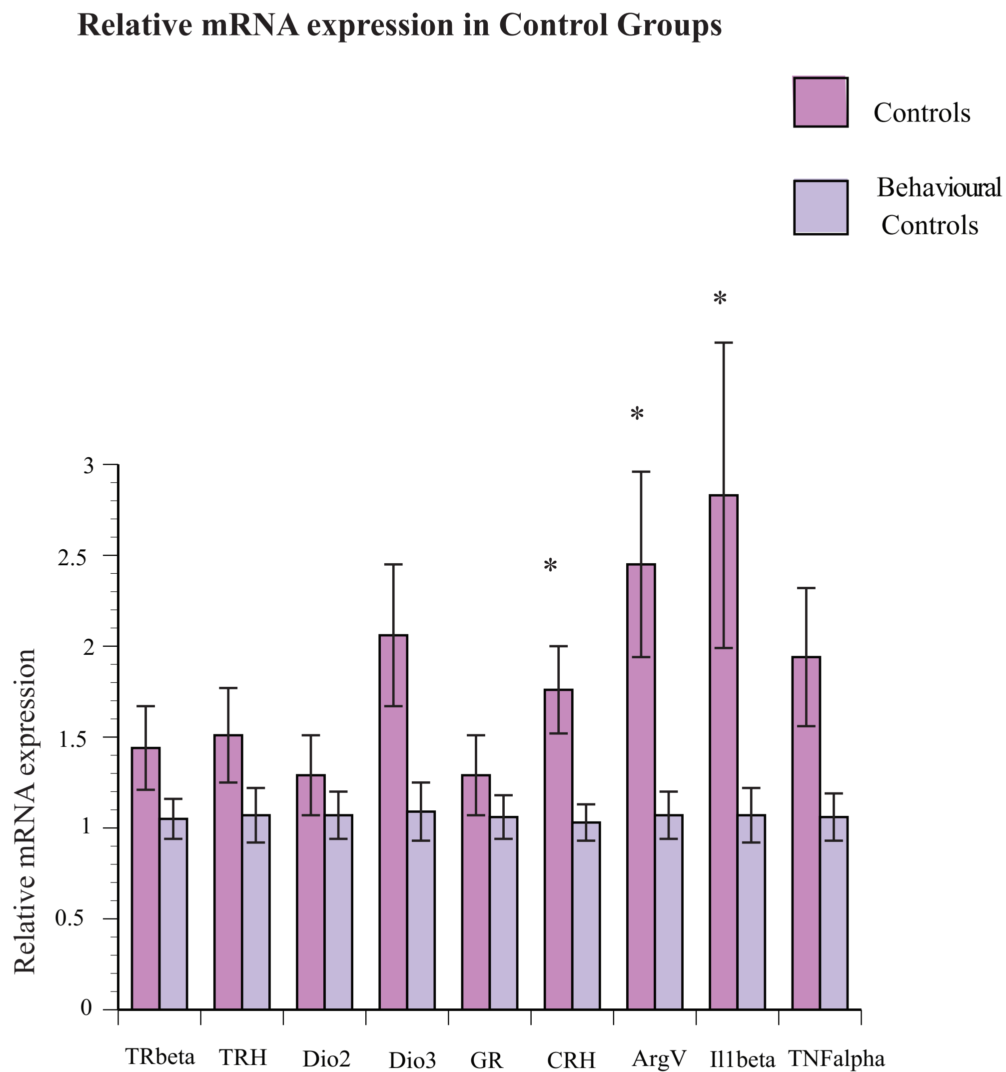



**Figure 5.3:** Histogram illustrating mRNA expression relative to 18S (±SEM) (normalised to the Behavioural Control group of rats) for the nine probes examined in the two groups of control rats, Control (C) and Behavioural Control (BC). All levels of relative mRNA expression were upregulated in the C group when compared to the BC group. The variance in values was greater in the C group. Significant differences in gene expression between the two groups are indicated: * $p < 0.05$ (Mann-Whitney-U test).

### 5.4.5 *Relative gene expression – HPT axis*

The histograms in Figure 5.4a and 5.4b illustrate the mRNA expression relative to 18S for the genes tested relating to the function of the HPT axis, normalised to the BC group of animals.

#### 5.4.5.1 *Thyroid Hormone Receptor β – TRβ*

There were no significant differences between any of the injured rats and the behavioural control group for mean relative expression of mRNA for TRβ: ND rats 1.18±0.10; PD rats 0.96±0.08: TD rats 1.20±0.16; BC 1.05±0.11 ($p>0.1$).

#### 5.4.5.2 *Thyrotrophin Releasing Hormone – TRH*

Mean TRH mRNA was down regulated in the PD group of nerve injured rats compared to the BC rats: the relative gene expression for PD rats, mean 0.67± 0.05 (Md=0.680) was significantly different to that of the BC rats, mean 1.05±0.15 (Md=1.000), $p=0.009$ (Mann-Whitney U test). TRH mRNA was also down regulated in ND and TD rats (0.84±0.14 and 0.85±0.26 respectively) but these levels of expression were not significantly different to the BC group ($p>0.1$).

#### 5.4.5.3 *Deiodinase 2 – Dio2*

Relative expression of mRNA for Dio2 was down regulated in the PD rats compared to the BC rats (0.68±0.06 Vs 1.07±0.13); a Mann-Whitney U test showed the PD group (Md=0.700) was significantly different to the BC group (Md=1.060), $p=0.023$). Relative expression for the ND and TD groups did not differ from the BC group (0.99±0.10 and 1.18±0.12 respectively, $p>0.1$).

#### 5.4.5.4 *Deiodinase 3 – Dio3*

Relative expression of mRNA for Dio3 was up regulated in the PD rats compared to the BC rats (2.28±0.23 Vs 1.09±0.16); the PD group (Md=2.090) was significantly different to the BC group (Md=0.940), $p=0.003$). The mRNA for Dio3 did not differ from the BC group for the other nerve-injured rats (ND, 1.44±0.26 and TD, 0.98±0.21, $p>0.1$). When the PD group was compared to the ND group, there was also a significant up regulation ($p=0.037$).

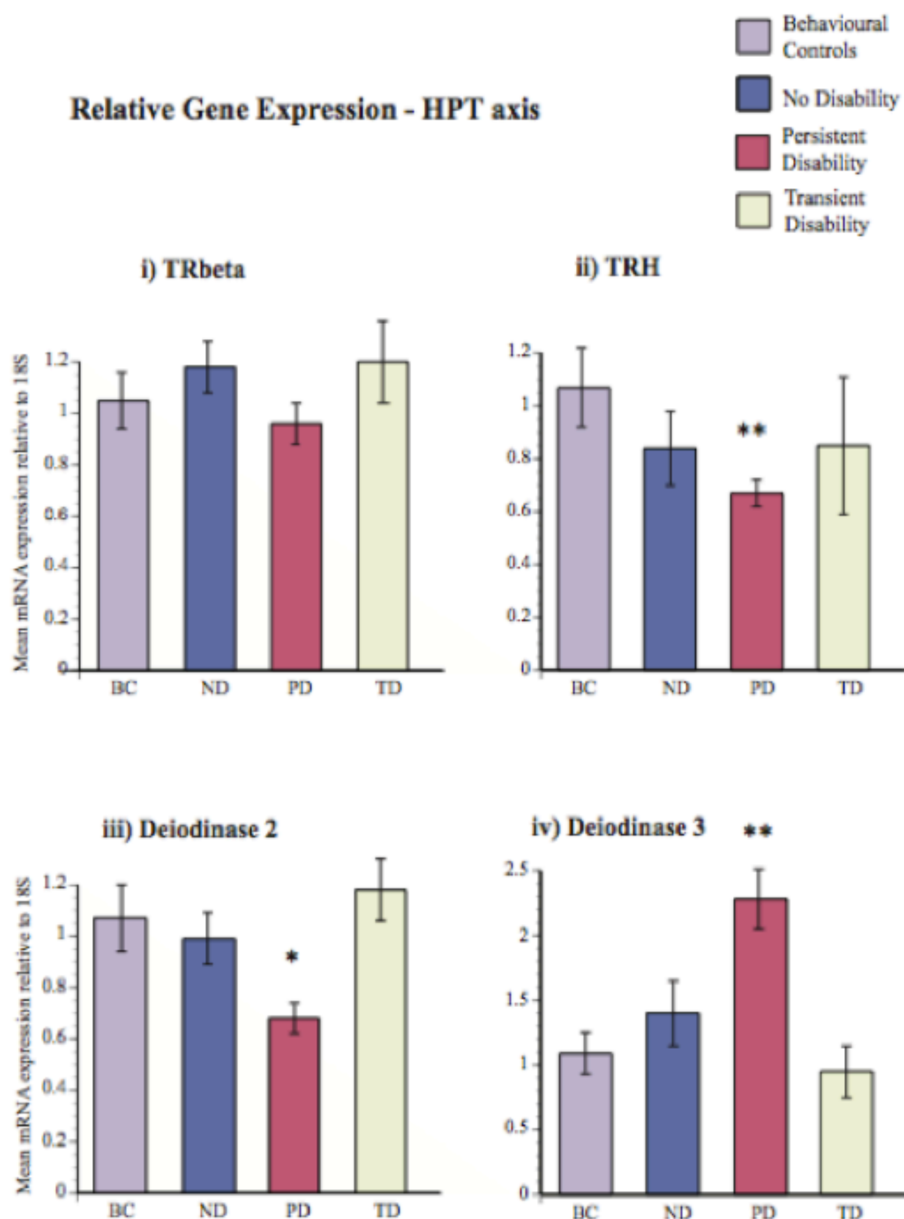



**Figure 5.4a:** Histogram illustrating mean mRNA expression relative to 18S (±SEM) for i) Thyroid Receptor beta (TRbeta), ii) Thyrotrophin Releasing Hormone (TRH), iii) Deiodinase 2 (Dio2) and iv) Deiodinase 3 (Dio3) for Behavioural Control (BC), No Disability (ND), Persistant Disability (PD) and Transient Disability (TD) rats. Relative gene expression is normalised to the Behavioural Control group of rats. Relative mRNA expression significantly different to the Behavioural Control group is indicated: *$p<0.05$; **$p<0.01$ (Mann-Whitney-U test).

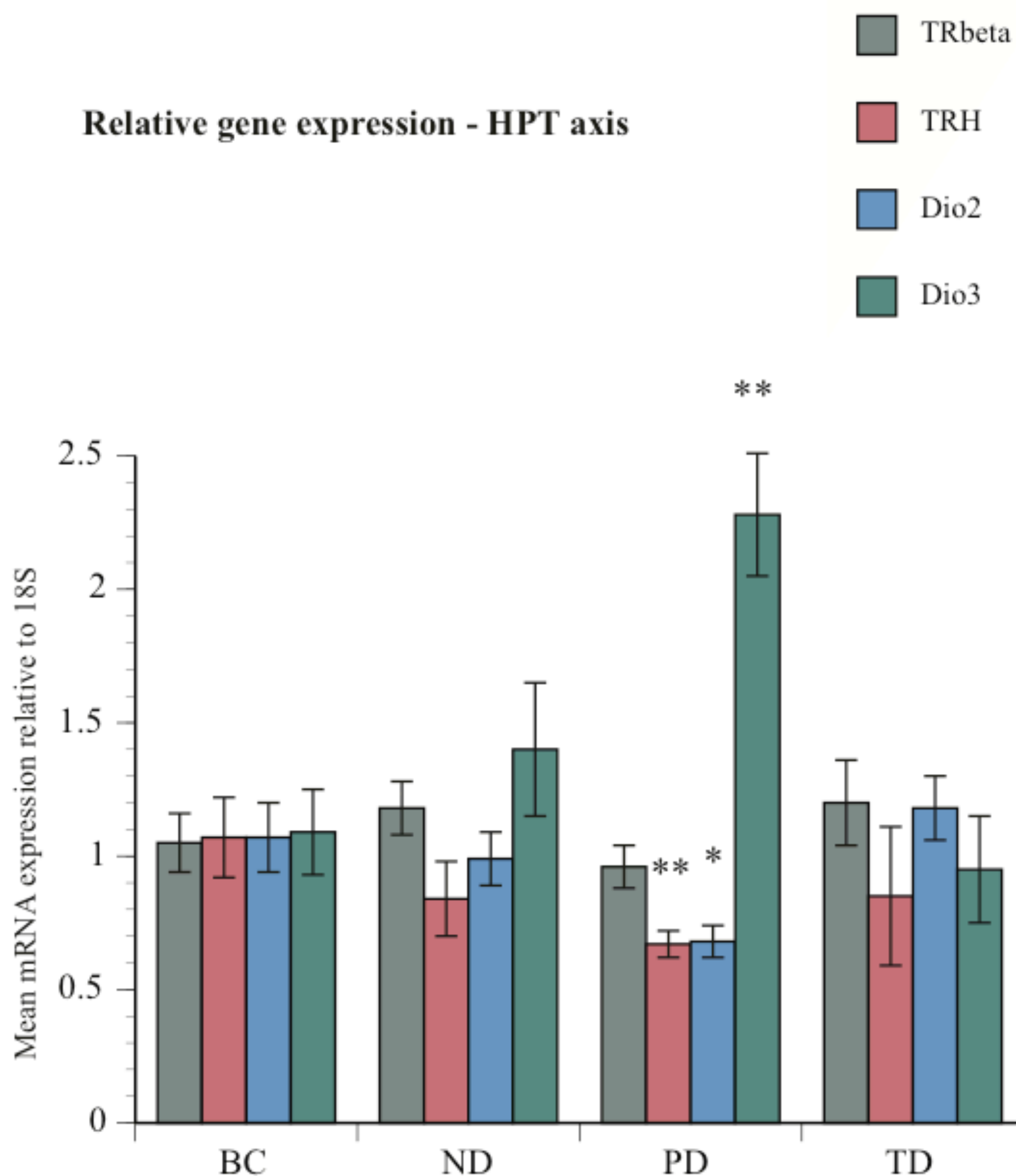



**Figure 5.4b:** Histogram illustrating mean mRNA expression relative to 18S (±SEM) for Thyroid Receptor beta (TRbeta), Thyrotrophin Releasing Hormone (TRH), Deiodinase 2 (Dio2) and Deiodinase 3 (Dio3) for Behavioural Control (BC), No Disability (ND), Persistant Disability (PD) and Transient Disabiloity (TD) rats. Relative gene expression is normalised to the Behavioural Control group of rats. Relative mRNA expression significantly different to the Behavioural Control group is indicated: *$p<0.05$; **$p<0.01$.

### *5.4.6 Relative gene expression – HPA axis*

The histogram in Figure 5.5 illustrates the mRNA expression relative to 18S for the genes tested relating to the function of the HPA axis, normalised to the BC group of animals. The values for the group caged control rats are included for comparison.

#### *5.4.6.1 Glucocorticoid Receptor – GR*

Relative mRNA expression for GR was down regulated in the PD rats compared to the BC animals but this difference failed to reach significance (0.79±0.06 Vs 1.06±0.12, $p=0.077$). There was no difference between relative expressions of GR mRNA for the ND and TD rats (1.01±0.08 and 1.15±0.18 respectively, $p>0.1$).

#### *5.4.6.2 Corticotrophin Releasing Hormone – CRH*

There was no significant difference in mean relative expression of mRNA for CRH between the BC group and any group of injured animals (BC, 1.03±0.10; ND, 1.30±0.14: PD, 1.11±0.06; TD, 1.12±0.17: $p>0.1$).

#### *5.4.6.3 Arginine-Vasopressin*

There was no significant difference in relative expression of mRNA for Vasopressin between the BC group and any group of injured animals (BC, 1.07±0.13; ND, 1.28±0.21: PD, 1.27±0.14; TD, 1.17±0.40: $p>0.1$).

### *5.4.7 Relative gene expression – cytokines*

Figure 5.6 illustrates mean relative cytokine mRNA expression for the four groups of rats; the values for the group caged control group are included for comparison.

Mean relative gene expression for IL-1β was not different to the BC group for any of the injured animals (BC, 1.07±0.15; ND, 1.38±0.25: PD, 0.96±0.08; TD, 1.36±0.15, $p>0.1$).

