THE EFFECTS OF STRESS ON β1 THYROID HORMONE RECEPTOR IMMUNOREACTIVITY IN BRAIN

by

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A thesis submitted in conformity with the requirements
for a degree of Master of Science
Graduate Department of Pharmacology
University of Toronto

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0-612-46138-6
ACKNOWLEDGEMENTS

I am deeply indebted to my supervisor, Dr. José Nobrega, for the encouragement, guidance, patience, support and enlightening discussions he provided throughout the course of my M.Sc. programme. I would also like to thank Dr. Nobrega for providing the lab facilities and technical advice that I needed to complete my present work.

Furthermore, I would like to thank Roger Raymond for his training and guidance in essentially all of the technical details required to complete my work. His work ethic and drive to output first rate work will remain with me for the rest of my years. I am also grateful to Yael Friedman for her contribution of RIA results which were used in my thesis. Special thanks to my advisor, Dr. Jane Mitchell, for her wise advice and to Lori Dixon for her kindness throughout my post graduate training.

I wish to thank my mother and father for the constant support they provided me throughout my academic career, and to my good friend Rohana Dharmakumar for her help and support. Finally, I would like to express gratitude for the University of Toronto Open Fellowship which was awarded to me during the course of my M.Sc. studies.
The Effects Of Stress On β1 Thyroid Hormone Receptor Immunoreactivity In Brain, Master of Science degree 1999, Ravi Bacchus, Department of Pharmacology, University of Toronto

The goal of this project was to determine whether stressful conditions could produce changes in β1-like immunoreactivity in rat brain. Three stress models were used. First, social isolation was examined, but produced no changes in β1-like immunoreactivity in the brain regions examined. Second, uncontrollable foot shock, a model of depression and a more intense form of stress, was utilized but also suggested a lack of plasticity in the β1 receptor subtype. Third, learned helplessness was used and resulted in increased β1-like receptor immunoreactivity in the locus coeruleus, cerebellum and superior olivary nucleus.

The upregulation of the β1 receptor subtype in the locus coeruleus is a potentially important finding, since it is a major adrenergic centre and a target for the actions of tricyclic antidepressants (TCAs) suggesting that changes in β1 like immunoreactivity are not a result of stress per se, but may be associated with differential, depressive-type responses to stress.
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<td>KDa</td>
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<td>LBD</td>
<td>Ligand Binding Domain</td>
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<td>mRNA</td>
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<td>μg</td>
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<td>N-CoA1</td>
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<td>N-CoR</td>
<td>Nuclear Receptor Corepressor</td>
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<tr>
<td>NE</td>
<td>Noradrenaline (norepinephrine)</td>
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<tr>
<td>ng</td>
<td>nanogram</td>
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<tr>
<td>OCT</td>
<td>Optimal Cutting Temperature embedding medium</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PF</td>
<td>Paraformaldehyde</td>
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<td>pg</td>
<td>picogram</td>
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<tr>
<td>PPAR</td>
<td>Peroxisome Proliferator-Activated Receptor</td>
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<tr>
<td>PRTH</td>
<td>Pituitary Resistance to Thyroid Hormone</td>
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<td>RIA</td>
<td>Radioimmune Assay</td>
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<td>Receptor-Interacting Protein 140</td>
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<tr>
<td>RNA</td>
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<td>RPM</td>
<td>Revolutions per minute</td>
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<td>RTH</td>
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<td>rTR</td>
<td>Rat Thyroid Hormone Receptor</td>
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<td>RXR</td>
<td>Retinoic Acid Receptor</td>
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<td>T4</td>
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<td>Tricyclic Antidepressant</td>
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<td>Transcription Factor 2B</td>
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<td>Transcriptional Intermediary Factor 1</td>
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<td>TSH</td>
<td>Thyroid Stimulating Hormone</td>
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1. INTRODUCTION

1.1 Thyroid Hormones: Synthesis, Regulation and Physiology

*Synthesis*

The thyroid hormones, triiodothyronine (T3) and thyroxine (T4, tetraiodothyronine), have a variety of different effects on body tissues at various stages of development. The structure of thyroid hormones consists of two six-carbon aromatic rings linked via an ether bridge and possessing aliphatic side chains (Figure 1). The number of iodine substitutions on the rings distinguishes the structure of T3 (3 iodines) from T4 (4 iodines). T3 is considered to be the biologically active hormone and is approximately four times as potent as T4 (Schimmer and George, 1989). Thyroid hormones exert the majority of their effects via interaction with nuclear thyroid hormone receptors (see section 1.3).

The thyroid gland secretes much more T4 than T3. This gland produces thyroid hormones from a larger 670 KDa precursor molecule known as thyroglobulin via an elaborate series of biochemical reactions (Guyton, 1992). Before exerting any effects on tissues, most

![Thyroxine (T4)](image)

![Triiodothyronine (T3)](image)

*Figure 1. The structure of thyroid hormones.*
of the circulating T4 is deiodinated to T3. In peripheral organs, at least 70% of the active hormone arises from deiodination of T4 (Schimmer and George, 1989) (see Figure 2). However, in the brain of a euthyroid individual, approximately 80% of T3 derives from the intracerebral monodeiodination of T4 to T3 (Crantz et al., 1982; Larson, 1982). Hence, the main determinant of cerebral thyroid hormone levels is plasma T4, whereas the main determinant of thyroid hormone levels in the peripheral organs is plasma T3 (see Figure 3). Understanding the differences in thyroid hormone metabolism in the brain versus the periphery is of importance because the differential regulation suggests that measurements of thyroid hormone levels in the periphery may not be representative of that found in the brain.