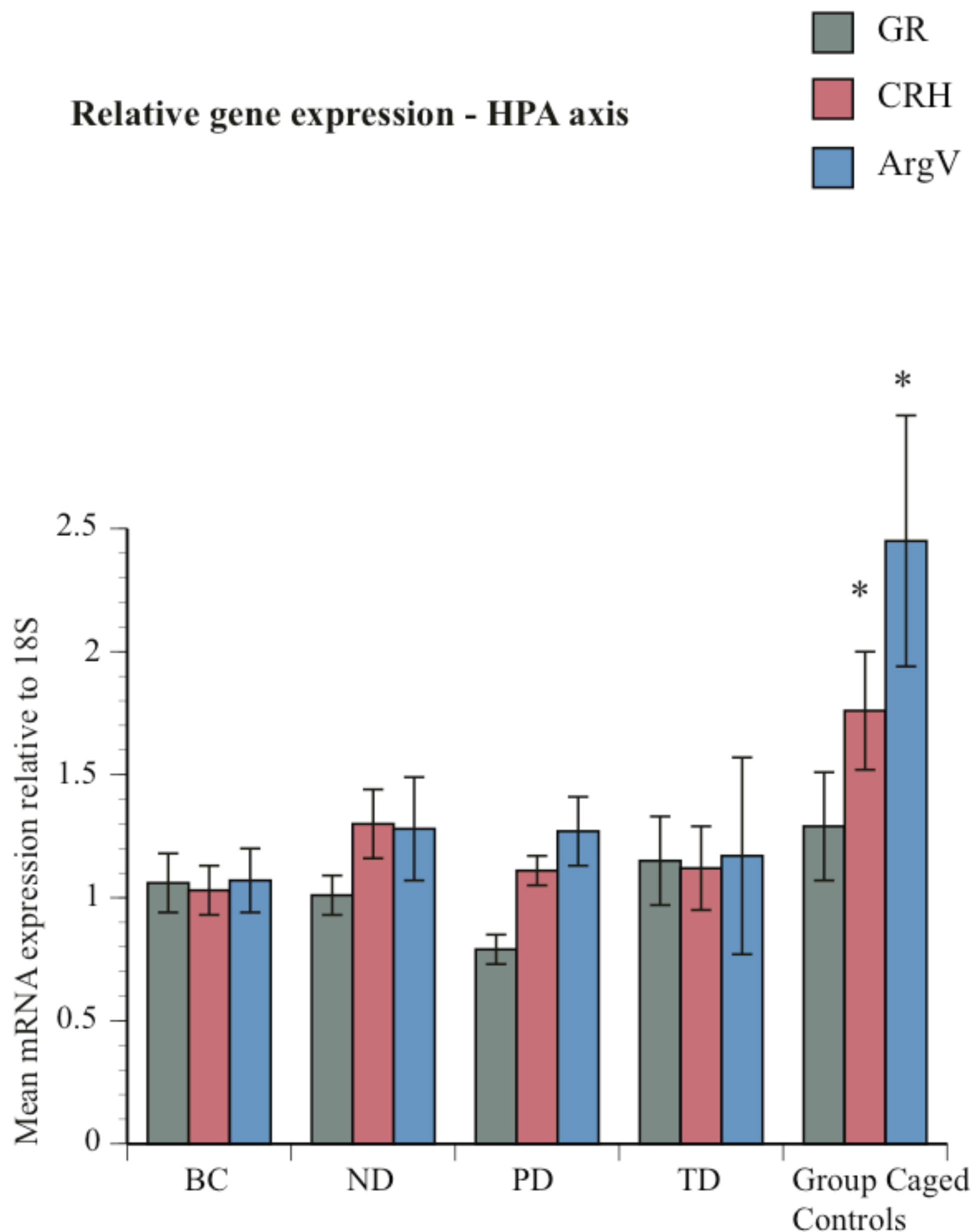



**Figure 5.5:** Histogram illustrating mean mRNA expression relative to 18S (±SEM) for Glucocorticoid Receptor (GR), Corticotrophin Releasing Releasing Hormone (CRH) and Arginine Vasopressin (ArgV) for Behavioural Control (BC), No Disability (ND), Persistant Disability (PD), Transient Disabiloity (TD) and Group Caged Control (C) rats. Relative gene expression is normalised to the Behavioural Control group of rats. Relative mRNA expression significantly different to the Behavioural Control group is indicated: *$p<0.05$.

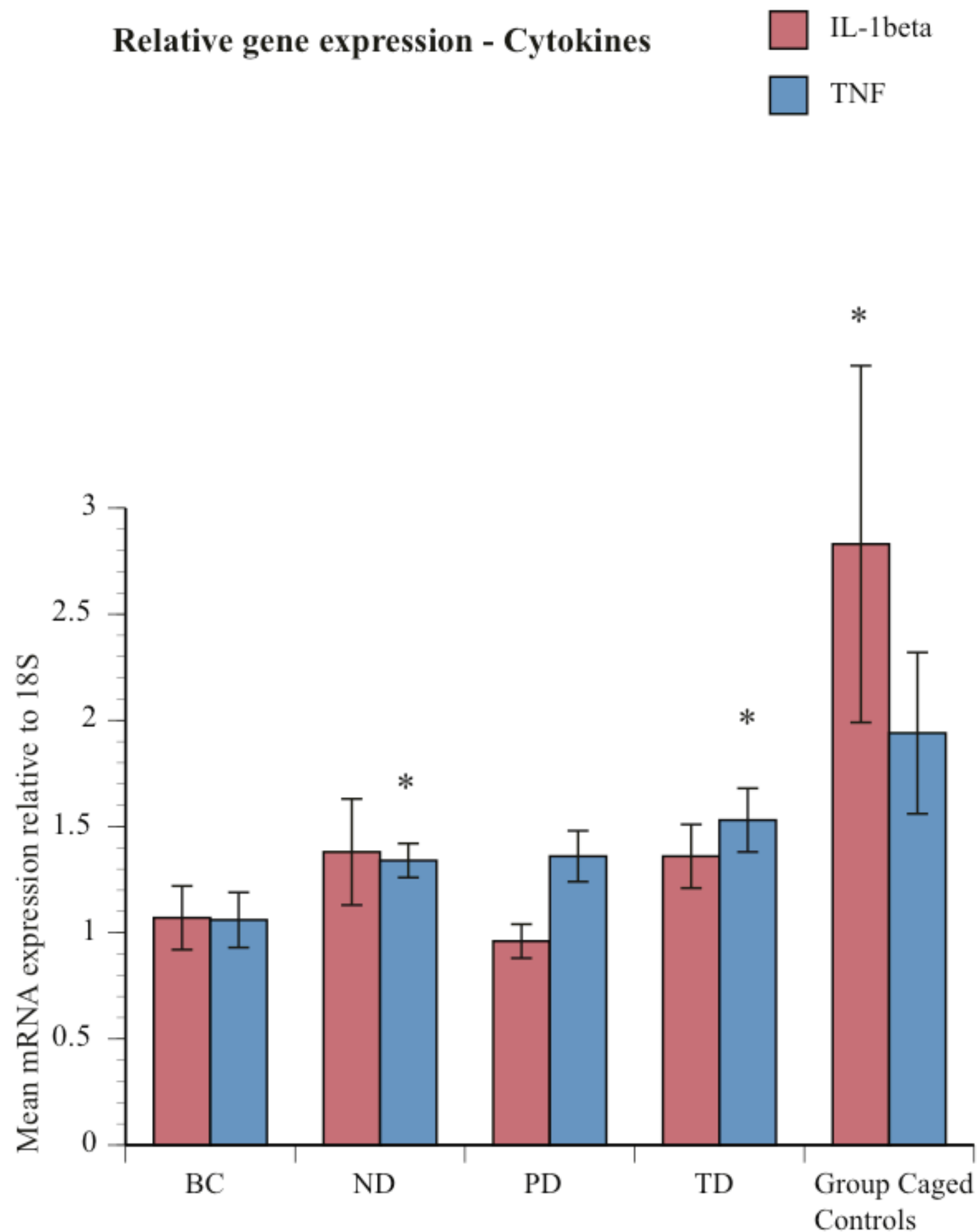



**Figure 5.6:** Histogram illustrating mean mRNA expression relative to 18S (±SEM) for Interleukin-1beta (IL-1beta) and Tumour Necrosis Factor (TNF) in Behavioural Control (BC), No Disability (ND), Persistent Disability (PD), Transient Disability (TD) and Group Caged Control (C) rats. Relative gene expression is normalised to the Behavioural Control group of rats. Relative mRNA expression significantly different to the Behavioural Control group is indicated: $*p<0.05$.

There was up regulation in relative expression of mRNA for TNF in the ND and TD groups when compared to the BC group (ND, 1.34±0.08: TD, 1.53±0.15 Vs BC, 1.06±0.13, p=0.033 and p=0.019 respectively). The similar up regulation in the PD rats was not significantly different to the BC group (PD, 1.36±0.12, p=0.079). The mRNA for both cytokines is however up regulated in each instance except for relative expression of IL-1β in the PD group.

## 5.5 Discussion

The aim of this study was to determine the relative mRNA expression of a number of hypothalamic molecules following chronic constriction injury of the sciatic nerve in rats. Using this stressor, behavioural change has been demonstrated in a sub-population of the rats. As detailed in Chapter 4, following injury, this sub-group have decreased peripheral thyroid hormones with lowered TSH levels suggesting altered hypothalamic regulation of the HPT axis. Altered corticosteroid levels following injury implicate the HPA axis in the post-injury state.

### 5.5.1 Behavioural Analysis

The tissue used to assess changes to hypothalamic factors was obtained from rats used in previous experiments and stored for varying amounts of time. The rats selected by behaviour as belonging to the ND, PD and TD groups were behaviourally tested, injured and euthanased in 2004-5; the behavioural control group tested and euthanased in 2009 and the control group euthanased in 2010. Multiple individuals performed the behavioural testing of these animals. As shown in Figure 5.1, the ND, PD and TD groups of animals (randomly selected based on testing and classification by others using standard criteria) conform to the criteria for behavioural classification. There is no difference between groups for any of the four behaviours pre-injury. Post-injury, the ND group maintain dominance; the TD group show a transient decrease in dominance on days 1-3 and the PD group show persistently changed behaviour with dominance scores significantly lower than the ND rats on days 1-3 and 4-6. The injured groups of animals behaviourally resemble those used to measure peripheral hormones as reported in Chapter 4.

The pre-anaesthetic behaviour of the BC group was however different to that of the other groups: social and non-social behavioural scores were both significantly different and dominance scores lower (though not significantly) compared to the pre-injury scores of the animals that were later injured. A suggested explanation for this finding was given in Table 3.2 and sections 3.4.8 and 3.5.5: namely, that there has been a change in the source rat population over time with a progressive decrease in dominance and social behaviour and an increase in non-social behaviour. Alternatively, there may have been a shift in the way in which the behavioural criteria are interpreted by different researchers over time. However, all are trained with the same training video and criteria are regularly discussed with new researchers learning from those with more experience. With reference to the scores detailed in Table 3.2, the first three groups were all scored by this researcher and a trend is apparent for S and NS behaviours; the two BC groups are very similar but scored by different individuals: four different individuals scored the experimental animals used in this experiment with higher D scores. However a subtle change in the way criteria have been applied over time cannot be excluded. Another possibility is that these animals, for some reason, took longer to establish dominance than the other groups. D scores after the intervention are not different to those of the ND rats (Figure 5.1).

The important question however, is whether or not these rats serve as an appropriate control group for the experimental rats? The question of appropriate control group will be further explored below. However, the breeding of the supplied rats is outside the researcher's control and the rescoring of animals used in others' experiments, after the fact, would be inappropriate. Within these limitations, the use of this group as a control group was deemed appropriate for the following reason. With respect to dominance behaviour, although lower than for the other groups, once established, that dominance behaviour is maintained after the intervention. In this respect, this BC group is similar to the ND group and different to the TD and PD groups.

### 5.5.2 *Reference Genes*

Measurement of gene expression using quantitative real-time PCR is an enabling technology allowing for sensitive and specific evaluation of relative expression of genes across many samples from varying sources (Bustin et al., 2009). However this technology is completely dependent on the identification of appropriate reference genes. Evaluation of gene expression is determined relative to a reference gene that is known to be stable across the experimental paradigm. Moreover, the reference gene must be stable in the specific tissue in which the evaluation is made.

Since the introduction of RT-qPCR, a number of genes have been commonly used as reference genes including GAPDH, beta-actin (ACTB) and ribosomal mRNAs including 18S. Recently there have been a number of publications questioning the use of some of these genes (Gubern et al., 2009) (Ropenga et al., 2004). The use of reference genes that are not stable will result in potentially misleading measures of gene expression. The need for greater validation of reference genes in specific experimental situations and in specific tissues is called for to ensure the valid use of the technology (Bustin et al., 2009).

Validation of reference genes in a model of neuropathic pain (dorsal root ganglion (DRG) compression), with gene expression determinations made on DRG samples, concluded that GAPDH was not a suitable reference gene whereas ACTB and a number of ribosomal proteins were stable in their paradigm (Wan et al., 2010). In our laboratory, using the CCI model of neuropathic pain: GAPDH was found to be stable in the nucleus accumbens (Austin et al., 2010) and GAPDH and 18S in the periaquaductal grey (PAG) (Mor et al., 2010). In the hands of this researcher, GAPDH was not stable in hypothalamic tissue; the mean raw level of expression for GAPDH for the PD group of rats was significantly different to that of the other injured rats and the behavioural controls. Iodothyronines have been shown to regulate GAPDH activity in the rat (Lombardi et al., 2000). Once two outliers were removed, raw expression values for 18S however were stable across the paradigm; thus it would seem to obey the

requirements of a reference gene and relative expression levels obtained using this reference gene should be valid. In light of work that has been published since this work was commenced, it would have been desirable to measure at least one other reference gene, possibly ACTB. This is however a new and evolving technology and calls for further regulation of the way experiments are conducted, with for example the inclusion of three validated reference genes, can only improve the quality of future work (Bustin et al., 2009).

### *5.5.3 Control Groups*

That control animals are an essential component of any experimental procedure is a given. What is less readily apparent is which components of the experimental animals' experiences need to be controlled in order that the control group is an appropriate one. This question is perhaps most pertinent in experiments where behaviour and hormonal change, including responses to stress, are investigated. Group housed rats are commonly used as control groups yet individual housing has for example been shown to lower corticosterone levels in male rats (Barrett and Stockham, 1963). Group housing exposes rats to social situations where aggression of cage mates and social defeat are common experiences (Sgoifo et al., 1999). However the degree and type of stress experienced by individual members of the group varies. The HPA axis in the rat may be suppressed or stimulated depending on whether stress is acute, repeated, chronic or novel (Jaferi et al., 2003) (Bhatnagar et al., 2006).

As shown in Figure 5.3, the group caged control (C) rats varied in relative mRNA expression for a number of the genes tested. Both CRH and Vasopressin were up regulated in the C group relative to the BC group. These findings are consistent with the up regulation of the HPA axis seen in repeated social stress (Bhatnagar et al., 2006) and also with increased arginine-vasopressin secretion reported in chronic stress situations (Treschan and Peters, 2006). These findings confirmed the view that, with respect to the HPA axis, this group of animals were not an appropriate control group. Moreover, the C group were different to the BC group in relative expression of deiodinase 3 and both cytokines tested, reinforcing the need for a different control group for assessing all the genes of interest.

The experiences of the behavioural control group were identical to those of the experimental animals except for the surgery to perform the CCI. A sham operated, behaviourally tested control group may have constituted a superior control group. The inclusion of yet another control group was not contemplated for logistical reasons related to cost.

With regard to control of experimental variables, perhaps the true control group for the animals of interest, the rats where social behaviour changes and thyroid hormone levels fall following injury, are the ND rats. These rats have identical experiences in terms of housing, anaesthetic, surgery, exposure to intruder animals, method of euthanasia and pathological findings post mortem (Hu et al., 2007). There were no differences between the BC group and the ND group for any of the genes tested related the HPT or HPA axes.

### *5.5.4 Relative gene expression - HPT axis*

There was evidence of altered activity in the HPT axis in the animals where behaviour changed following injury, but not in the animals where dominance behaviour was unchanged (ND) or returned to normal by days 4-6-post injury (TD) (Figure 5.4b).

#### *5.5.4.1 Thyroid Hormone Receptor β – TRβ*

The isoforms of TRβ are known to be central to the T3 transcriptional repression of TRH in rodents (Abel et al., 2001) (Guissouma et al., 2006). It is therefore possible that altered expression of this receptor might be involved in altered HPT function. However, the finding in this study, that there was no difference between the animal groups in mean mRNA expression for TRβ, concurs with other findings in animal models that have investigated HPT axis changes following inflammatory stress. In LPS induced illness in mice, chronic localised inflammation in mice and chronic illness in rabbits, mRNA determinations for TRβ in the hypothalamus were all unchanged when compared to control groups (Boelen et al., 2004) (Boelen et al., 2006) (Mebis et al., 2009). In chronic localised inflammation, this lack of change in TRβ expression was specific to the PVN (Boelen et al., 2006). While this does not negate a role for TRβ in the

changes that occur, changes to mechanisms that regulate T3 concentrations appear to offer a more likely explanation.

#### *5.5.4.2 Thyrotrophin Releasing Hormone – TRH*

The production of TRH in response to changes in peripheral thyroid hormones has been shown to be the critical regulator of TSH production by the pituitary (Nikrodhanond et al., 2006). Down regulation of mTRH in the PVN has been reported in patients who died with NTI and is the proposed mechanism to explain the fall in T4 that occurs in fatal and prolonged illness (Fliers et al., 1997) (Van den Berghe et al., 1998) (De Groot, 2006). Significant down regulation, in the PD rats, of TRH mRNA (relative to control animals) suggests altered hypothalamic regulation and offers an explanation for the failure of these animals to increase TSH levels and maintain peripheral thyroid hormone levels as detailed in chapter 4. We have previously reported the same finding in another group of animals following CCI (Kilburn-Watt et al., 2010). Down regulation of TRH mRNA has been reported in the other animal models: both in the turpentine injected mice and the chronically ill rabbits this down regulation was shown to be specific to the PVN (Boelen et al., 2006) (Mebris et al., 2009). This demonstration that the down regulation was specific to the region of the hypophysiotrophic TRH neurones was important since there are other regions of the hypothalamus which contain TRH producing cells. Thus the finding that TRH mRNA was unchanged in LPS mice, using whole hypothalamus derived mRNA, does not necessarily rule out down regulation in the cells that control the pituitary thyrotrophs (Boelen et al., 2004). Whole hypothalamus was also used for mRNA determinations in this study, and down regulation of TRH mRNA was only demonstrated in the PD group. A result where mRNA was unchanged would still have been of interest since in this group, like the LPS mice, peripheral hormones fell. The tissue used is taken at a specific point in the time course of the process, in this case seven days after injury. The ND and TD animals have also experienced activation of the HPT axis with increased TSH released in the first few days after injury (chapter 4); a down regulation in these rats could represent re-establishment of the pre-injury equilibrium. For the PD group, that TRH mRNA is decreased relative to control animals, even in whole hypothalamus determinations, at a time when T3, T4 and

TSH are all below pre-injury levels strongly suggests change to the hypothalamic set-point for TRH in this sub-group.

#### *5.5.4.3 Deiodinase 2 – Dio2*

Deiodinase 2 is the enzyme responsible for conversion of T4 to T3 in the hypothalamus and is thus one of the factors regulating the tissue concentration of T3 in that region of the brain. Highest concentrations of the enzyme are found in the medial basal hypothalamus (MBH) where it is found in glial cells, both astrocytes and the tanycytes that line the 3rd ventricle (Fliers et al., 2006) (Courtin et al., 2005). An increase in Dio2 resulting in a localised hyperthyroidism in the hypothalamus has been proposed as a possible mechanism to explain the down regulation of TRH in the face of low peripheral thyroid hormones (Fekete et al., 2004). The PD group of rats showed a significant decrease in Dio2 mRNA compared to BC rats while the ND and TD groups did not differ from the controls. This finding is not consistent with the findings in other studies. Relative expression of Dio2 mRNA was up regulated in LPS injected mice and chronically ill rabbits when whole hypothalamus extracted RNA was used (Boelen et al., 2004) (Mebis et al., 2009). Dio2 mRNA was transiently increased in the turpentine-injected mice when whole hypothalamus and when Arcuate Nucleus (AN) tissue only was used but was not significantly different to controls (Boelen et al., 2006). *In situ* hybridisation techniques demonstrated that the up regulation occurs specifically in the MBH, in the floor and walls of the 3rd ventricle (Mebis et al., 2009) (Fekete et al., 2004). However hypothalamic Dio2 enzyme activity was not increased in the sick rabbits nor were T3 and T4 levels in the hypothalamus elevated leading to the conclusion, that in the chronic condition, local elevated T3 does not appear to be the mechanism responsible for the down regulation of TRH (Mebis et al., 2009). This interpretation does not allow for localised differences in enzyme activity and hormone concentrations within sub-regions of the hypothalamus.

The time point of observation may be all important when assessing whether or not there has been change in the regulation of a gene; up regulation may be followed by a compensatory decrease in expression. It would have been interesting to

measure the gene expression for Dio2 at 1-2 days post-CCI; the reported peak increase in Dio2 gene expression in the LPS animals was measured at 12 hours after injection. These animals were acutely ill. However the increase in Dio2 expression in the turpentine-injected mice peaked at 48 hours after injection and after 120 hours was not very different to control animals albeit still elevated over pre-injury levels. The increased Dio2 expression in the rabbit model was measured at seven days, as in this study, but the rabbits were in a critical state whereas these rats were not 'sick'. Nonetheless, the isolated finding of a significant down regulation of Dio2 mRNA in this study is not easily explained in the context of the findings in other models or the hypothesised hypothalamic hyperthyroidism mechanism.

#### *5.5.4.4 Deiodinase 3 – Dio3*

Deiodinase 3 is the enzyme responsible for the metabolism of T3 and T4 and thus also of importance in regulation of tissue T3 levels. A decrease in Dio3 activity might be an additional or alternate mechanism causing localised PVN hyperthyroidism and TRH down regulation (Koenig, 2008). However the PD group of rats had significant up-regulation of Dio3 mRNA in the hypothalamus seven days after injury while the ND and TD rats were not different to the BC rats in relative expression of Dio3. A decrease in Dio3 mRNA, specifically in the PVN, was reported in the chronic inflammation model in mice (Boelen et al., 2006). The gene expression was lowest at 48 hours after injection and by the subsequent time point, at 5 days, was not different to controls. The time course for the Dio3 mRNA closely resembled that for TRH mRNA post injection with TRH mRNA levels lowest at 48 hours and returning to control animal levels at the same time as T3 and T4 levels are returning to normal (5 days) (Boelen et al., 2006). Although suffering an inflammatory injury, the majority of rats in the CCI study did not have significant falls in T3 and T4 and TSH levels were elevated 2-3 days post injury; it is only the PD group which have had suppression of the HPT axis and they were still experiencing this state on day six whereas in the turpentine mice, equilibrium had been restored. Direct comparisons are therefore difficult without additional time point measurements. Increased Dio3 activity (as well as Dio2 activity) was found in hypothalamic extracts from the chronically ill rabbits after seven days and hypothalamic T3 was not elevated (Mebis et al.,

2009). An increase in Dio3 does not agree with the hypothesis of hypothalamic thyrotoxicosis as the mechanism for suppression of TRH and changes in thyroid hormone transporters MCT10 and OATP1C1 found in this model were suggested as implicated in the perpetuation of the HPT axis suppression in the chronic critical state (Mebis et al., 2009).

The isolated finding of up regulation of Dio3 mRNA in the PD group in this study is not easily explained based on the literature. However, the issue as to whether or not this finding is related to the sample used in the determination needs clarification. Moreover, up regulation of a gene does not necessarily result in increased protein production if translational processes are inhibited. Measurement of enzyme activity or localisation of the protein using immunohistochemical techniques would be useful to further explore this finding.

### *5.5.5 Relative gene expression - HPA axis*

The histogram in Figure 5.5 illustrates that, at seven days post-injury, there is little evidence for a role for the HPA axis in the continuing down regulation of the HPT axis. There were no significant differences between the BC and PD groups of rats nor any of the groups of injured rats at this time. The group that were significantly different were the group caged control animals where relative expression of both CRH and Vasopressin mRNA was significantly elevated relative to the BC group. These findings suggest that the group caged animals experienced greater levels of chronic stress than the individually caged animals despite the experience of resident-intruder testing: this concurs with reports that show resident-intruder testing, while stressful for the intruder rats, is not stressful for the residents (Monassi et al., 2003).

The relative expression of GR mRNA for the PD group was of interest although not significantly different to the BC group. Again, it would be of interest to examine earlier time points following injury. As reported in Chapter 4, all groups of injured rats had increased levels of corticosterone following injury: the PD group had the least increase while the increase in the TD rats was significantly greater than for the other two groups. Corticosteroids have been implicated in the

down regulation of the HPT as discussed previously, however it is pathological levels and pharmacological doses that are reported in this context. Physiological variations in corticosteroid secretion have been implicated in the diurnal variation in TSH secretion but the role that endogenous fluctuations have in regulating the HPT axis are not clear (Samuels, 2000). Moreover the suppression of the HPT axis seen in rats following LPS injection has been shown to be independent of corticosterone levels (Kondo et al., 1997) (Sanchez et al., 2008). Following CCI, the animals with greatest increases in corticosteroids, the TD rats, do not have suppression of the HPT axis (Chapter 4).

Rather than the widely accepted concept of HPT suppression by corticosteroids, of interest is whether or not a failure of response in the HPA axis may be a contributing factor in the HPT suppression in the PD rats. Working with the same model, it has been shown that relative to ND and control rats, the PD group shows evidence of altered hypothalamic regulation, with a decreased numbers of both GR and CRH immunoreactive cells in the paraventricular nucleus of the hypothalamus (Sosa, 2008). Peripheral corticosterone was not measured in the TD group, however, as in the present study: while both ND and PD rats had elevated corticosterone post injury, the PD group had decreased adrenocorticotrophic hormone (ACTH) levels relative to the ND group (Sosa, 2008).

### *5.5.6 Relative gene expression – Cytokines*

Increased levels of IL-1β mRNA were demonstrated in both whole hypothalamic and PVN extracts in the chronic inflammatory (turpentine abscess) model of HPT suppression (Boelen et al., 2006). Both IL-1β and TNFα have been suggested as possible causative factors in the acute (LPS injected) model (Kakucska et al., 1994) (Fekete et al., 2005). Numerous studies have implicated these and other cytokines in the NTI syndrome as previously discussed. At day 7 after injury there was up regulation of hypothalamic TNF mRNA in the ND and TD rats relative to the BC group. A similar mean mRNA expression in the PD group was not significantly different to the BC group. The expression of IL-1β was also up regulated in the ND and TD groups, although not significantly, while that of the

PD group was not. The up regulation in relative mean mRNA expression for both cytokines was however much greater in the group caged control rats than the injured rats (Figure 5.6). It is therefore difficult to assess the significance of these results in terms of factors implicated in the ongoing HPT repression of the PD rats. The peak in IL-1β mRNA in the turpentine model occurred at 24 hours – thus again, an earlier time point would have been useful. With respect to the PD group, the most interesting observation may be a lack of response in relative expression of IL-1β in the hypothalamus following injury.

## *5.6 Summary*

Significant down regulation of TRH mRNA in the injured rats with reduced Dominance behaviour supports the hypothesis of a change in hypothalamic set point in this sub-group. This in turn offers an explanation for the failure of these animals to increase pituitary TSH and maintain peripheral thyroid hormone levels. However the hypothesis that this down regulation is due to a localised hypothalamic thyrotoxicosis due to increased Dio2 and/or decreased Dio3 enzymes was not supported by results reported here. There were however significant changes in the gene expression of both these enzymes which distinguished this group from the other injured rats. Further investigations as to protein expression of these enzymes, is it a sampling issue, would be useful in clarifying these results. There is no supportive evidence for a role for the HPA in the continuing HPT suppression in the PD rats. The significance of the two cytokines measured was inconclusive and further information for a causative role for these molecules would require an experiment where earlier time points following injury were investigated.

# *CHAPTER SIX*

# *Evidence for Altered Hypothalamic Regulation – Protein Expression in the Paraventricular Nucleus following Injury*

## *6.1 Introduction*

In a number of the animal models used to investigate the HPT changes seen following inflammatory stimuli, failure of the TRH producing cells to respond to low peripheral T4 has been reported (Kakucska et al., 1994) (Boelen et al., 2004). In these investigations, findings on whole hypothalamus extracts to determine change in gene expression were reported. Other studies, where anatomically specific techniques have been employed, have demonstrated down-regulation of mRNA for TRH specifically in the PVN in inflammatory stress (Kondo et al., 1997) (Boelen et al., 2006) (Mebis et al., 2009).

The down-regulation of mRNA for TRH in the PD group reported in Chapter 5, suggests that this sub population of rats may have altered regulation of the HPT axis. That down regulation of relative mRNA for TRH was demonstrated using whole hypothalamus preparations might well reflect that this sub-group of animals, identified behaviourally, has responded, biologically, differently to the total population of rats. This conclusion is supported by the fall in peripheral hormones seen in this sub-group as reported in chapter 4. However, in this study, as in others reported, changes in gene expression, while supportive of altered regulation, do not necessarily translate to changes in protein expression.

Similarly, studies that report changes in hypothalamic Dio2 and/or Dio3 levels in inflammatory stress models differ in methodology (Fekete et al., 2004) (Boelen et al., 2006) (Sanchez et al., 2008) (Mebis et al., 2009). Thus some studies report changes in gene expression while others measure enzyme activity. Some are

anatomically specific to the PVN while others report whole hypothalamus findings, as were the changes in these enzymes documented in chapter 5.

Immunohistochemistry techniques, in using antibodies to target specific proteins, offer a means of determining whether altered gene expression results in altered protein expression. The technique allows for anatomically specific regions of the hypothalamus to be examined for the presence of specific proteins.

## *6.2 Aim*

To use immunohistochemical techniques to label proteins involved in regulation of the HPT axis. To determine the distribution of these proteins in the PVN and specifically in the sub-regions of the PVN where the hypophysiotrophic TRH neurons have been shown to be located. To correlate these findings with the behavioural responses demonstrated by rats following chronic constriction injury of the sciatic nerve.

## *6.3 Materials and Methods*

### *6.3.1 Experimental animals*

The rats used for this experiment, Group B, were subjected to Experimental Paradigm 1 as detailed in section 2.4.1. That is, after environmental acclimation, indwelling catheters were inserted into the external jugular veins of all experimental rats before commencing behavioural testing. Blood sampling was carried out as described in section 2.4.2 (sampling 1 hour before lights ‘on’) and behavioural testing as in section 2.4.3. CCI was performed after pre-CCI baselines were established as before (section 2.3.3). The experience of these rats was identical to the experience of the rats reported on in chapter 4. The rats were behaviourally categorised as previously described. The behavioural data for the whole cohort of Group B (n=28) were presented in chapter 3; pre-CCI behaviour for the group is shown in Figure 3.2b and post-CCI behaviour included in the data presented for the total (n=59) rats used in the experiments reported in chapters 4 and 6.

On day seven post-CCI, the rats were euthanased, perfused with paraformaldehyde and the brains removed, post fixed and stored as detailed in section 2.4.4.3. Ten animals were behaviorally tested as per section 2.4.3.4 and were used as behavioural controls for the experiment.

The brains of all of the rats categorised as PD (n=6), seven ND rats and seven control rats were used for immunohistochemistry procedures.

### *6.3.2 Immunohistochemical procedures*

The specific immunohistochemical procedures used are detailed in section 2.8.4 and 2.8.5. In brief, antibodies to TRβ, synthetic TRH, Dio2 enzyme and Dio3 enzyme were used with free-floating sections of hypothalamus from injured and control rats to determine localisation of these proteins in the PVN.

### *6.3.3 Section identification and counting procedures*

Section 2.9.4 describes the methodology employed in the identification of relevant hypothalamic sections and the criteria used to identify immunohistochemical profiles. Briefly, hypothalamic sections at approximately -2.1, -1.9, -1.7 and -1.5 mm to bregma were identified using serial Nissl-stained sections from each animal. Using the relevant Nissl sections and guided by schematic diagrams adapted from Geerling *et al.,* and the rat brain atlas of Paxinos and Watson (Figure 2.2) ( Geerling et al., 2010) (Paxinos and Watson, 2005) regions of interest were drawn on photoshop images captured with a light microscope. The regions identified for quantifying stained profiles were the medial (PaMP), ventral (PaV) and dorsal (PaDP) parvocellar regions of the PVN. Profiles were counted in each of these regions separately, bilaterally and two sizes were identified: > 10 μm and < 10 μm.

## *6.4 Results*

### *6.4.1 Behavioural analysis*

The pre-injury (or pre-anaesthetic) behaviours for the 20 rats used for immunohistochemistry are shown in Figure 6.1a. There is no difference in pre-injury levels in the four different scored behaviours for the two groups of injured animals or the control rats ($p>0.05$, independent samples t-test). The seven BC rats chosen for the procedure were not different to the total group

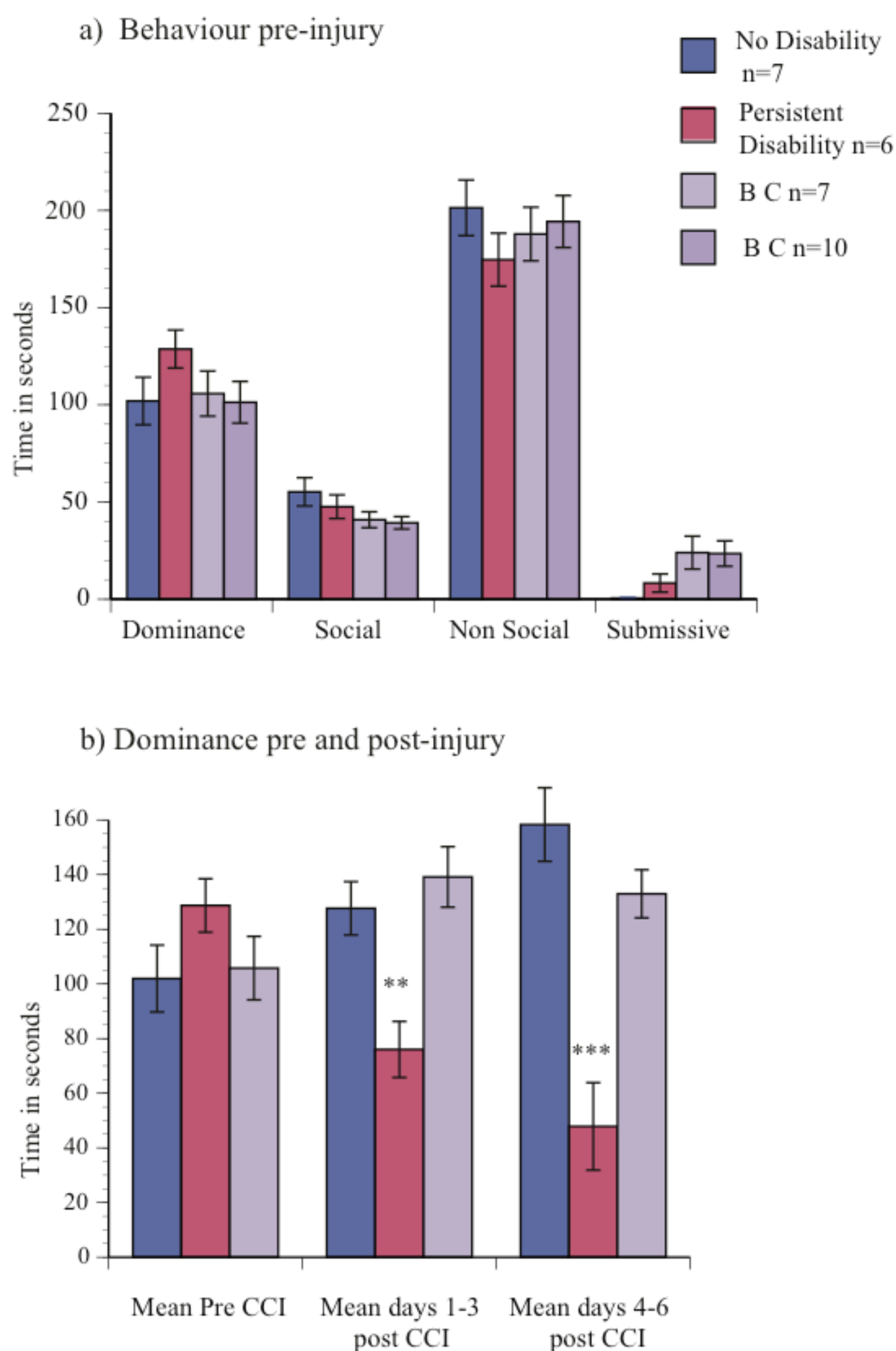



**Figure 6.1:** (a) Histogram illustrating mean pre-injury levels (± SEM) of behaviour (Dominance, Social, Non Social and Submissive) in the rats used in the immunochemistry studies No Disability (ND), Persistent Disability (PD) and Behavioural Controls n=7 and n=10. (b) Post-injury levels of Dominance behaviour for the ND (n=7), PD (n=6) and BC (n=7) rats . Changes that are significantly different to the ND group are indicated: *** $p<0.001$ and ** $p<0.01$.

of BC rats. The seven randomly chosen ND rats were not different to the total group of ND rats before injury (mean pre-injury D, 102.0 ± 12.2 seconds (n=7); D, 100.9±9.7 seconds (n=20)).