Figure 2. Schematic representation of thyroid hormone metabolism. Thyroid hormones are released in the blood and circulate to many different tissue types. Before utilization, the majority of T4 is converted to T3 which may enter the nuclear compartment of the cell. T3 can then proceed to bind the thyroid hormone receptors in the nucleus to modify the transcriptional activity of the cell.
Figure 3. Schematic representation of thyroid hormone regulation in brain. The regulation of thyroid hormones in the brain differs from that in peripheral tissues because T3 is too polar to cross the hydrophobic blood brain barrier allowing T4 to be the main determinant of brain T3. Upon entering the brain T4 is converted to T3 which can then bind the thyroid hormone receptors within the brain. The β1 subtype is considered to be the predominant thyroid hormone receptor within the brain.

*Physiology and Regulation*

Thyroid hormones are known to be involved in the growth and development of Central Nervous System (CNS) in the first few years of post-natal life and maturation of tissues and bones in youth (Schimmer and George, 1989). In adults, the general effect of thyroid hormone is to increase nuclear transcription of a large number of genes causing virtually every cell of the body to increase its numbers of protein enzymes, structural proteins, transport proteins, and many other substances via its actions on transcriptional machinery. The end result of enhanced protein synthesis is a generalized increase in functional activity throughout the body (Guyton, 1992).

Until recently it was assumed that the actions of thyroid hormones on the CNS ceased at the end of the developmental period. However, as discussed in Section 1.2, a considerable amount of evidence suggests that thyroid hormones have significant effects on mature brain function.
Peripheral Regulation of Thyroid Hormones

Thyroid hormone concentrations are tightly regulated within the circulation. Precisely the right amount of hormone must be released into blood for proper body function (Schimmer and George, 1989). This regulation over secretion and plasma concentrations of thyroid hormone is accomplished by feedback mechanisms which operate at the level of the hypothalamus and pituitary involving the hormones of the thyroid axis (Guyton, 1992) (See Fig. 4).

Figure 4. Schematic representation of the hypothalamic-pituitary-thyroid axis. Thyrotropin Releasing Hormone (TRH) is secreted from the hypothalamus and stimulates the secretion of thyroid stimulating hormone (TSH) from the pituitary gland. Somatostatin, also released from the hypothalamus inhibits the pituitary release of TSH, which then inhibits the release of triiodothyronine (T3) and thyroxine (T4) from the thyroid gland. T3 and T4 can feedback to both pituitary and hypothalamic levels to inhibit the release of TSH and stimulate the release of somatostatin.

TRH (pyroglutamyl-histidyl-proline-amide) is produced in the hypothalamus and released to directly affect the anterior pituitary cells by increasing their output of TSH (thyroid stimulating hormone). TSH, in turn, acts on the thyroid gland to increase the secretion of the thyroid hormones (Guyton, 1992). Circulating and free T3 and T4 inhibit the anterior
pituitary release of TSH by feedback inhibition (Schimmer and George, 1989). The mechanisms by which inhibition is accomplished are unclear, but it is thought that, at least at the level of the anterior pituitary, thyroid hormones decrease the number of TRH receptors and thus decrease the ability of TRH to induce TSH release (Guyton, 1992). The hypothalamic hormone, somatostatin, has inhibitory effects on the pituitary release of TSH.

1.2 Alterations In Thyroid Hormone Level In Stress And Depression

Stress

A commonly used definition of stress is "any demand (physical or psychological) that is outside the norm and that signals a disparity between what is optimal and what actually exists" (Checkley, 1996). Stressors include personal life changes and physical or psychological traumas, both actual and perceived.

According to Koob (1989), the effectiveness of stressors in provoking pathology depends on the organism's ability to cope with these aversive experiences. In this respect a number of mechanisms are available to the organism in order to diminish the impact of the stressor. Not only will organisms rely on behavioural and psychological responses for buffering the stress effect using socioenvironmental resources, but several physiological changes will also occur whose function is primarily one of meeting environmental demands. Some of the physiological changes act to blunt the immediate physical and psychological attributes of the stressor, whereas others will protect the organism from potential health threats. At the same time, the directed mobilization of resources may leave the organism disposed toward, or at least unprotected from the impact of other physical or psychological stresses. Moreover, if the stress is severe enough, exhaustion of the resources necessary to contend with it may render the organism relatively unable to deal with further environmental issues (Anisman and Zacharko, 1982).

In recent times, interest has focused on the contribution of aversive stress to both physiological and psychological pathologies (Selyé, 1973). There is evidence indicating that stress can contribute to the onset of depression (Akiskal et al, 1973; Anisman and Zacharko, 1982), schizophrenia, and anxiety disorders (Herbert, 1997), in addition to gastrointestinal ulceration (Weiss, 1971) and heart disease (Glass, 1977).
With regards to psychological pathology, stressful events are known to influence turnover of a variety of central neurotransmitters, including norepinephrine (NE), dopamine (DA), serotonin (5-HT), and acetylcholine (ACh) (Zacharko and Anisman, 1991). Stress also affects cortisol, dehydroepiandrosterone (DHEA) levels (Checkley, 1996; Herbert, 1997), gene expression (Lechin et al, 1996; Dishman, 1997) and several components of the hypothalamic-pituitary-adrenal (HPA) axis (Horger and Roth, 1996; Heit et al, 1997; DeSouza, 1995). Several of these changes are also involved in reward processes and thus stress is often thought to play a role in reward processes (Anisman and Zacharko, 1982). Controllability of stressful stimuli appears to be a particularly important feature in eliciting biochemical changes. This is discussed further in the Introduction to Section 4.