Figure 6.1b illustrates the change in mean dominance behaviour following intervention for the three groups of animals. Compared to the ND rats, the PD group shows decreased D on days 1-3 and days 4-6 post-injury ($p<0.01$ and $p<0.001$ respectively). The BC group however, like the ND animals, maintains dominance behaviour over the 6 days following the injury/anaesthetic.

### *6.4.2 Analysis of protein expression*

As no consistent difference was found between right and left hemi-hypothalamus sections other than what was explainable by asymmetry (a issue related to cutting of sections), the numbers of profiles for each animal were determined as total counts in each section counted. Two brains were excluded from the analysis as the tissue was too degraded for quantitative analysis: both these brains were from BC animals.

#### *6.4.2.1 Localisation of TRβ-like protein*

##### *6.4.2.1.1 Qualitative analysis*

Specific staining indicating the presence of TRβ-like protein was found in numerous anatomical structures: in general, staining was found in regions of cellular elements and not in fiber tracts. Immunoreactive profiles greater than 10 μm were found in the PVN and in other hypothalamic nuclei. Staining was seen in the thalamus, the hippocampus and the cortex. In Figure 6.2 some representative micrographs showing staining of TRβ-like protein are given. The images of the PVN in Figure 6.2a and 6.2b are from a BC rat and a PD rat respectively.

##### *6.4.2.1.2 Statistical analysis of immunoreactive profiles*

The results obtained for each behavioural group for total profiles counted in four sections (-2.1, -1.9, -1.7, -1.5 mm to bregma) are given in the following table; large and small profiles were counted as described above. The results obtained

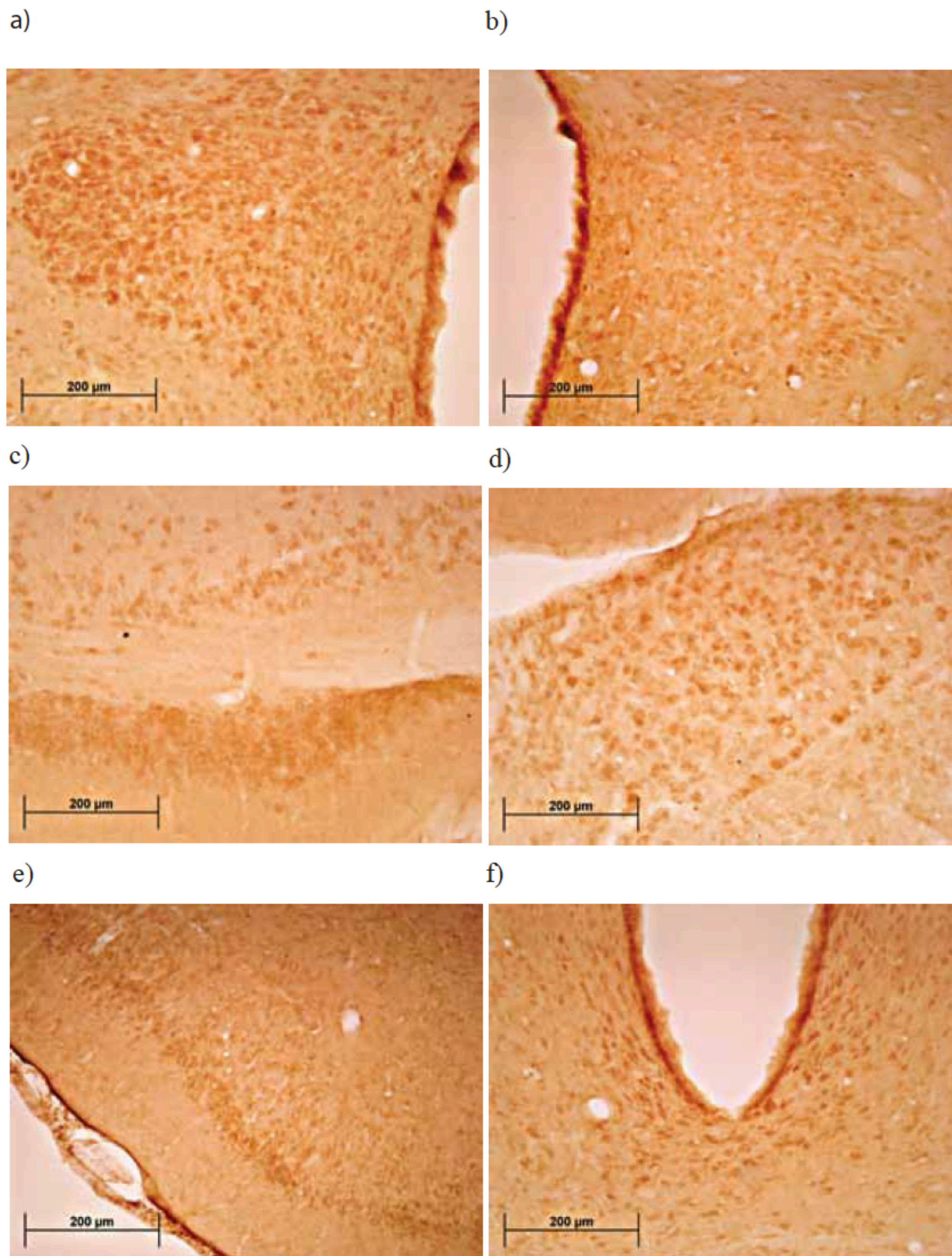



**Figure 6.2:** Representative micrographs of immunoeactive profiles obtained using an antibody to TRbeta. Sections were observed under magnification of x200: scale bars as indicated.
a) and b) Paraventricular nucleus at -1.7 mm to bregma in a BC rat and a PD rat respectively. c) Hippocampus in a BC rat d) Anterodorsal thalamus in a BC rat
e) Cortex in a BC rat and f) Periventricular hypothalamic nucleus in a BC rat all at -1.9mm to bregma.

for the total large profiles in two sections (-1.9 and -1.7 mm to bregma) are also given.

**Number of immunoreactive profiles using an antibody to TRβ**

| Behavioural Group | Total All Sections | Large >10 μm | Small < 10 μm | Large > 10 μm bregma sections -1.9 and -1.7 only |
|---|---|---|---|---|
| Behavioural Controls | 1102 ± 59.3 n=5 | 770 ± 39.7 | 332 ± 43.1 | 555 ± 35.0 n=5 |
| No Disability | 867 ± 115.0 n=6 | 624 ± 70.9 | 242 ± 46.7 | 433 ± 51.2 n=7 |
| Permanent Disability | 780 ± 91.2 n=6 | 547 ± 68.6 | 233 ± 37.0 | 411 ± 55.5 n=6 |

**Table 6.1**: Mean numbers (±SEM) of immunoreactive profiles, using an antibody to TRβ, for each behavioural group of rats in the four sections of PVN counted: total numbers of profiles and large and small numbers of profiles are shown separately. Mean numbers (±SEM) of large profiles in the -1.9 and -1.7 mm to bregma sections only, are also given. The number of rats that were included in the data is indicated.

Differences between behavioural groups in mean numbers of immunoreactive profiles were evaluated using the Mann-Whitney U test. The distribution of total profiles for the ND group (Md=869) was not different to the BC group (Md=1136, p>0.05) whereas there was a difference between the PD group (Md=758) and the BC group (p=0.018). When the numbers of large profiles only were evaluated, there was no difference between the BC and ND groups (BC, Md=744; ND, Md=651; p>0.05); there was a just significant difference between the BC and PD groups (PD, Md=496; p=0.045, Mann-Whitney U test). There is no difference between the distributions of small profile numbers when comparing the BC group to the ND group or the PD group (p>0.05). The difference between the groups is maintained when the numbers of large profiles in the -1.9 and -1.7 mm to bregma sections only are evaluated: the BC group (Md=555) is not different to the ND group (Md=488; p>0.05) whereas there is a difference between the BC (Md=555) and PD (Md=390) groups (p=0.045, Mann-Whitney U test).

Compared to the BC group, the variance within the groups of injured rats is large. Figure 6.3a displays the individual data points for the numbers of large profiles in the -1.9 and -1.7 mm to bregma sections. While the variance is large within the ND group, the data is not skewed. In the PD group however, one animal is a clear outlier; for both, numbers of total large profiles and those in the -1.9 and -1.7 mm to bregma sections only, the values for rat #107 are greater than 3 SD units above the mean of the other 5 rats. If this outlier is removed, the mean numbers of profiles for the PD group are as shown in Table 6.2. With the outlier removed, the differences between the BC group and the PD group are as follows: for total profiles PD (Md=738; p=0.016); for large profiles (Md=481; p=0.009); and for large profiles in sections -1.9 and -1.7 mm to bregma only (Md=375; p=0.009, Mann-Whitney U test).

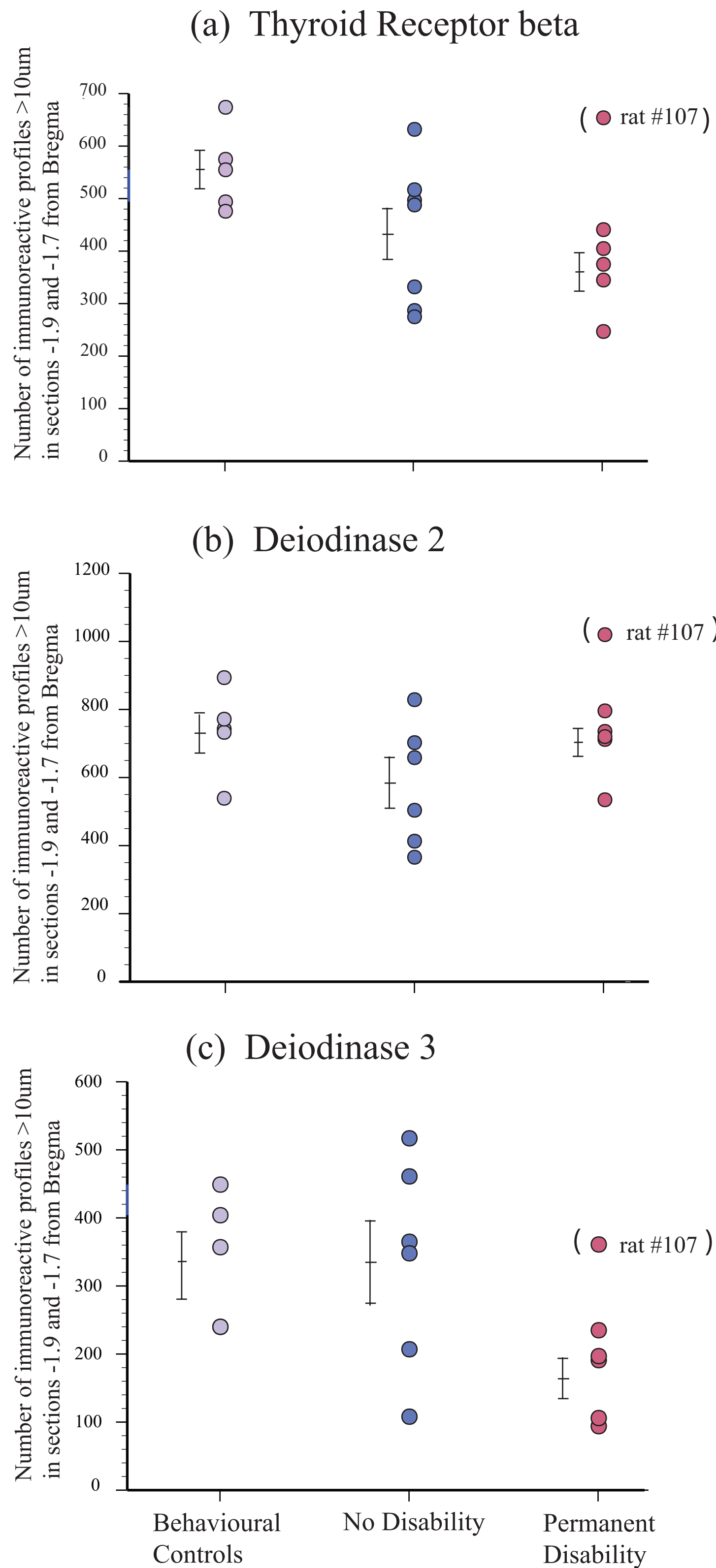



**Figure 6.3:** Scattergrams illustrating the spread of data points (mean +/- SEM) for numbers of immunoreactive profiles (>10um) in the hypothalamic sections Bregma -1.9 and -1.7, for each group of rats using antibodies for (a) Thyroid Receptor beta (b) Deiodinase 2 and (c) Deiodinase3. Behavioural Controls n=5: No Disability rats n=6 or 7; Permanent Disability rats n=6. Mean for the PD group in each case excludes rat #107: values for this rat are >3SD from the mean of the other 5 rats.

**Number of immunoreactive profiles using an antibody to TRβ in the PD group after removal of data related to rat #107**

| Behavioural Group | Total All Sections | Large >10 μm | Small < 10 μm | Large > 10 μm bregma sections -1.9 and -1.7 only |
|---|---|---|---|---|
| Permanent Disability Excluding rat#107 | 713 ± 76.9 n=5 | 486 ± 38.7 | 228 ± 44.9 | 363 ± 33.0 n=5 |

**Table 6.2:** Mean numbers (±SEM) of immunoreactive profiles for the PD group with data for the outlier (rat #107) removed. For the four sections of PVN counted: total numbers of profiles and large and small numbers of profiles are shown separately. Mean numbers (±SEM) of large profiles in the -1.9 and -1.7 mm to bregma sections only, are also given. The number of rats that were included in the data is indicated.

A histogram illustrating the numbers of immunoreactive profiles obtained using an antibody to TRβ (large, small and total) in the four sections counted as well as the numbers of large immunoreactive profiles seen in the -1.9 and -1.7 mm to bregma sections only, in the different behavioural groups is given in Figure 6.4.

### *6.4.2.2 Localisation of TRH-like protein*

#### *6.4.2.2.1 Qualitative analysis*

Although some specific staining was obtained using an antibody to synthetic TRH the quality of staining was inferior to that obtained with the other antibodies.

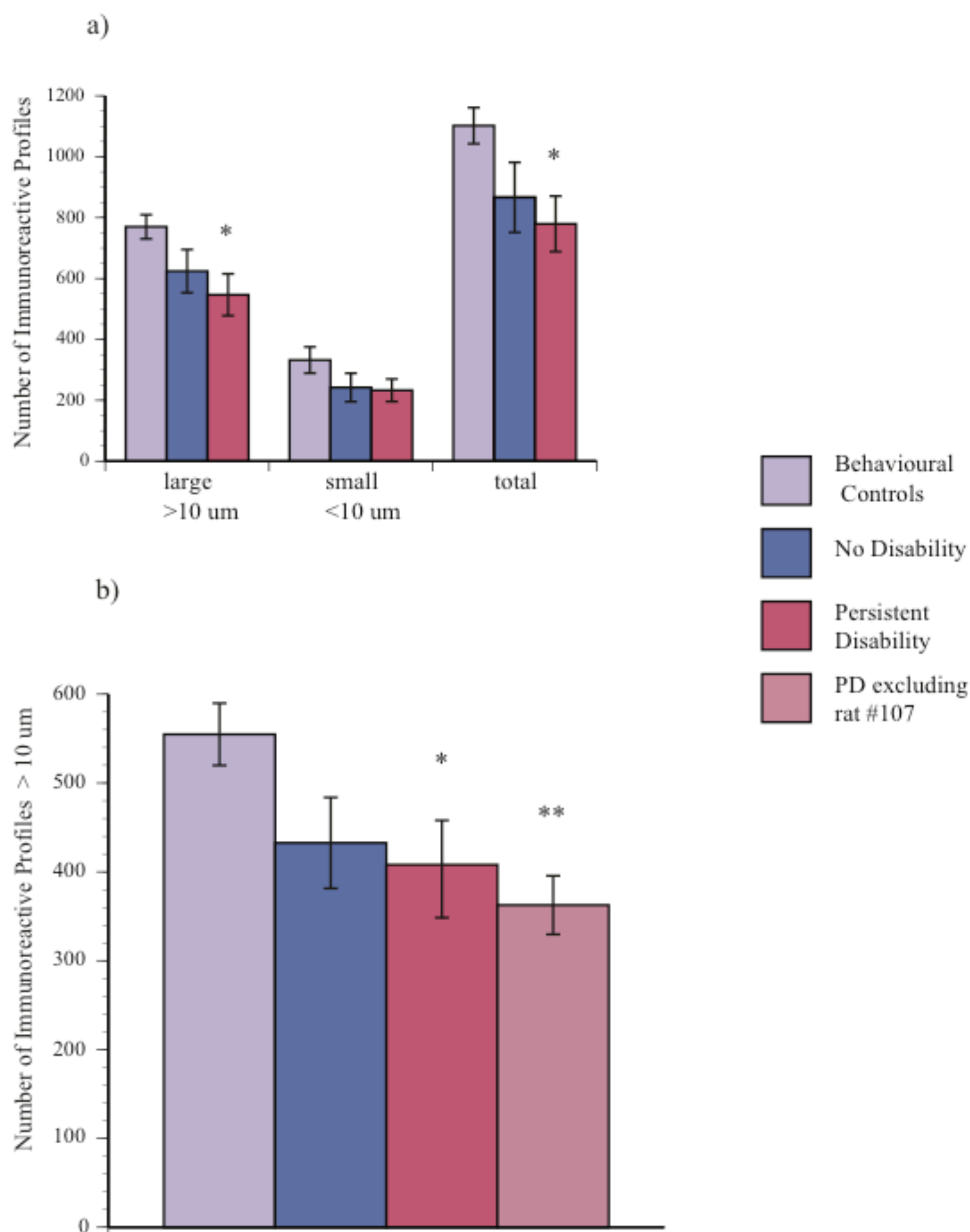



**Figure 6.4:** Histogram illustrating the number of immunoreactive profiles in the Paraventricular Nucleus (PVN) using an antibody to Thyroid Receptor beta.
a) Large (>10 um), small (<10 um) and total profiles in four sections of PVN -2.1, -1.9, -1.7 and -1.5 mm to bregma.
b) Number of large (>10 um) profiles in sections -1.9 and -1.7 mm to bregma.
Numbers of profiles significantly different to the Behavioural Control group are indicated: *p<0.05; **p<0.01 (Mann-Whitney U test).