**Stress and Thyroid Hormones**

In humans, a relationship between stress and thyroid hormone alterations is suggested in a condition known as Euthyroid Sick Syndrome (ESS), where abnormalities in thyroid function tests are observed in patients with systemic nonthyroidal illnesses (NTIs). NTIs are associated with several forms of stress including pain associated with disease, trauma and fear (Pinn et al, 1998). Most NTIs include physical or injurious stressors such as starvation, brain injuries, sepsis, liver and kidney disease, brain tumors, extreme thermal exposure, infection, serious acute illness, hemorrhagic shock, surgery, diabetes mellitus and fasting, among others (Chopra, 1997; Zaloga and O’Brien, 1985). The most common specific changes in serum concentrations of thyroid hormones in NTIs are low levels of T3 and T4 (Chopra, 1997).

Several stressors are known to cause changes in levels of thyroid hormones in the mature rat. Stressors such as uncontrollable footshock (Berl, 1996; Josko, 1996; Pollard et al, 1979; Pollard et al, 1976), immobilization stress (Langer et al, 1983; Turakulov et al, 1994) and cold exposure (Bernal and Escobar del Rey, 1975; Pollard et al, 1976) have all been shown to alter circulating levels of thyroid hormones in the mature rat, although the nature of the changes varies depending on the details of the experimental situations.

Hence human and rat studies suggest that stress can have an effect on thyroid status. However, it should be noted that all of these studies examined *peripheral* rather than brain levels of thyroid hormones. Little is known about the relationship between neuronal thyroid
hormone levels and stressors. Recent studies, including work performed in our laboratory, suggest that brain thyroid hormone levels are sensitive to stress (Friedman et al, 1999; Bauer et al, 1994).

*Central vs. Peripheral Thyroid Hormone Functions in Maturity*

The role of thyroid hormones in the periphery includes effects on mitochondria, cell membrane ion transport, carbohydrate metabolism, fat metabolism, basal metabolic rate, body weight, heart rate, respiration, gastrointestinal function and muscular function (Guyton, 1992). Additionally, thyroid hormones are critically important for the maturation and development of neuronal systems. While much less is known about their roles in mature brain, several lines of evidence now suggest that thyroid hormones have a direct and important effect on mature brain function. Thyroid hormones are concentrated in discrete brain regions and particularly in synaptosomes, suggesting that they are regulators or modulators of synaptic activity (Dratman et al, 1976, 1983). T3 receptors, the site of initiation of hormonal action, have been detected in mature brain. Finally, in vivo and in vitro studies suggest that the brain is responsive to minimal alterations in thyroid state and that, even with extremes of thyroid hormone availability, reciprocal alterations in cerebral and hepatic thyroid hormone metabolism serve to maintain brain thyroid hormone levels within very narrow limits (Dratman et al, 1983; Leonard and Safran, 1994; Leonard et al, 1981). It has been suggested that disruption of this fine regulation leading to the smallest alterations in brain thyroid hormone levels may lead to changes in mental state such as mood, behaviour and cognition (Joffe et al, 1994).

*Stress and Depression*

There is a strong association between stress and depressive disorder, or more precisely, between depression and the occurrence of stressful “life events”. A review of current studies (Herbert et al, 1997) reveals that a large percentage of patients with recent onset of depression are found to have experienced a stressful life event involving some sort of loss (i.e., death of family member or divorce) six months prior to the depressive episode. Hence, the organism’s ability to manage stress and the type of stressor are key in determining whether an individual develops depressive symptoms. Likewise, in experimental animals,
stress may induce behavioural, neurochemical and hormonal alterations (Zacharko and Anisman, 1989; Makara et al, 1986) which are reminiscent of those in human depression, but which may also vary depending on specific features such as controllability of the stress (Anisman, 1984)

Depression

Depression is a psychiatric disorder characterized by significant and long-term changes in mood and cognitive function (Koob, 1989). One of the most important symptoms of a major depressive episode is a failure to obtain pleasure from activities that previous brought enjoyment (Klein, 1974). This is a motivational disturbance termed “anhedonia” which is defined as “the diminished capacity to experience pleasure of any sort” (Fawcett et al, 1983). The mood of the individual is characterized as depressed, sad, hopeless and discouraged.

Clinically, depression is classified according to the Diagnostic and Statistical Manual of Mental Disorder (DSM-IV) of the American Psychiatric Association. It can be subclassified into a spectrum ranging from minor depression (neurotic depression) through major depression to melancholic depression (endogenous depression and psychotic depression). This discussion will focus on the findings for major unipolar depression.

It is widely accepted that depression is associated with dysfunction of neurochemical activity. Two principal hypotheses have been advanced. One of these attributes depression to a functional deficiency in norepinephrine activity (Schildkraut, 1970, 1978) while the other assigns a primary role to serotonin (Murphy et al, 1978). Other central transmitters including acetylcholine and dopamine may also be associated with the depressive symptomatology (Bunney et al, 1979; Janowsky et al, 1972).

Depressive Symptoms

Generally, 90% of depressed people experience prolonged bouts of sadness, discouragement, or a sense of “not caring anymore”. Other major depressive symptoms include depressed mood, anhedonia, anxiety, sleep disturbances, appetite disturbance, loss of energy, decreased libido, psychomotor retardation, psychomotor agitation, loss of interest in usual activities, feelings of hopelessness and helplessness, suicidal thoughts or acts, sense of guilt, worthlessness, low self esteem, difficulty in concentrating, psychosis, and somatic
complications (bodily complaints including headaches, backaches, muscle cramps, nausea, vomiting, constipation, heartburn, shortness of breath, hyperventilation and chest pain) (DSM IV).

\textit{Mood Disorders and Abnormalities of The Thyroid Axis}

Many systems and hormonal levels are altered in depressive conditions including DHEA, serotonin, and elements of the HPA axis (Checkley, 1996; Herbert, 1997). Elements of the thyroid axis are also changed in patients with depression. There is an association between clinical hyper- and hypothyroidism with some of the psychiatric symptoms seen in depression. In patients with clinical hyperthyroidism, depression, anxiety and emotional disturbances, profound cognitive impairment, psychotic symptoms, and frank mania have been documented (Haddad, 1998). Clinical hypothyroidism, defined as a decrease in thyroid hormones below the euthyroid range, is associated with several features of depression, particularly lethargy, fatigue, depressed mood and cognitive impairment (Dugbartey, 1998; Adlin, 1998).

The psychiatric symptoms associated with clinical thyroid disorders usually resolve with treatment of the underlying thyroid disorder (Maccrimmon et al, 1970; Hall, 1983; Whybrow and Prange, 1981). Since the symptoms associated with clinical hypothyroidism are also commonly found in depression, it has been suggested that hypothyroidism predisposes to depression and that increased thyroid function promotes recovery from depression. According to Joffe et al (1990) the relationship is considerably more complex than that, because: a) there is no direct relationship between the severity of depression and the degree of abnormality of thyroid measurement; b) resolution of psychiatric symptoms is not synchronized with achievement of the euthyroid state -- that is, psychiatric symptoms may persist beyond restoration of normal thyroid function and may require antidepressants or electroconvulsive therapy for a response; c) psychiatric symptoms may respond to therapies, but the patient may still have evidence of thyroid axis abnormalities; d) treatment of the thyroid disorder may precipitate the onset of additional psychiatric symptoms; and e) depressive symptoms accompany, both, hyperthyroidism and hypothyroidism.
Subclinical Disorders of the Thyroid Axis

Subclinical hypothyroidism is characterized by the occurrence of very few or no clinical symptoms, normal thyroid hormone levels, but abnormal TSH measures. This disorder has been shown to be associated with abnormalities of mood and cognition, particularly if occurring over a prolonged period of time. The disorder thus suggests that even minor disturbances of thyroid function can affect mental state (Joffe et al, 1994).

Peripheral Levels of Thyroid Hormones in Depressed Patients

There are several consistent findings with respect to disturbances of the thyroid axis in depression. The majority of patients with depression have thyroid hormone levels in the normal range. However, in adult depressives the levels of serum T4 and/or free T4 can be as much as 15 to 20% higher than in normal controls and recovery from depression appears to be associated with concurrent decreases in the levels of T4 and free T4 (Sokolov et al, 1996). Although the reductions are significant, they are limited so that the levels before and after antidepressant treatment fall within the euthyroid range (Joffe et al, 1994). The findings for circulating T3 are less consistent than those for T4 in that the majority of studies found no change in circulating T3 levels in depressed patients, although a few have reported decreases (Joffe et al, 1985; Orsulak et al, 1985) or increases in free T3 (Kirkegaard and Faber, 1986).

TSH levels are of importance in subclinical hypothyroidism because they are the only abnormality of the thyroid axis in this disease. TSH levels may also be important in depression in that subclinical hypothyroidism may occur in up to 10% of patients with major depression (Gold et al, 1981; Joffe and Levitt, 1992). There is evidence that the occurrence of subclinical hypothyroidism may modify the clinical manifestations of depressive disorder and make patients more refractory to antidepressant treatments (Joffe and Levitt, 1992). Also, elevated TSH levels may be pertinent to bipolar affective disorder, particularly the rapid cycling form, as several studies have reported a high prevalence of clinical and subclinical hypothyroidism in patients with this illness. This observation has led to the introduction of high dose T4 to treat patients with this disorder, because T4 (in addition to T3), by feedback inhibition, decreases TSH serum levels. There has been documentation of a circadian variation in the secretion of TSH from the pituitary in healthy subjects, but the TSH circadian pattern has been reported to
be flattened in subjects with depression (Duval et al, 1990), and to normalize with clinical recovery (Kjellman et al, 1984; Souetre et al, 1986). The blunting of the TSH response to TRH (TRH test) is the most commonly described thyroid related abnormality in patients with depressive illness (Loosen and Prange, 1982; Loosen, 1985). This is a widely used endocrine procedure that involves measurement of TSH levels following i.v. administration of a single dose of TRH. The blunting of the TSH response to TRH is found in approximately 45% of depressed patients (Sullivan et al, 1997). This test is very sensitive to feedback from circulating levels of T4 and T3 and it is possible that a blunted TRH response may represent a form of subclinical hyperthyroidism (Sawin and Hershman, 1976; Schlote et al, 1992).