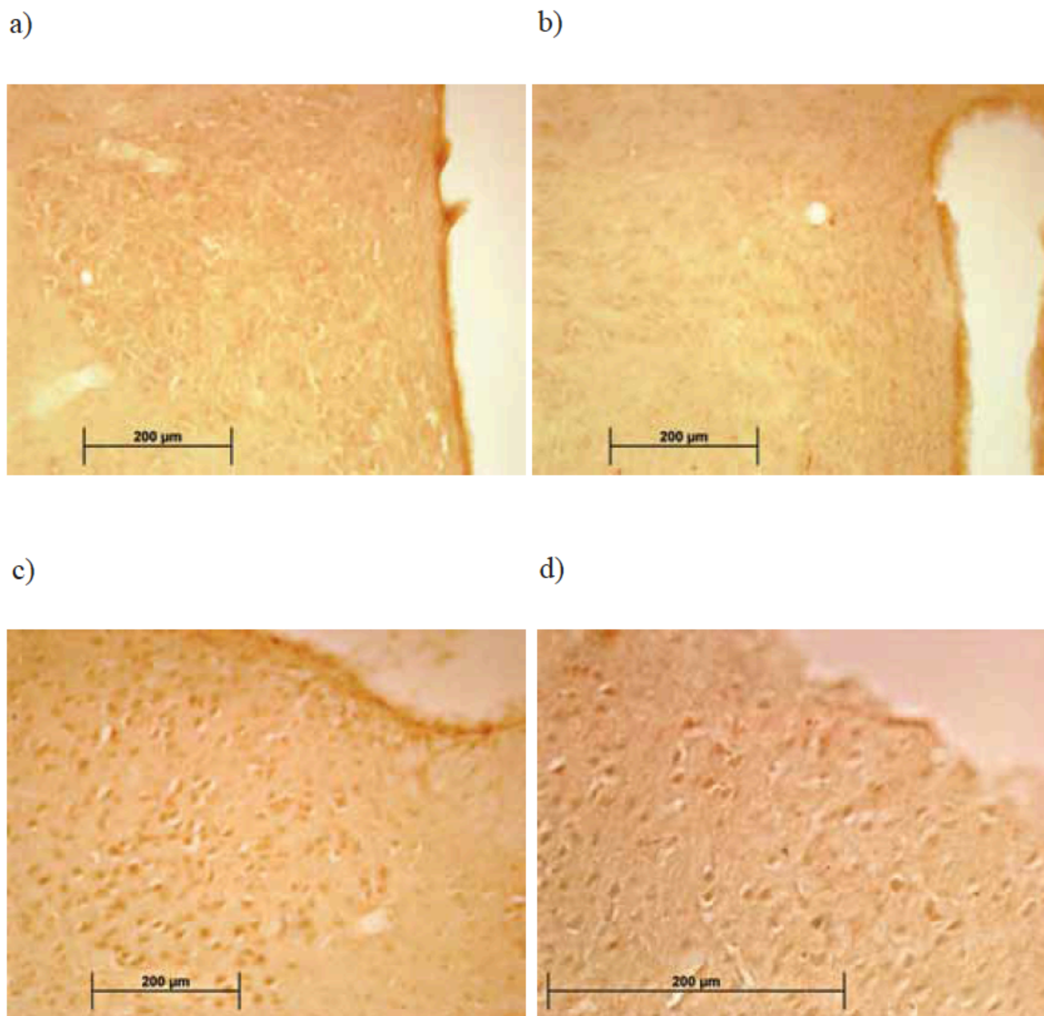



**Figure 6.5:** Representative micrographs of immunoreactive profiles obtained using an antibody to synthetic TRH. Sections were observed under magnification of x200 or x400: scale bars as indicated.
a) and b) Paraventricular nucleus at -1.9 mm to bregma in a BC rat and a PD rat respectively; staining was minimal in the PVN using this antibody.
c) Anteriodorsal thalamus in a BC rat at -1.9 mm to bregma and d) Cortex in a PD rat at -1.9 mm to bregma.

Immunoreactive profiles were observed in thalamus and cortex and some in hypothalamic nuclei. In the PVN however, the background staining was high and few profiles could be said to satisfy the criteria for inclusion of twice background intensity. In Figure 6.5 some representative micrographs showing staining of TRH-like protein are given. The images of the PVN in Figure 6.5a and 6.5b are from a BC rat and a PD rat respectively.

The numbers of immunoreactive profiles in the PVN were low for all groups of rats and numbers were such that no attempt could be made to compare data between groups. As will be discussed below, this finding is consistent with reports in the literature using immunohistochemistry techniques to localise TRH in euthyroid rats.

### *6.4.2.3 Localisation of Deiodinase 2-like protein*

#### *6.4.2.3.1 Qualitative analysis*

Specific staining indicating the presence of Dio2-like protein was found in numerous anatomical structures: in general, staining was found in regions of cellular elements and not in fiber tracts. Immunoreactive profiles greater than 10 μm were found in the PVN, and in other hypothalamic nuclei. Staining was seen in the thalamus, the hippocampus and the cortex. In Figure 6.6 some representative micrographs showing staining of Dio2-like protein are given. The images of the PVN in Figure 6.6a and 6.6b are from a BC rat and a ND rat respectively. Intense staining was observed in the ependymal cell layer in some animals and these profiles were not included; only profiles that were clearly separated from the ependymal layer and lay fully within the PVN were counted.

#### *6.4.2.3.2 Statistical analysis of immunoreactive profiles*

The results obtained for each behavioural group for total profiles counted in the four sections (-2.1, -1.9, -1.7, -1.5 mm to bregma) are given in the following table; large and small profiles were counted as described above. The results obtained for the total large profiles in two sections (-1.9 and -1.7 mm to bregma) are also given.

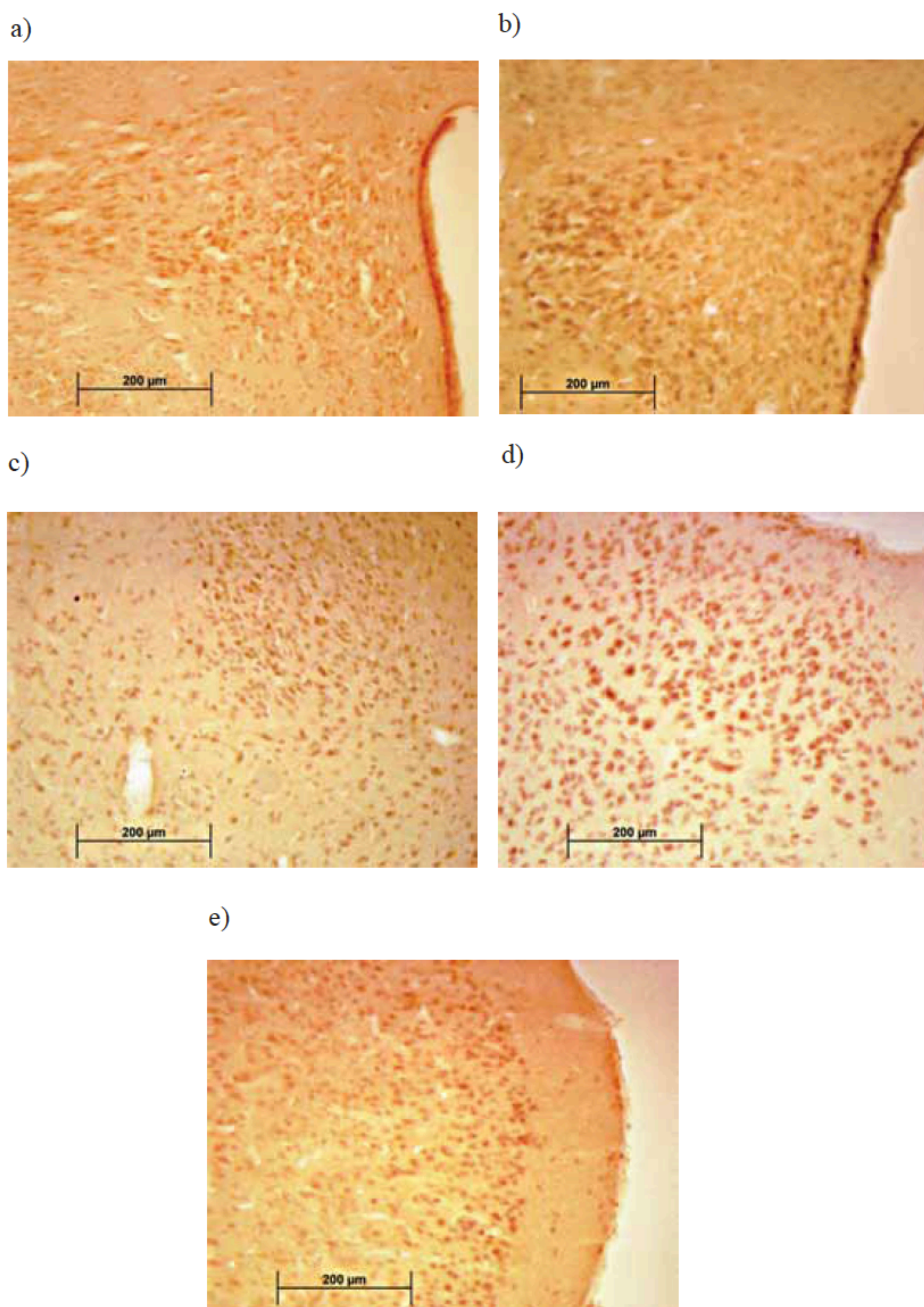



**Figure 6.6:** Representative micrographs of immunoreactive profiles obtained using an antibody to Deiodinase 2. Sections were observed under magnification x200: scale bars as indicated.
a) and b) Paraventricular nucleus at -1.9 mm to bregma in a BC and ND rat respectively. c) Ventromedial hypothalamic nucleus in a PD rat d) Anterodorsal thalamus in a PD rat and e) Cortex in a PD rat all at -1.9 mm to bregma.

**Number of immunoreactive profiles using an antibody to Dio2**

| Behavioural Group | Total All Sections | Large >10 μm | Small < 10 μm | Large > 10 μm bregma sections -1.9 and -1.7 only |
|---|---|---|---|---|
| Behavioural Controls | 1944 ± 60.8 n=4 | 1117 ± 136.1 | 827 ± 150.7 | 737 ± 56.8 n=5 |
| No Disability | 1225 ± 185.1 n=5 | 776 ± 94.9 | 449 ± 103.3 | 582 ± 73.6 n=6 |
| Permanent Disability | 1626 ± 178.6 n=5 | 1067 ± 122.9 | 558 ± 94.9 | 758 ± 69.4 n=6 |

**Table 6.3**: Mean numbers (±SEM) of immunoreactive profiles, using an antibody to Dio2, for each behavioural group of rats in the four sections of PVN counted: total numbers of profiles and large and small numbers of profiles are shown separately. Mean numbers (±SEM) of large profiles in sections -1.9 and -1.7 mm to bregma only, are also given. The number of rats that were included in the data is indicated.

Differences between behavioural groups in mean numbers of immunoreactive profiles were evaluated using the Mann-Whitney U test. The distribution of total profiles for the ND group (Md= 1114) was not different to the BC group (Md=1945) (p=0.05) nor was there a difference between the PD group (Md=1470) and the BC group (p>0.05). When the numbers of large profiles only were evaluated, there was no difference between the BC and the ND and PD groups (BC, Md=1176; ND, Md=773; PD, Md=1000; p>0.05, Mann-Whitney U test). There was no difference between the distributions of small profile numbers when comparing the BC group to the ND group or the PD group (p>0.05). Similarly, there were no differences between the groups when the numbers of large profiles in the -1.9 and -1.7 mm to bregma sections were evaluated: the BC group (Md= 739) is not different to the ND group (Md=592) or PD group (Md=729) (p>0.05, Mann-Whitney U test).

Compared to the BC group, the variance within the groups of injured rats is again large. Figure 6.3b displays the individual data points for the numbers of large profiles in the -1.9 and -1.7 sections. While the variance is large within the ND group, the data is not skewed. In the PD group however, one animal is a clear outlier, again rat #107. For numbers of large profiles in the -1.9 and -1.7 mm to bregma sections only, the values for rat #107 are greater than 3 SD units above the mean of the other 5 rats. If this outlier is removed the mean number of profiles for the PD group are 699 ± 43. With the outlier removed there is still no difference between the BC group (Md=739) and the PD group (Md=725) (p>0.05 (Mann-Whitney U test).

A histogram illustrating the numbers of immunoreactive profiles obtained using an antibody to Dio2 (large, small and total) in the four sections counted as well as the numbers of large immunoreactive profiles seen in the -1.9 and -1.7 mm to bregma sections only, in the different behavioural groups is given in Figure 6.7.

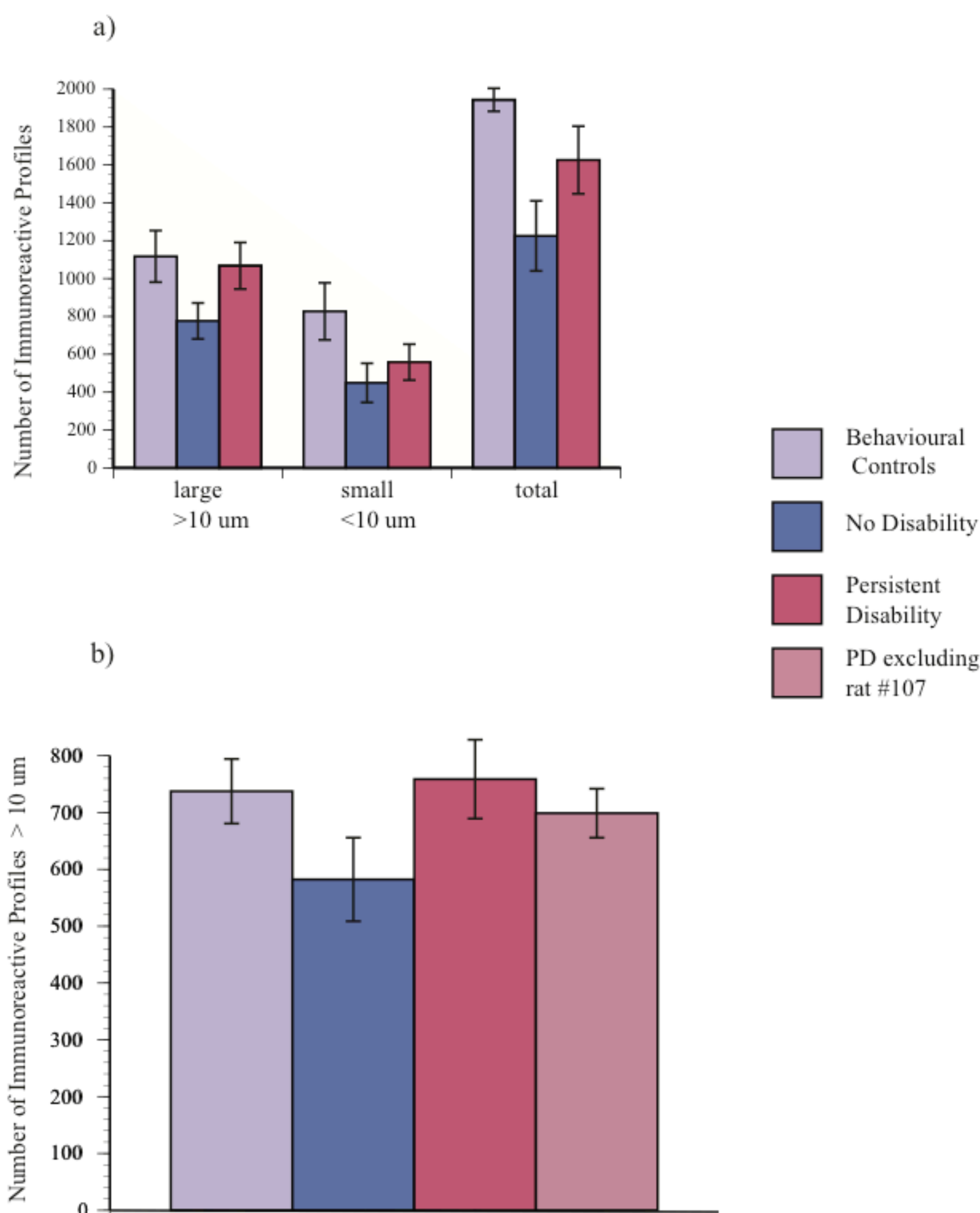



**Figure 6.7:** Histogram illustrating the number of immunoreactive profiles in the Paraventricular Nucleus (PVN) using an antibody to Deiodinase 2.
a) Large (>10 um), small (<10 um) and total profiles in four sections of PVN -2.1, -1.9, -1.7 and -1.5 mm to bregma
b) Number of large (>10 um) profiles in sections -1.9 and -1.7 mm to bregma. There were no differences in numbers of profiles between the BC group and any other group: $p>0.05$ (Mann-Whitney U test).