Studies which have examined the association between TRH and depression have found that TRH levels of depressed patients are particularly high when compared with other patient groups and are especially high in depressed patients who show a tendency towards violent and suicidal behaviours (Banki et al, 1988).

**Antidepressants Effects on the Thyroid Axis**

The most consistent thyroid findings in depressed patients upon treatment with antidepressants are those concerning T4. Responders to antidepressant treatments consistently have greater reductions in T4 and free T4 when compared with nonresponders (Brady and Anton, 1989; Mason et al, 1989; Joffe and Singer, 1990; Hoflich et al, 1992). This effect has been seen with mood stabilizers such as carbamazepine (Roy-Byrne et al, 1984; Unden et al, 1986; Gibbons et al, 1960), TCAs (Baumgartner et al, 1988; Ferrari, 1973; Muller and Boning, 1988), electroconvulsive therapy (Kirkegaard et al, 1975; Kirkegaard and Faber, 1986; Kirkegaard et al, 1977; Kirkegaard and Faber, 1981) and cognitive therapy (Board et al, 1959; Whybrow et al, 1972; Yamaguchi et al, 1975).

**Thyroid Hormones as Adjuncts to Therapy**

The importance of thyroid hormones in depression is further emphasized by their use as adjuncts usually after the antidepressant alone fails in treating patients with depression (Nakamura and Nomura, 1992). T3 appears to have limited clinical use, but the combination of T3 and an antidepressant has substantial clinical efficacy. When 25 to 50 μg of T3 have been used in combination with TCAs, there is an acceleration in mood improvement in
euthyroid depressed women (Feighner et al, 1972). Even more impressive is the ability of T3 to potentiate the response to TCAs in nonresponders to treatment. Studies have shown that approximately 30% of depressed patients fail to respond to traditional antidepressants, and 67% of these nonresponders can benefit from the coadministration of thyroid hormones (Henley and Koehnle, 1997). Studies also indicate that this potentiation is independent of antidepressant type, sex, bipolar/unipolar diagnosis, or baseline thyroid status of patients. The mechanism by which T3 accomplishes this potentiation is thought to involve an increase in β-adrenergic receptor activity but this has not been conclusively demonstrated (Joffe et al, 1994). T3 potentiation of antidepressant drug response has contributed to the view that depression is a state of relative thyroid hypofunction, as discussed in the next section.

T4 is not considered to be as effective as T3 as an adjunct to antidepressant treatments (Joffe et al, 1994). This may appear surprising as one might imagine that T4 would eventually be converted to T3 in the blood to enhance antidepressant response. However, it has already been mentioned that the main determinant of tissue T3 is plasma T3, whereas the main determinant of brain T3 is brain T4. Hence, exogenous administration of T3 and T4 may have different effects on brain thyroid hormone levels, which result in a different efficacy in augmenting antidepressant response. Neither TRH nor TSH administration have ever been found to have consistent effects on depressive illness (Kastin et al, 1972; Prange et al, 1972; Coppenet al, 1974).

The Involvement of Thyroid Hormones in Depression – Two Hypotheses

The first hypothesis by Whybrow and Prange (1981) suggests that depression is a state of relative thyroid hypofunction and that increases in thyroid hormone levels are required to promote antidepressant response. According to this hypothesis, the relative increases in T4 observed in depressed patients are a compensatory physiological response to the depressed state. In response to being depressed, patients mobilize T4, which enhances recovery. In this way, relative thyroid hyperfunction in depression is a response to the disorder, rather than a component of the illness itself. The reductions in T4 seen after antidepressant treatment are explained as the body mounting a more effective thyroid response to depression which is then used to aid in recovery. A strength of this hypothesis is that it is consistent with the strong
association between depression and clinical hypothyroidism. Also supportive are the data on T3 augmentation of treatment response in unipolar and bipolar patients. A weakness of this hypothesis is that it does not address the finding that T3 may be more effective than T4 in augmentation of antidepressant response.

A second hypothesis (Joffe et al., 1994) suggests that depression is a state of relative thyroid hyperfunction. This hypothesis suggests that the relative increases in T4 observed in depressed patients are a pathological finding and that the decrease in T4 observed with antidepressant treatment is required for antidepressant response. Evidence to support this hypothesis includes the findings of relative increases in T4 that decrease with antidepressant response, the diminished TSH response to TRH, the blunted circadian variation in TSH levels, and the elevated TRH levels in cerebrospinal fluid. However, this hypothesis does not account for the findings in patients with clinical hypothyroidism.

In summary, the most consistent findings relating to the thyroid axis in patients with depression are: a) increased levels of circulating T4; b) increased cerebrospinal fluid levels of TRH; c) blunting of the circadian variation of TRH; and d) blunted TSH response to TRH. Additionally, T3 has been shown to be beneficial as an adjunct to antidepressant treatments. Conflicting hypotheses have been suggested to account for the data and for the role of thyroid hormones in depression. These hypotheses have been difficult to test in the clinic because the hypothesized changes are postulated to occur not in the peripheral circulation, but in brain. Animal models may contribute important information in this regard.