### *6.4.2.4 Localisation of Deiodinase 3-like protein*

#### *6.4.2.4.1 Qualitative analysis*

Specific staining indicating the presence of Deiodinase 3-like protein was found in numerous anatomical structures: in general, staining was found in regions of cellular elements and not in fiber tracts. Immunoreactive profiles greater than 10 μm were found in the PVN and in other hypothalamic nuclei. Staining was seen in the thalamus, the hippocampus and the cortex. In Figure 6.8 some representative micrographs showing staining of Dio3-like protein are given. The images of the PVN in Figure 6.8a and 6.8b are from a BC rat and a PD rat respectively.

#### *6.4.2.4.2 Statistical analysis of immunoreactive profiles*

The results obtained for each behavioural group for total profiles counted in the four sections (-2.1, -1.9, -1.7, -1.5 mm to bregma) are given in the following table; large and small profiles were counted as described above. The results obtained for the total large profiles in two sections -1.9 and -1.7 mm to bregma are also given.

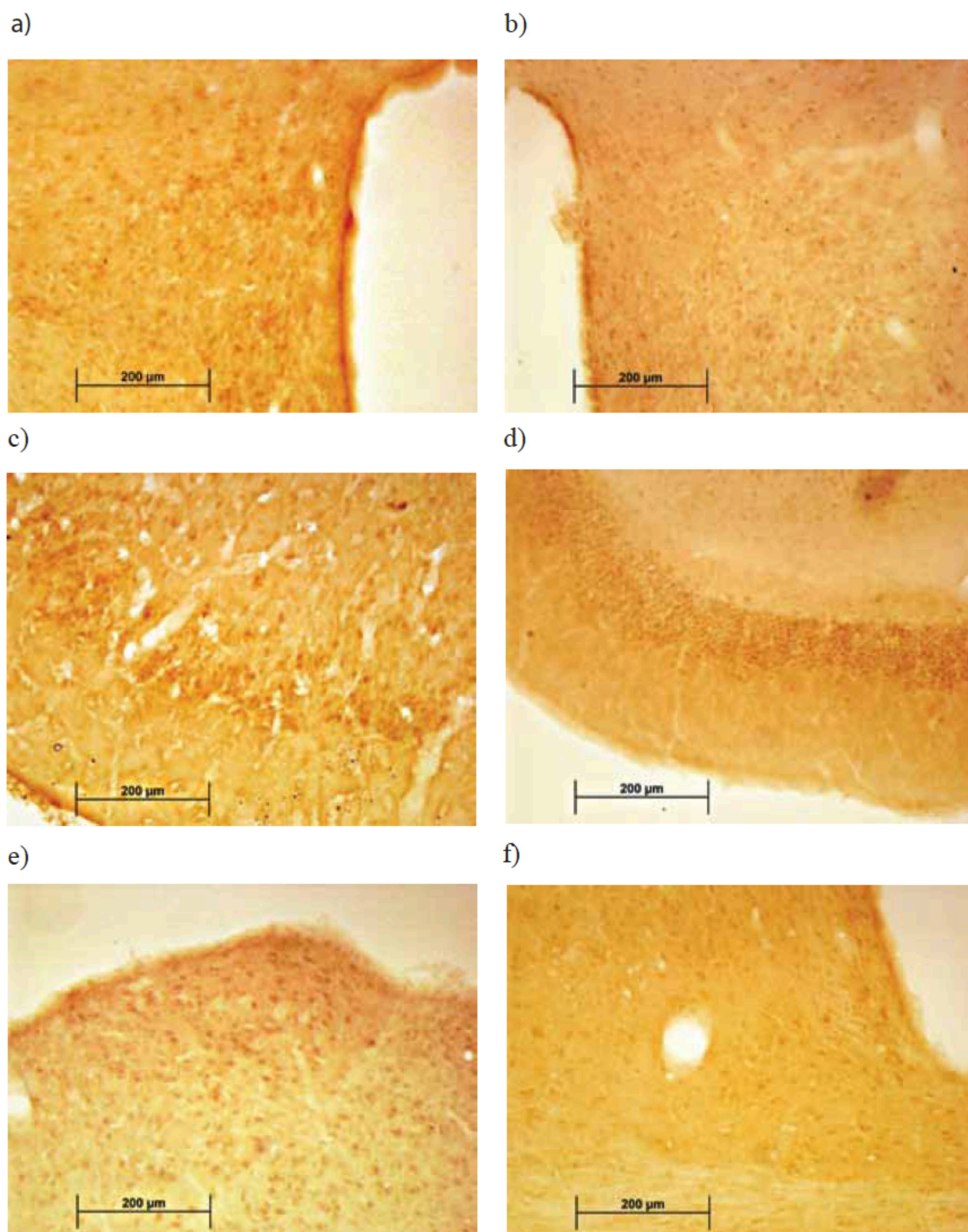



Figure 6.8: Representative micrographs of immunoreactive profiles obtained using an antibody to Deiodinase 3. Sections were observed under magnification of x200: scale bars as indicated.
a) and b) Paraventricular nucleus at -1.9 mm to bregma in a BC rat and a PD rat respectively. c) Cortex in a BC rat d) Hippocampus in a BC rat e) Anterodorsal thalamus in a BC rat and f) Arcuate nucleus in a BC rat all at -1.9 mm to bregma.

**Number of immunoreactive profiles using an antibody to Dio3**

| Behavioural Group | Total All Sections | Large >10 μm | Small < 10 μm | Large > 10 μm bregma sections -1.9 and -1.7 only |
|---|---|---|---|---|
| Behavioural Controls | 1062 ± 130.4 n=4 | 491 ± 66.5 | 572 ± 83.2 | 338 ± 42.6 n=5 |
| No Disability | 1011 ± 171.7 n=6 | 442 ± 79.1 | 570 ± 105.4 | 336 ± 53.0 n=7 |
| Permanent Disability | 773 ± 80.0 n=6 | 295 ± 54.6 | 478 ± 50.8 | 197 ± 39.7 n=6 |

**Table 6.4**: Mean numbers (±SEM) of immunoreactive profiles, using an antibody to Dio3, for each behavioural group of rats in the four sections of PVN counted: total numbers of profiles and large and small numbers of profiles are shown separately. Mean numbers (±SEM) of large profiles in the -1.9 and -1.7 mm to bregma sections only, are also given. The number of rats that were included in the data is indicated.

Differences between behavioural groups in mean numbers of immunoreactive profiles were evaluated using the Mann-Whitney U test. The distribution of total profiles for the ND group (Md=1029) and the PD group (Md=734) were not different to the BC group (Md= 1085) ($p>0.05$). When the total numbers of large profiles were evaluated there was no difference between the BC group (Md=485) and ND group (Md=458) or the PD group (Md=282) ($p>0.05$, Mann-Whitney U test). There was no difference between the distributions of small profile numbers when comparing the BC group to the ND group or the PD group ($p>0.05$). There was no difference between the BC group (Md=357) and the ND group (Md=349) when the numbers of large profiles in the -1.9 and -1.7 mm to bregma sections only, were evaluated: however there was a significant difference between the BC group and the PD group (Md=194) ($p=0.028$, Mann-Whitney U test).

Compared to the BC group, the variance within the groups of injured rats is large. Figure 6.3c displays the individual data points for the numbers of large profiles in the -1.9 and -1.7 mm to bregma sections. While the variance is large within the ND group, the data is not skewed. In the PD group however, one animal is a clear outlier, again rat #107. For numbers of total large profiles and those in the -1.9 and -1.7 mm to bregma sections, the values for rat #107 are greater than 3 SD units above the mean of the other 5 rats. If this outlier is removed the mean number of profiles for the PD group are as shown in Table 6.5. With the outlier removed the differences between the BC group and the PD group are as follows: for total profiles (PD, Md=722; $p=0.05$); for large profiles (PD, Md= 249; $p=0.027$); for large profiles in sections -1.9 and -1.7 only (PD, Md=191; $p=0.009$; Mann-Whitney U test).

**Number of immunoreactive profiles using an antibody to Dio3 in the PD group after removal of data related to rat #107**

| Behavioural Group | Total All Sections | Large >10 µm | Small < 10 µm | Large > 10 µm bregma sections -1.9 and -1.7 only |
|---|---|---|---|---|
| Permanent Disability | 720 ± 72.5 n=5 | 251 ± 39.7 | 468 ± 61.1 | 165 ± 27.5 n=5 |

**Table 6.5:** Mean numbers (±SEM) of immunoreactive profiles for the PD group with data for the outlier (rat #107) removed (antibody to Dio3). For the four sections of PVN counted: total numbers of profiles and large and small numbers of profiles are shown separately. Mean numbers (±SEM) of large profiles in the -1.9 and -1.7 mm to bregma sections only, are also given. The number of rats that were included in the data is indicated.

A histogram illustrating the numbers of immunoreactive profiles obtained using an antibody to Dio3 (large, small and total) in the four sections counted as well as the numbers of large immunoreactive profiles seen in the -1.9 and -1.7 mm to bregma sections only, in the different behavioural groups is given in Figure 6.9.

##### *6.4.2.4.3 Dio3-like protein superior to the PVN*

No attempt was made to quantify numbers of immunoreactive profiles outside the PVN. However, intense staining just superior to the PVN was observed in some animals. In Figure 6.10 some representative micrographs showing staining of

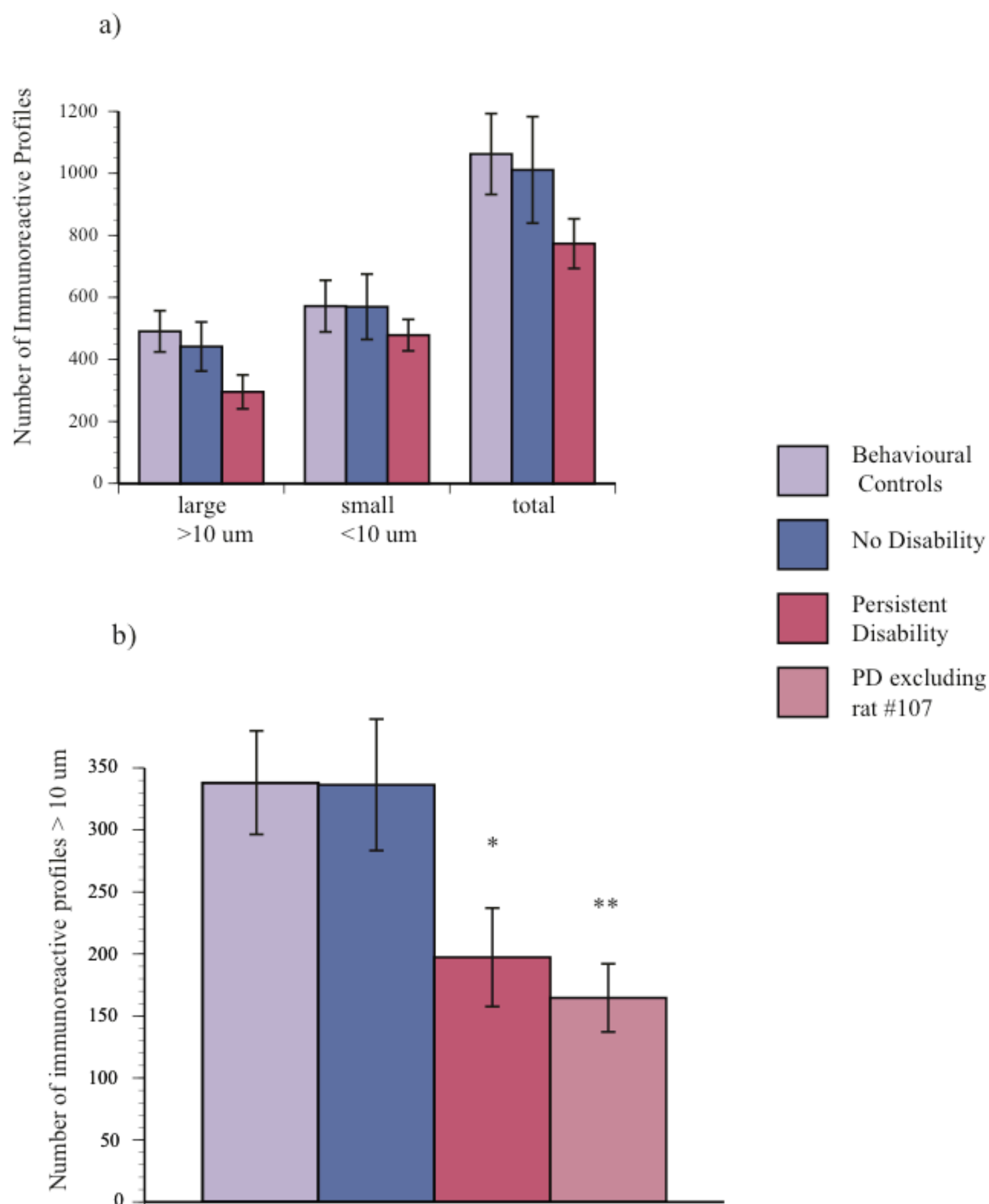



**Figure 6.9:** Histograms illustrating the number of immunoreactive profiles in the Paraventricular Nucleus using an antibody to Deiodinase 3.
a) Large (>10 um), small (<10 um) and total profiles in four sections of PVN -2.1,-1.9,-1.7 and -1.5 mm to bregma.
b) Number of large (>10 um) profiles in sections -1.9 and -1.7 mm to bragma. Numbers of profiles significantly different to the Behavioural Control group are indicated: *p<0.05; **p<0.01 (Mann-Whitney U test).

Dio3-like protein outside the PVN are given. The images of the region superior to the PVN in Figure 6.10 (a) and (c) are from BC rats, those in (b) and (d) from PD rats and (e) from a ND rat respectively.

## *6.5 Discussion*

The aim of this study was to determine the relative distribution within the PVN of proteins that may be involved in altered HPT regulation following chronic constriction injury of the sciatic nerve in rats. This was an exploratory first pass analysis with the focus on determining if there were differences in numbers of stained profiles within the region of the PVN where the TRH hypophysiotrophic neurons have been shown to be located (Fekete and Lechan, 2007).

Decreased peripheral thyroid hormones, an inadequate TSH response and down regulation of mRNA for TRH have been reported in previous chapters in the sub-group of rats with altered social behaviour. These findings are supportive of HPT dysregulation in this sub-group similar to that reported in other animal models in response to inflammatory stress. The mechanism hypothesised as responsible for HPT suppression is a local thyrotoxicosis, specific to the region of the hypophsiotrophic TRH neurons. Evidence for this local increase in tissue T3 comes from studies that have demonstrated increased deiodinase 2 and/or decreased deiodinase 3 gene expression in the hypothalamus (Boelen et al., 2004) (Fekete et al., 2004) (Boelen et al., 2006) (Mebis et al., 2009). The results presented in this chapter, within the limitations discussed, are generally supportive of a localised thyrotoxicosis as the mechanism responsible for the down-regulation of TRH in the rats with altered behaviour.

### *6.5.1 Behavioural analysis*

As shown in Figure 6.1, the ND and PD groups of animals used in this experiment conform to the criteria for behavioural classification. There is no difference between groups for any of the four behaviours pre-injury. Post-injury, the ND group maintain dominance and the PD group show persistently changed behaviour with dominance scores significantly lower than the ND rats on days 1-3 and 4-6. The injured groups of animals behaviourally resemble those used to measure

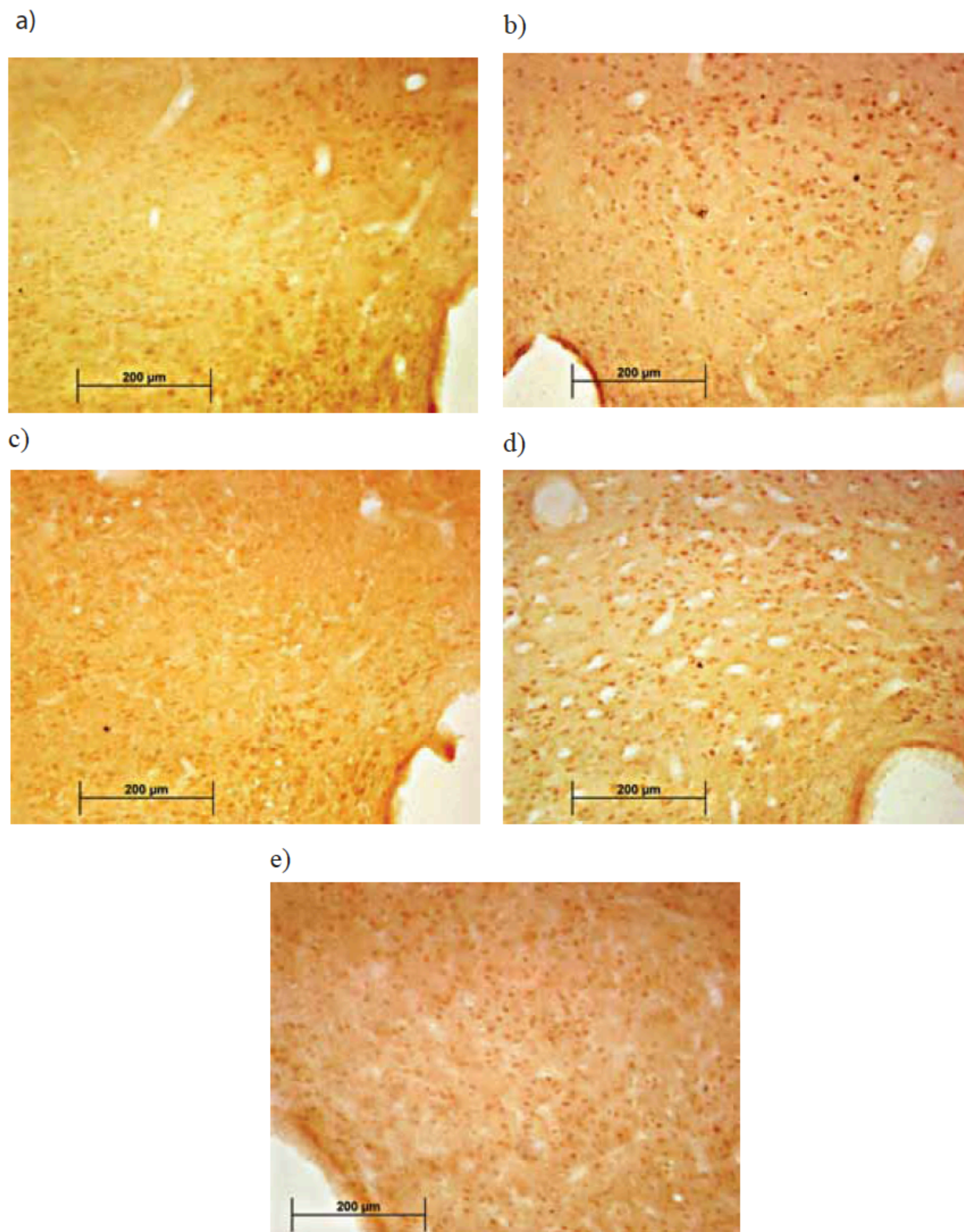



**Figure 6.10:** Representative micrographs of immunoreactive profiles obtained using an antibody to Deiodinase 3. Sections were observed under magnification of x200: scale bars as indicated.
All of the images are taken of the region superior to the Paraventricular nucleus at -1.9 mm to bregma. a) and c) are from two different BC rats. b) and d) are from two different PD rats. e) is from a ND rat.

peripheral hormones as reported in Chapter 4. The rats used as controls were behaviourally no different to the ND rats.

### *6.5.2 Thyroid Hormone Receptor β*

There was a barely significant decrease in staining using the antibody to TRβ in the PD rats when compared to the control rats: with rat #107 removed from the analysis the difference between the number of large immunoreactive profiles in the two groups was significant ($p<0.009$) both looking at total large profiles and those confined to the -1.9 and -1.7 sections only.

This was a surprising finding. There was no difference between the PD group and the BC group for relative gene expression for TRβ as reported in chapter 5. Moreover, others investigating the HPT axis changes seen in inflammation have not reported change in gene expression of TRβ. No change was found using whole hypothalamus extracts in an acute illness model (Boelen et al., 2004) nor specifically in the PVN in the chronic (turpentine) model (Boelen et al., 2006) nor in the chronic (burns) rabbit model (whole hypothalamus) (Mebis et al., 2009). However, in all these studies gene expression was investigated rather than protein expression.

It is possible however that the sub-population defined by behaviour is different and would not have been identified within the findings in the other studies as they represent only 20% of the population. TRβ is expressed in neurons and is essential for TRH regulation (Abel et al., 2001) but is found in astrocytes and possibly in oligodendroglia (Carlson et al., 1994) (Trentin, 2006). In this exploratory study, no attempt was made to identify cell type.

An alternate explanation for a decreased expression of TRβ-like protein is that the antigen is less available to react with the antibody (Cuello, 1993) (Saper, 2009). A possible reason for this reduced sensitivity of the antibody for the TRβ-like protein is that there could be more ligand bound to the receptor making it less available for binding, possibly due to conformational change (Saper, 2009).

When T3 binds to its receptor there is conformational change of chromatin locally and binding of TREs (Boelaert and Franklyn, 2005). Thus it seems possible that increased local concentration of T3 could result in less binding of antibody to the TRβ protein. If indeed the PD rats have a local thyrotoxicosis in the PVN, as has been hypothesised, as the mechanism for down regulation of TRH in inflammation, then there would be greater binding of T3 to the TRβ in these rats.

### *6.5.3 Thyrotrophin Releasing Hormone*

Although specific binding of the antibody to TRH-like protein was seen in a number of regions outside the PVN, little specific staining of significant intensity was seen in the PVN of the rats. No qualitative differences was observable between different groups of rats and no quantification was possible to assess whether there was lower protein expression for TRH in the PD rats to match the down regulation of TRH mRNA reported in chapter 5.

While these immunohistochemical observations did not lend support to the PCR data, they are not unexpected. In all of the models reporting down regulation of TRH following inflammation, gene expression has been investigated either using RT-qPCR or *in situ* hybridization techniques. Identifying TRH protein in this region is difficult as has been discussed in section 1.8.5: specifically in the PVN, immunoreactive TRH protein is only identified in hypothyroid animals or in animals treated with colchicine to prevent the neurons releasing the protein, there is little cellular staining in euthyroid animals (Nishiyama et al., 1985) (Kreider et al., 1985) (Cintra et al., 1990) (Espinosa et al., 2007). It would be expected that the BC rats were euthyroid; the ND rats are in a state of reestablishing equilibrium after HPT axis stimulation and the PD rats were relatively hypothyroid, without increased TSH; so poor visualisation of TRH protein in the region of the TRH hypophysiotrophic neurons was to be expected. Additionally, the antibody used was not a specific rat antibody; it was made against a synthetic TRH and was not necessarily optimal. Some staining was observed outside the PVN however; this is in agreement with other reports of TRH protein throughout the CNS (Kreider et al., 1985) (Chung et al., 1989) (Fekete and Lechan, 2007).

### *6.5.4 Deiodinase 2*

There were no differences between groups of rats for staining for Dio-2-like protein in the PVN. The literature describes the primary location of Dio-2 mRNA as being in the MBH and ME (Tu et al., 1997) (Diano et al., 2003) and no attempt to quantify differences in those regions was made here. In the turpentine mice, increased mRNA for Dio2 was found in whole hypothalamus extracted RNA but not in that specifically from the PVN (Boelen et al., 2006). There is however, no support for an increase in Dio-2 protein locally in the PVN (resulting in a local increase in T3) as an explanation for the down regulation of TRH mRNA reported in chapter 5. At least, not at day seven following injury. An experimental design that allowed for assessment of hypothalamic factors earlier in the post-injury period would be useful to clarify whether increases in Dio-2 enzyme occurred in any of the rats using this model.

If some animals were euthanased after two days, any early changes in Dio2 could be determined, although when assessing protein expression, the MBH and ME would be more interesting than the PVN. However, because of the way in which behavioural groups are classified, while the ND group could be identified after two days, the PD group is not distinguished from the TD group at this time. The TD group can be distinguished from the PD groups at day 2 post-injury if peripheral hormones are measured, however given the size of these two sub-groups, 10% and 20% of the population respectively, large numbers of rats would need to be used to find statistically significant differences.

Rather than an increase in Dio2-like protein in the PVN, many of the rats in the ND group appear to have a decrease, although wide variance within the group means that the group as a whole does not differ from the BC group ($p=0.05$) (Figure 6.3b). Decreased Dio2 enzyme would be consistent with animals that are somewhat hyperthyroid according to the proposed model of hypothalamic feedback (Fliers et al., 2006). However, the significance of the protein in the PVN is not clear.

The finding that the PD group do not differ from the BC group for Dio2-like protein in the PVN is not surprising given the findings reported in the turpentine mice (Boelen et al., 2006) that showed no difference for Dio2 mRNA in the PVN. What is still not clear however is why the PD group had significantly less Dio2 mRNA in the whole hypothalamus extracts as reported in chapter 5. Future quantification of the Dio2-like staining in the MBH may reveal if there is a difference between the groups in this region.

### *6.5.5 Deiodinase 3*

Staining of Dio3-like protein in the PVN of the PD rats, specifically in the region of the hypophysiotrophic TRH neurons, was significantly less than in the control rats. This finding is consistent with the down regulation of Dio3 mRNA in the PVN seen in the turpentine abscess model at 24 hours post-injection (Boelen et al., 2006). This difference in the PD rats however, is occurring at seven days after injury at a time when TRH mRNA is down regulated. Decreased local Dio3 activity would result in an increased local T3 concentration providing a mechanism for down regulation of TRH mRNA (Koenig, 2008). It seems possible that a change in gene expression, seen in a total population in the first days following inflammatory stress (Boelen et al., 2006), has persisted in this behaviourally identified sub-group.

There is a possible explanation for the seemingly contrary finding of increased mRNA for Dio3 in the PD group reported in chapter 5. This finding was in whole hypothalamus extracted RNA and Boelen *et al.,* reported that their finding of decreased mRNA was specific to the PVN with no difference in whole hypothalamus mRNA for Dio3 (Boelen et al., 2006). While no attempt was made to quantify staining for Dio3-like protein other than in the PVN, staining in the region just superior to the PVN was seen in some animals. As shown in the micrographs in Figure 6.10, the staining in some animals is much more intense than in the PVN. Clearly the results of gene expression for Dio3 mRNA in total hypothalamus could well be different to that for the PVN alone. The intensity of the staining appears to be greater in the experimental animals when compared to the BC rats as examples from both a PD rat and a ND rat show. Further

evaluations of regions of the hypothalamus other than the PVN are required to attempt to fully clarify these results. It would seem that the staining for Dio3-like protein seen in both groups of experimental rats could be related to the inflammatory stress if there is indeed a difference between these animals and the control animals. Dio3 enzyme is found in neurons but is also found in astrocytes and oligodendroglia (Tu et al., 1999) (Courtin et al., 2005) and its up regulation is increasingly being reported associated with inflammatory cells (Boelen et al., 2009) (Simonides et al., 2008).

## *6.6 Summary*

Decreased numbers of immunoreactive profiles were found in the PVN of the PD group of rats for both TRβ-like protein and Dio3-like protein when compared to the control animals. There was no difference in numbers of immunoreactive profiles for Dio2-like protein between injured rats and control rats. Within the limits of this exploratory study, the findings support a persistent decrease in Dio3, specifically in the region of the hypophsiotrophic TRH neurons, as providing an explanation for the down regulation of TRH mRNA seen in this behaviourally defined sub-group. The decrease in TRβ-like protein could represent a less available antigen, due to increased ligand binding, which would be consistent with the Dio3 finding. Unfortunately, confirmation of decreased TRH-like protein in the PVN of the PD rats could not be made. Further exploration of the distribution of immunoreactive profiles in regions of the hypothalamus other than the PVN are required to fully clarify the differences in findings using RT-qPCR techniques and those obtained using immunohistochemistry approaches.

# *CHAPTER SEVEN*

# *General Discussion and Conclusion*

## *General Discussion*

A number of animal models have been employed in the investigation of the changes to the HPT axis seen in humans in acute illness and in particular following acute infection. These changes are said to be adaptive and therefore intervention not warranted. However, with increasing severity of the condition and decreasing thyroid hormone levels there is increased morbidity, resulting in considerable controversy as to when or whether treatment should be implemented (De Groot, 1999) (Utiger, 1995) (Stathatos et al., 2001) (Van den Berghe et al., 1998). In animal models, where the animals are made acutely ill, similar peripheral indices are seen as in acutely ill humans (falls in T3 and T4 with no compensatory rise in TSH). Decrease in systemic deiodinase 2 results in decreased peripheral conversion of T4 to T3, the low T3 syndrome (Adler and Wartofsky, 2007). However, there are also central changes, a number of investigators have documented down regulation of TRH gene expression in the PVN and up-regulation of deiodinase 2 in the MBH (Boelen et al., 2004) (Fekete et al., 2004) (Mebis et al., 2009). The hypothesised explanation is as follows: increased Dio2 expressed by astrocytes and tanycytes in the hypothalamus, in response to cytokines, overrides the normal HPT feedback regulatory mechanism. Increased conversion of T4 to T3 creates a local thyrotoxicosis in the PVN, down-regulating TRH gene expression in the hypophysiotrophic neurons (Lechan and Fekete, 2006) (Fliers et al., 2006). The resultant state is one of tertiary or hypothalamic hypothyroidism.

### *Chronic inflammatory stress*

The aim of the experiments detailed in this thesis was to explore whether HPT axis changes occurred in a model of less severe, chronic inflammatory stress. Further, to evaluate whether there were differences between animals whose

behaviour changed post-inflammatory stress compared to those where normal social behaviours were maintained. While 'sickness behaviour' is seen as adaptive in the acutely sick animal (Hart, 1988) (Konsman et al., 2002) it is more likely maladaptive in non-life threatening chronic conditions and, in humans, is associated with poorer health scores and outcomes (Moussavi et al., 2007).

The findings from these experiments were that the group of rats with changed social behaviour had significantly reduced peripheral thyroid hormones with no compensatory rise in TSH; in the six days after injury, the TSH response was delayed, blunted and inadequate to maintain thyroid hormone levels; hormone levels were still below pre-injury levels after six days. Gene expression for TRH in the hypothalamus was down regulated in these animals and deiodinase gene expression in the hypothalamus differed significantly from the rats that maintained normal behaviour. Techniques identifying protein expression support a reduction in deiodinase 3-like protein specifically in the region of the TRH neurons that control the TSH producing cells of the pituitary. Moreover, corticosteroid levels were not elevated relative to rats that maintained social behaviour and there was no evidence of HPA axis activation in these animals.

There are a number of points for discussion arising from these findings: the significance of the findings using this model in the context of other models examining altered HPT axis regulation following inflammatory stress; possible changes to the HPT equilibrium following nerve injury; mechanisms that may explain altered hypothalamic regulation of TSH in the PD rats and a possible moderating role for corticosterone in this dysregulation. In addition, the relationship between decreased thyroxine and changed behaviour warrants discussion.

### *Models of chronic inflammatory stress*

In most of the studies evaluating changes following inflammation, the animals have been acutely ill. Following lipopolysaccharide injection, the findings reported occur in the first 24 hours of illness, modeling the changes seen following acute illness seen in humans (Boelen et al., 2004) (Fekete et al., 2004).

There are two animal models that have been used to evaluate HPT axis changes over a longer time period in order to investigate the situation in chronic conditions. Mebis *et al.,* (Mebis et al., 2009) devised a model that mimics the situation seen in long-term intensive care patients: while the time frame in this model is of the same order as that in the model studied here, the rabbits are severely debilitated. Thus, while the animals are chronically ill they also display behaviours indicating that they are very 'sick' (Mebis et al., 2009) (Hart, 1988). In the CCI model reported on here, the animals are not and do not appear 'sick'. While the stress inflicted on these rats clearly causes distress as seen by the altered gait and altered pain threshold, the rats engage in normal activities. Allowing for recovery from surgery, they eat, drink, groom, nest build, move around their cages and explore their environment as do control animals. They show social interest in and vigorous dominating behaviours towards an intruder despite their injury: this includes chasing and fighting behaviours, even in PD rats, albeit in a reduced fashion. Thus the CCI model provides insight into the situation in a chronic condition rather than chronic illness as in the case of the burned rabbits. The model was developed as a model for the condition of chronic neuropathic pain but findings can possibly be generalized to other chronic inflammatory conditions. Although in this study the rats were only observed for 6 days, the changed pain thresholds and altered behaviour in the sub-group (PD) have been documented for 16 days (Monassi et al., 2003).

In another model that examines the state in a chronic condition, subcutaneous injection of turpentine in the hind limbs of mice results in sterile abscess formation which causes serious discomfort to the animals (Boelen et al., 2005, Boelen et al., 2006). Decreased peripheral thyroid hormones, changes in gene expression of the deiodinase enzymes and of TRH were all reported. However, the majority of these findings had normalized by day 5 after injection of the turpentine (Boelen et al., 2006). The time course of events after injection was followed with animals sacrificed at 8, 24, 48 and 120 hours post-injection. The strength of this study is that the observations made follow both the acute and chronic phases of the inflammatory response. Decreased peripheral T3, T4 and PVN TRH mRNA at 24 and 48 hours revert to control levels by 120 hours. Likewise increased Dio2 mRNA in the AN and decreased Dio3 mRNA in the

PVN normalized by 120 hours (Boelen et al., 2006). While the authors cite their findings as support for changed T3 levels in the PVN, due to both increased Dio2 and decreased Dio3, as providing a mechanism for down regulation of TRH, their findings reflect initial, acute, changes in a chronic condition (Boelen et al., 2006). There is no behavioural description of the mice in these studies other than 'the abscess results in serious discomfort for the mice' (Boelen et al., 2005) (Boelen et al., 2006) and no indication of the state of the animal after five days. In the 2005 study, increased staining for Dio3-like protein was shown in the inflammatory cells (predominantly granulocytes, lymphocytes, and macrophages) surrounding the turpentine-induced abscess, but the time point for the finding (whether at 24, 48 or 120 hours) is not given. However, all time points were examined using the immunohistochemical technique and so it must be assumed that the mice had an ongoing abscess at 120 hours after injection and a chronic inflammatory condition (Boelen et al., 2005). Importantly, the studies in the turpentine model demonstrated that the diminished food intake seen in sick animals does not explain the illness-induced alterations in hypothalamic Dio2 and Dio3 gene expression in inflammation (Boelen et al., 2006).