**Animal Models for Thyroid Studies**

A significant limitation in the study of thyroid hormones in depression is the lack of access to human brain material. However, animal models can provide valuable initial information which can serve as an important basis for future human and animal studies.

Most of the available animal models involve stress manipulations. In this thesis we used three different stress models, namely social isolation, uncontrollable footshock stress and learned helplessness, in order to assess changes in thyroid hormone receptors in brain. As discussed in the introduction to Sections 3, 4 and 5, respectively, each of the three stress models has been associated with features of human depressive illness.
1.3 The Thyroid Hormone Receptor

It is now accepted that thyroid hormones produce their actions via interaction with nuclear receptors (Puymirat, 1992). Thyroid hormone receptors are ligand modulated transcription factors which are members of a large superfamily of c-erbA related nuclear receptors for molecules such as steroids, vitamin D3, retinoic acid, prostaglandins, terpenoids, farnesoids, fatty acids, and orphan receptors (Farsetti et al, 1997). Thyroid hormone receptors are found with chromatin (DNA) in the nucleus of cells with a weight of between 47 and 57 KDa and a sedimentation coefficient of 3.7S (Degroot et al, 1989). These receptors are able to bind T3 and T4, but have approximately 10 times higher affinity for T3 than T4 (Schimmer and George, 1989). In addition to ligand dependant activation and repression of gene transcription, these receptors are capable of ligand independent repression and stimulation of transcription. It appears that repression is the default state of the receptor and is relieved by binding to T3 or T4 (Degroot et al, 1989).

The Thyroid Hormone Receptor Genes

Thyroid hormone receptors are produced by two distinct genes, α and β, which are located on human chromosomes 17 and 3, respectively (Ting and Cheng, 1997). The receptor’s DNA sequence is homologous to v-erbA, an oncogene present in the genome of the avian erythroblastosis virus which is involved the production of avian sarcomas. Both thyroid hormone receptor genes encode multiple receptor types (see Figure 5).
Figure 5. Schematic processing of the \( \alpha \) and \( \beta \) genes. Indicated are the RNA, molecular weights, and whether the gene produced can bind T3. Note that since \( \alpha_2 \) and Rev-erbA do not bind T3, they are not considered to be thyroid hormone receptors.

The \( \alpha \) gene gives rise to thyroid hormone receptors \( \alpha_1 \) and \( \alpha_2 \) via alternate splicing. The only difference between the \( \alpha_1 \) and \( \alpha_2 \) gene products is the carboxy tail, but only \( \alpha_1 \) is considered to be a true thyroid hormone receptor as \( \alpha_2 \) does not bind thyroid hormones. V-erbA represents a mutated form of the \( \alpha \) receptor subtype that behaves as a constitutive transcriptional repressor of thyroid responsive genes (Munoz and Bernal, 1997). Additionally, via expression of the alternate strand of RNA (non-coding strand of the \( \alpha \) gene), rev-erba(\( \alpha \)) is produced and it has been suggested that this gene could suppress expression of thyroid hormone receptor \( \alpha_2 \) by formation of antisense mRNA (Degroot et al, 1989). The \( \beta \) gene produces \( \beta_1 \) and \( \beta_2 \) thyroid hormone receptors via alternative promoters and these receptors differ only in their amino terminal sequence. Despite having a high degree of homology, the two \( \beta \) receptors have different tissue expression and biological activities (see section 1.3).

**Thyroid Hormone Receptor Response Elements**

The T3 receptors modulate transcription by binding to specific DNA acceptor sites
known as T3 response elements (T3REs). T3REs are located in the promoter regions of target genes and confer ligand-dependent transcriptional activity to them. Thyroid receptors have a great deal of flexibility when interacting with T3REs. Sequence and organization of T3REs within the target genes tend to differ depending on the gene. That is, T3REs consist of two half sites of 6 or 8 nucleotides each and each half-site has variable degrees of degeneracy from a consensus hexameric sequence reported to be AGGTCA, or the optimized octameric element (T/C)(A/G)AGGTCA respectively (Tagami et al, 1998). Additionally, this main recognition motif can be present as a single half-site, as two half-sites arranged as a direct repeat spaced by 4 base pairs (i.e. "DR4"), as a palindrome, or as an inverted palindrome. Thyroid hormone receptors may bind T3REs as monomers, homodimers, or as heterodimers, as commonly occurs with retinoic X receptors (Tagami et al, 1998) (See Figure 6).

Figure 6. Model for dimerization of the thyroid hormone receptor. Before exerting any effects on the transcriptional machinery of the cell, the thyroid hormone receptor dimerizes with another receptor. Zn indicates the placement of the zinc fingers used in DNA binding. Adapted from DeNayer, 1992.

Receptor Complexes

In addition to complexing with retinoic X receptors (RXR), thyroid hormone receptors
can form heterodimers with other receptors including vitamin D3 and peroxisome proliferator-activated receptor (PPAR) (Munoz and Bernal, 1997). The coupling of receptors from different signaling systems within a cell produces elaborate interactions. For instance, it is known that since RXR is a receptor which can form dimers with a host of other receptors, complexing to T3 may modify RXR's availability to modify other cellular responses. This is also the case for vitamin D3 signaling (Munoz and Bernal, 1997). The ability of thyroid hormone receptors to heterodimerize with receptors from other systems can, in part, account for diversity of effects that thyroid hormones have on the body.

Corepressors and Coactivators

Thyroid receptor heterodimers are able to exert an influence on cells via interactions with small adapter molecules known as corepressors and coactivators. These regulate receptor heterodimer complexes via their interaction with the transcriptional systems of cells. Corepressors are proteins that mediate the ligand-independent function of the thyroid hormone receptor by means of inhibitory activity within the transcription machinery. Two corepressor molecules have been described, N-CoR (nuclear receptor corepressor, p270) and SMRT (silencing mediator for RXR’s and TR’s or p165) (Munoz and Bernal, 1997). When the heterodimer complex is bound by a ligand, the corepressors leave, removing their depression on the transcriptional machinery, and coactivators are recruited, causing the activation of gene transcription (see Figure 7). Little is known about how repressors function, but it has been suggested that the N-CoR corepressor represses transcriptional activity by forming a complex with histone deacetylase thus making DNA inaccessible to transcription (Munoz and Bernal, 1997).
Coactivators are proteins that function as bridging factors between the receptors and the basal transcription factors. Several coactivators for steroid/thyroid receptors have been described such as thyroid receptor-interaction protein 1/transcriptional intermediary factor 1 (Trip 1/TIF 1), receptor-interacting protein 140 (RIP140), steroid receptor co-activator-1/nuclear-coactivator-1 (SRC-1/N-CoA1), TRAP complex, mouse CREB-binding protein (CBP) and the closely related human p300. Coactivators interact with many other transcription factors, such as phosphorylated CREB, c-Jun, c-fos, c-myb etc. It has been suggested that some coactivators including CBP or p300 have histone acetyltransferase activity and that their role is to destabilize nucleosomes through an increased acetylation of
histones, making DNA more accessible to transcription factors (Munoz and Bernal, 1997).

**The Structure of Thyroid Hormone Receptors**

There are four main structural domains in the thyroid hormone receptor (See Fig. 8). These are designated A/B (Amino terminal domain), C (DNA binding domain), D (hinge domain), and E/F (ligand binding carboxy terminal domain).

![Diagram of thyroid hormone receptor domains](image)

Figure 8. Schematic representation of the domain structure of the thyroid hormone receptor molecule. Shown are the locations of the classical A-F domains of nuclear receptors. The transactivation domains are located at the amino terminus (hormone-independent, AF-1) and at the carboxy terminus (hormone dependent, AF-2). The DNA binding C domain contains the two zinc (Zn) fingers and the T and A boxes. The DNA recognition helix P-box is located at the C-terminal portion of the first zinc finger and is important in the specific association with hexamer sequences. The D-box is involved in dimerization and spacing recognition. The T and A boxes participate in RXR heterodimerization and in the recognition of the two nucleotides located at the 5'-end of octamer binding sequences respectively. The D domain contains the binding site for corepressors. The E domain is involved in ligand binding and receptor dimerization. It includes the conserved \( \tau_i \) region. The F domain is responsible for the hormone-dependent transactivation, containing the binding site for coactivators. The numbers above the boxes refer to the amino acid sequence starting with the first in-frame methionine. Adapted from DeNayer, 1992 and Puymirat, 1991.

The A/B domain is thought to be involved in transcriptional activation. The sequence is not conserved between \( \alpha \) and \( \beta \) receptor subtypes nor between the \( \beta \) receptor isoforms. Via interaction with transcription factor 2B (TFIIB) of the basal transcription machinery, the amino terminal domain is involved in ligand-independent transcriptional activation (Munoz and Bernal, 1997). This domain also plays a role in the modulation of functional interactions with corepressors. There is evidence to suggest that the amino-terminal domain may play some role in the recognition of DNA (Munoz and Bernal, 1997) as well as targeting of the receptor for the nucleus of the cell (Degroot et al, 1989). Finally, comparison of the sequence of
cDNAs of placenta, testis, liver, kidney and brain shows that there is much sequence variability in the N-terminus and it may, therefore, play a role in tissue selectivity (Fukuda et al, 1988).

The DNA binding domain is the most conserved region among nuclear receptors. The DNA binding sequence of thyroid receptors is described as being of the "C4" type. That is, it contains two copies of a motif which includes 4 cystine residues coordinated to a zinc atom which forms finger-like structures known as "zinc fingers". The first of the two zinc fingers fits into the major groove of the DNA α helix (Degroot et al, 1989). The C-terminal portion of this first zinc finger is an α helix known as the "DNA recognition helix" because it interacts with specific DNA sequences. This helix contains lysine and arginine residues that bind specifically to a sequence of nucleotides comprising the thyroid hormone response element. The second finger contacts the DNA in another manner, or may interact with other components of the transcriptional machinery (Fukuda et al, 1988).

Downstream of the second zinc finger there are two other regions of helical structure known as T and A boxes. The T box provides contact with the N-terminal region of the second zinc finger of the RXR. The A box stabilizes the DNA binding domain on the DNA with a more extensive recognition interface than for steroid receptors, conferring the thyroid receptor with the ability bind as monomers and to recognize octamer response elements (Munoz and Bernal, 1997). The A box determines the specific recognition of the two nucleotides T(A/G) at the 5' end of the octamer.

The hinge (D) domain is a stretch of amino acids that links the DNA and ligand binding domains. Its functions are not well known but studies have shown that it may play a role in nuclear targeting as well as participating in the association with corepressor proteins (Munoz and Bernal, 1997).