The studies in the turpentine-injected mice demonstrated peripheral and central changes similar to those seen in the LPS injected acutely ill animals, albeit with the addition of data related to Dio3, with normal thyroid indices by five days post-inflammatory stress. A deficiency in the present study was that while changes in hormone levels were followed for six days post-injury, data related to central changes was only obtained at day seven after injury. However the findings in the animals that did not change behaviour post-injury (ND rats) are not dissimilar to those reported by Boelen *et al.,* in their chronic inflammation model (Boelen et al., 2006). Differences in time points at which observations were made means direct comparison are difficult. The turpentine-injected mice were possibly initially 'sicker" than the CCI rats and hence the fall in peripheral hormones as per acutely ill animals. However, the hormone level measurements in the current study did not recommence until 48 hours after CCI surgery by which time the ND rats showed an elevated TSH indicating a response to thyroid hormone levels that had been inadequate (Figure 4.3). Peripheral hormone levels had normalised by day six post-CCI. The central indices determined at day seven showed no

significant difference on any measurement to the control animals and hence resemble the turpentine mice after five days (Boelen et al., 2006). Moreover, as in the turpentine mice, inflammatory changes were ongoing; inflammatory changes have been documented in dorsal root ganglia and spinal cord segments seven days after CCI (Hu et al., 2007).

However, the unique contribution of the work described in this thesis is to identify the existence of a sub-population of animals that have persistently altered peripheral thyroid hormones and hypothalamic regulation of the thyroid system in response to chronic inflammatory stress. As detailed in the preceding chapters, the rats identified by persistent behavioural change towards an intruder rat are different to the ND rats in terms of hormone response post-injury and different to control animals in terms of gene expression and protein expression in the hypothalamus. These animals represent only 20% of the total population and thus are not identifiable as different unless identified as a sub-group based on behavioural change.

### *Changes to the HPT equilibrium following nerve injury*

Changes seen to the HPT axis following acute inflammation in humans are seen as adaptive (Adler and Wartofsky, 2007). Sickness behaviour in animals is seen as adaptive: the lethargy, withdrawal, decreased grooming and anorexia are seen as an adjunct to fever in combating acute infection (Hart, 1988). It would seem reasonable that decreased thyroid hormones, associated with some of these same behaviours, would contribute to this adaptive syndrome and thus would be a beneficial response in the acutely ill animal.

However in the case of a lesser injury or non-life threatening situation, the role for thyroid hormone metabolism may be quite different. As previously discussed in section 4.5.1, a role for thyroid hormones in tissue repair has been proposed (Safer et al., 2005) (Anderson et al., 1998). Specifically following nerve injury in the rat, induction of deiodinase enzymes suggests a role for increase T3 requirement following the injury (Li et al., 2001b) (Li et al., 2001a). Where tissue repair is the primary metabolic drive, rather than survival, increased tissue requirements for T3 may need to be met by local changes in deiodination in the context of the

maintenance of thyroid homeostasis under conditions of increased metabolic need. Thus after an initial decrease in thyroid hormones, chronic stimulation of the HPT rather than suppression would benefit the animal while tissue repair is ongoing. The time course of peripheral hormones for the ND rats is suggestive of increased T4 production (mean T4 and fT4 levels are above baseline) with increased T3 utilisation (mean T3 and fT3 levels at or below baseline) (Figure 4.3). Although not statistically significant, the ND rats have less Dio2-like protein in the PVN (p=0.5 for total immunoreactive profiles) (Figure 6.7). The model of TRH regulation suggests that decreased Dio2 protein in the MBH would be consistent with animals that are somewhat hyperthyroid (Fliers et al., 2006) but the MBH was not assessed in this study and the relevance of Dio2 in the PVN is not clear. The failure to find significant difference in Dio2-like protein in the ND group is due to the great variance in this group; this is consistent however with the greater variance in all measures in this group and with the concept of this group as one of individuals experiencing dynamic change while moving towards recovery from their injury.

### *Altered hypothalamic regulation of TSH in the PD rats*

Thyroid hormone levels did not recover in the PD group of rats. The hormone levels remained below pre-injury levels after six days. The delayed, blunted and inadequate TSH response indicated an altered central response in this sub-group reminiscent of that seen in the models of acute inflammatory stress and in the first days after injection in the turpentine mice (Fekete et al., 2004) (Boelen et al., 2004) (Mebis et al., 2009) (Boelen et al., 2006). This was suggestive of a change in the ‘thyrostat’, resulting in a relative hypothyroid state existing post-injury in this group. Measurement of relative TRH mRNA expression in the hypothalamus showed significant down-regulation in this group only, seven days after injury. However, decreased TRH protein expression in the PVN was not confirmed. This was due either to technical failure or the documented difficulty in visualizing TRH protein in euthyroid rats, or both (Nishiyama et al., 1985) (Espinosa et al., 2007).

Inflammatory cytokines have been implicated in the aetiology of the low thyroid syndrome in both rodents and humans (Papanicolaou, 2000) (Boelen et al., 1995)

(Boelen et al., 1996). The limited assessment of possible cytokine involvement attempted here did not reveal any marked differences between behavioural groups. Indeed, while there was some up-regulation for the injured rats of relative gene expression for both IL-1β and TNF, the changes were small relative to those for the group caged control rats (Figure 5.6). The RT-qPCR measurements however were at seven days after injury; changes to cytokine levels might be better assessed earlier. In the turpentine chronic model, IL-1β mRNA was markedly increased over controls at 24 and 48 hours after injection and while still significantly above control levels at 120 hours, was lower than at the previous time points (Boelen et al., 2006). The optimal time to assess differences between groups for cytokine expression in the hypothalamus would probably be two days after injury. Then it might be possible to determine whether the failure of the PD group to show up-regulation of IL-1β mRNA is perhaps real.

Alterations in the deiodinase enzymes that control T3 levels in the brain and periphery have been reported in both acute and chronic inflammatory models in rodents (Boelen et al., 2004) (Boelen et al., 2006) (Fekete et al., 2004) (Fekete et al., 2005) (Mebis et al., 2009). The specific increase in hypothalamic Dio2 reported by others was not found in the CCI rats. This may again be due to the time at which assessments were made as suggested above; Dio2 mRNA levels in the turpentine mice returned to baseline by five days. At seven days however, the PD rats had significantly decreased Dio2 mRNA measured in whole hypothalamus extracts (Figure 5.4 a and b) while showing no difference to control rats in Dio2-like protein levels in the PVN (Figure 6.7). As the evidence supports the primary location of hypothalamic Dio2 in the MBH with little in the PVN (Diano et al., 2003) (Tu et al., 1997) these findings are consistent with the PD group differing from the other injured rats in expression of Dio2 elsewhere in the hypothalamus but does not implicate changes in this enzyme in the down-regulation of TRH found in this group.

Boelen and colleagues also report a decrease in deiodinase 3 mRNA specifically in the PVN with no difference in whole hypothalamus levels following turpentine injection. The Dio3 mRNA levels in the PVN returned to baseline by five days as

discussed above (Boelen et al., 2006). In the burned rabbits, Dio3 activity in whole hypothalamus was not significantly increased, whereas specific activity in the PVN was not assessed (Mebis et al., 2009). As discussed above, the model most similar to the CCI is that of the chronic abscess following turpentine injection. The PD rats show decreased Dio3-like protein in the PVN and specifically in the region of the TRH neurons (Figure 6.9) (Fekete and Lechan, 2007). This is demonstrated seven days after injury and suggests the possibility that an altered level of Dio3 activity has persisted in this sub-group of animals. As Dio3 is responsible for the metabolism of T3, a specific decrease in the activity of this protein would result in a local increase in T3 and provide an explanation for the down-regulation of TRH seen in this sub-group. Relative Dio3 mRNA levels in whole hypothalamus for this group were significantly up regulated (Figure 5.4 a and b). No attempt was made here to quantify immunoreactive profiles for Dio3 in regions of the hypothalamus external to the PVN, however as shown in Figure 6.10, there is considerable immunoreactive staining outside this region. It is possible that this subgroup have altered expression of this enzyme in other regions of the hypothalamus that contribute to the biological difference in coping with inflammatory stress. Dio3 up regulation has been found in various tissues following injury and specifically associated with inflammatory cells (Boelen et al., 2005) (Boelen et al., 2009) (Li et al., 2001a) (Simonides et al., 2008). Further studies are needed to determine the nature of the immunoreactive profiles external to the PVN and the relative distribution of these elements in the PD rats compared to the control and ND rats.

### *Possible role for corticosterone in HPT dysregulation*

A role for glucocorticoids in the altered HPT axis regulation in inflammatory stress has been suggested. However the early changes seen have been shown to occur independently of corticosterone levels in rodent models and in humans as discussed previously (Kondo et al., 1997) (Sanchez et al., 2008) (Monig et al., 1999). In chronic severe illness, however, there are undoubtedly multiple mechanisms involved and a role for glucocorticoids cannot be ruled out (Van den Berghe, 2002) (Mebis et al., 2009). The findings in this study do not support a role for corticosterone in the down regulation of TRH in the PD rats. There were no differences between the PD rats and the behavioural control animals in relative

mRNA expression for any of the HPA factors measured: these were assessed seven days after injury at a time when TRH mRNA was down regulated. Moreover, the increase in corticosterone post-injury in the PD rats was the lowest of the three behavioural groups; levels the day after injury were not measured however, and it is possible that there could have been a difference immediately post injury. The small sub-group of TD rats had significantly higher corticosterone secretion post-injury and while showing altered dominance behaviour, did not have changed thyroid hormones. This finding suggests a protective rather than a causative role for corticosterone post CCI.

### *Decreased thyroid hormone and changed behaviour*

As reported in chapter 4, the PD rats showed a correlation between decreased Dominance behaviour and decrease in both T4 and fT4 (Figure 4.4). The argument as to the significance of altered levels of thyroid hormones, below an individual's 'set point', but still within the normal range, is on going (Andersen et al., 2002) (Danzi et al., 2005) (De Groot, 2006). Similarly the arguments for treatment of mild hypothyroidism, in particular for cognitive and behavioural symptoms (Monzani et al., 1993) (Cooper, 2001) (Davis et al., 2003): treatment of mild hypothyroidism for cardiovascular health may however be an ultimately more powerful argument (Cooper et al., 1984) (Monzani et al., 2004) (Bunevicius et al., 2006). The long-held belief that thyroid hormone status does not impact on neural function, despite the reversal of psychiatric symptoms with treatment, seems to be fading under the weight of evidence (Whybrow and Hurwitz, 1976) (Konig and Neto, 2002) (Bernal, 2009) (Zhang et al., 2009).

Animal models are being used to explore behavioural aspects of thyroid hormone deficiency using measures of behaviour developed in models of depression (Kulikov et al., 1997) (Levine et al., 1990) (Pilhatsch et al., 2010). These investigators have shown changed behaviour in hypothyroid animals. Similarly in this model, changed social behaviour is demonstrated in a sub-group that are relatively hypothyroid when compared to pre-injury indices. The down regulation of TRH mRNA in these animals suggests that they have tertiary hypothyroidism. The entity of tertiary hypothyroidism is controversial (Esposito et al., 1997), central hypothyroidism (secondary and tertiary) is said to be rare (Ladenson et al., 2000). There are others who argue that TSH levels inappropriately low for the

thyroid hormone levels are evidence that it exists (De Groot, 1999) (De Groot, 2006). Mood and behavioural change is reported in a percentage of individuals with chronic disease (many of which have an inflammatory etiology) and the low thyroid syndrome is described in similar groups of patients (Moussavi et al., 2007) (Adler and Wartofsky, 2007). The findings in this study support a tertiary hypothyroidism following inflammatory stress in a sub-population only.

### *Tertiary hypothyroidism*

The diagnostic issue is that there are, at present, no acknowledged peripheral markers for this condition. The level of TSH in the blood is an indicator of primary hypothyroidism and thyroid hormone levels must be measured if central hypothyroidism is suspected (Ladenson et al., 2000). This is not necessarily useful for the individual with TRH suppression due to inflammatory stress. Thyroid hormones may fall below the normal set point for an individual while remaining within the wide 'normal' reference range (Andersen et al., 2002) (Danzi et al., 2005). Hypothalamic insufficiency can be evaluated using the TRH stimulation test as suggested by Kraus *et al.,* who demonstrated an exaggerated TSH response to TRH in a large percentage of depressed patients with TSH levels in the normal range (Kraus et al., 1997). This is however not a screening test and not inexpensive. Those who argue for the recognition of this condition are left with 'TSH levels that are too low for the thyroid hormone levels' (De Groot, 2006).

Interestingly, there is one group of patients with an inflammatory condition who do have a recognizable peripheral marker. Individuals presenting with chronic fatigue who have thyroiditis, inflammation of the thyroid gland itself, have anti-thyroid antibodies in the peripheral blood or on biopsy (Wikland et al., 2001) (Wikland et al., 2003). In this group of patients the favourable response to thyroxine treatment was unrelated to pre treatment TSH levels. The recognition that in this sub-group of individuals with behavioural symptoms, thyroid indices were not diagnostic has been useful in arguing for a lower upper threshold for the TSH reference range and increased testing for thyroid antibodies (Gharib et al. 2002). However, for individuals with other inflammatory conditions the problem remains.

Investigations have demonstrated differences between survivors and non-survivors of acute sepsis in components of the HPT axis and the enzymes involved in thyroid metabolism (den Brinker et al., 2005) (Joosten et al., 2000). Measurement of rT3 and TT3/rT3 ratios as used in these studies perhaps have potential for identifying differences in adaption to the acute syndrome which may prove useful in identifying those who appear to have a maladaptive response to chronic inflammatory stress, possibly a genetically susceptible sub-group.

Interestingly, there were two animals identified behaviourally that were statistically very different to the rest of the PD animals and were removed from analysis as outliers. One animal was identified in chapter 4 as rat #6. This animal showed greatly increased TSH following injury and despite this, the peripheral thyroid levels fell markedly. The Dominance behaviour was also markedly low. This animal would satisfy diagnostic criteria for primary hypothyroidism. In chapter 6, rat #107 was identified as an outlier based on numbers of immunoreactive profiles that were greater than 3SD outside the mean levels for the other PD rats. Unfortunately, the plasma levels were not measured in these rats so the thyroid status of this rat could not be assessed. While the resultant state is the same, thyroid hormone levels below pre-injury levels, there is a means of diagnosis and treatment available to one (possibly two) members of the sub-group behaviourally identified, but not to the others.

# *Conclusion*

A percentage of humans with chronic disease states develop a syndrome of mood and behavioural change that increases their burden beyond their primary condition (Moussavi et al., 2007). Likewise a 'low thyroid syndrome' has been identified in a percentage of patients with chronic disease that correlates with poor prognosis and mood and behavioural change (Bunevicius et al., 2006). Additionally, levels of inflammatory markers and measures of depression correlate with increased morbidity and mortality in chronic disease (Frasure-Smith et al., 2007).

Bennett and Xie developed chronic constriction injury of the sciatic nerve in the rat as a model to assist in the study of pain syndromes (Bennett and Xie, 1988). Moreover, as demonstrated initially by Monassi *et al.,* it more closely parallels the pain syndromes seen in humans in that, despite no difference in 'pain', a sub-population of rats shows changes in complex social behaviours and in their sleep-wake cycle (Monassi et al., 2003). In a more general sense, however, CCI is a model of inflammatory stress. That only a sub-population of rats demonstrates behavioural change following injury parallels the finding of mood and behavioural change in only a proportion of chronically ill humans.

The experiments detailed in this thesis have shown that the behaviourally identified sub-group has relatively suppressed peripheral thyroid hormones post-injury. In this way they resemble the subjects in other animal models of inflammatory stress used to investigate the mechanisms involved in the 'low thyroid syndrome'. Similarly, these rats show evidence of changed HPT axis regulation, a down regulation of TRH gene expression, a change in the hypothalamic set point or 'thyrostat'. Additionally, there appears to be a decreased expression of deiodinase 3-like protein, specifically in the PVN, supporting the finding in another model of chronic inflammation (Boelen et al., 2006). Decreased metabolism of T3 in the local environment of the TRH neurons could result in a localised hypothalamic thyrotoxicosis, providing an explanatory mechanism for the TRH down regulation.

The unique contribution of this model is that the HPT changes occur only in a sub-group of animals. Importantly, the sub-group is only identifiable behaviourally. Peripheral thyroid indices post injury would not identify these animals; the change is relative to the pre-injury state and post-injury hormone levels would lie within broad 'normal' reference ranges.

In addition, these observations were made, unlike in other models of inflammatory stress, in animals that are not overtly 'sick'. This is reminiscent of clinical presentations that are a diagnostic challenge because they are largely behavioural in nature. We still lack the understanding and the technology to investigate the biological mechanisms underlying 'subclinical' syndromes. However, it seems likely that this sub-group, genetically or phenotypically susceptible to this mechanism, would be subject to further HPT dysregulation with multiple or recurrent stress. Failure to recognise these individuals early may result in a progressive 'disability' beyond that of the inflammatory disease. While controversial, there is the possibility of the treatment of relatively suppressed thyroid levels, with readily available hormones.

# CHAPTER EIGHT

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## *Appendix*

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