The E/F domain is the ligand binding domain, or LBD. It functions to recognize and bind the specific ligand. Thyroid receptors function differently than other steroid receptors in that they (and the related v-erb A gene product) bind to specific DNA sites in the absence of bound hormone and act as transcriptional repressors at those sites (Degroot et al, 1989). Binding of T3 to the thyroid receptor overcomes this constitutive repressor function of the receptor. The LBD has been found to be buried deep within the receptor protein within a
hydrophobic pocket suggesting that the conformational changes associated with ligand binding affects the surface of the receptor, exposing critical residues involved in protein-protein interactions important in transcriptional activation (Munoz and Bernal, 1997).

**Thyroid Hormone Receptor Mechanism of Action**

The details of the thyroid hormone receptor mechanism of action are still not entirely clear. What is known about its mechanism is that the receptor remains in the nucleus of cells and is always associated with chromatin. Bound to the receptor are corepressors (ex. SMRT and p270) which mediate ligand-independent suppression of activity. T3 or T4 can enter the receptor’s hydrophobic binding pocket causing a conformational change within the receptor such that the receptor can be readily phosphorylated by specific kinases. Receptor phosphorylation causes corepressor binding to become unfavourable and they are replaced with coactivators (ex. TRIP, TIF1, RIP140, SRC1-N-CoA1, TRAP). This binding allows the formation of homodimers (with other thyroid hormone receptors) or heterodimers (predominantly with retinoic acid receptors). The dimer then binds response elements in thyroid hormone responsive genes to affect the transcriptional machinery that assembles in the promoter region such that transcription is facilitated (Fukuda et al, 1988)

**Thyroid Hormone Receptor Regulation**

In addition to regulation via binding to steroid receptors, coactivators and corepressors, thyroid hormone receptors may also be regulated via protein kinase-directed phosphorylation. Upon binding T3 or T4, the receptor’s change in conformation facilitates protein kinase phosphorylation of serine and threonine residues leading to increased interaction between subunits of the receptor in addition to increased resistance to proteolysis. Phosphorylation has also been shown to lead to increased binding of auxiliary proteins and increased heterodimer formation with retinoic acid receptors and hence increased transcriptional activity (Ting et al, 1996).

Information on synthesis and kinetics of thyroid receptors is sparse. The receptors are believed to be translated from mRNA in the extranuclear cell compartment, and to move into the nucleus under control of signal sequences which may be in the N-terminal domain or the
hinge region of the molecule. Receptor turnover in the nucleus has been estimated to have a \( t_{1/2} \) of 2-6 hours (Degroot et al, 1989).

**The Significance of Multiple Receptors**

The significance of multiple forms of thyroid hormone receptors is unclear. It has been speculated that different types of receptor could be expressed in certain organs or in specific types of cells and that these may control different classes of genes, or may interact with each other in the control of transcription. Tissue specificity in expression of individual thyroid receptors has been reported (see below).

Human thyroid hormone receptors are found in many (if not all) human tissues. Each of the thyroid hormone receptor's mRNA has been identified in kidney, liver, placenta, tonsil, spleen, brain, prostate and thyroid with an abundance of signal in brain, prostate and thyroid (Degroot et al, 1989). Additionally, \( \beta_1 \)- and \( \beta_2 \)-like immunoreactivity has been identified in kidney, liver, placenta, tonsil, spleen, brain, prostate and thyroid (Degroot et al, 1989).

Rat thyroid hormone mRNA has also been identified in many tissues. \( \alpha_1 \) mRNA has been found in brain, testis, brown adipose and skeletal (excluding cardiac) tissues (Degroot et al, 1989). \( \alpha_2 \) mRNA has been found in liver in addition to all of the tissues that \( \alpha_1 \) mRNA has been identified (Degroot et al, 1989). Also, \( \beta_1 \) mRNA has been found throughout the brain, especially in regions of cortex, cerebellum, and hypothalamus (Lechan et al, 1993). Specific antibodies have also detected \( \alpha_1 \), \( \alpha_2 \) and \( \beta \) thyroid hormone receptor proteins in various rat tissues as liver, kidney, thyroid, anterior pituitary, posterior pituitary, brain, adrenal cortex, heart, striated muscle, testis and spleen (Macchia et al, 1990; Puymirat 1992; Nobrega et al 1997).

**Resistance to Thyroid Hormone (RTH) Syndrome and Thyroid Hormone Receptors**

Resistance to thyroid hormone (RTH) is a rare disorder characterized by elevated circulating levels of free thyroid hormones due to reduced target tissue responsiveness and normal, or elevated, levels of thyroid-stimulating hormone (TSH) (Kopp et al, 1996; Macchia et al, 1997). It is sometimes subdivided into generalized resistance to thyroid hormone (GRTH) and pituitary resistance to thyroid hormone (PRTH) which are based on the absence (GRTH) or presence (PRTH) of symptoms and signs suggestive of thyrotoxicosis (Hayashi et
Patients with RTH usually present with goiter and an euthyroid or mildly hypothyroid metabolic state. Thus, PRTH results in hypersecretion of TSH, which compensates, at least in part, for hormone resistance in peripheral tissues. Despite this compensation, clinical effects of RTH can include short stature, delayed bone maturation, hyperactivity, mental retardation, tachycardia, attention-deficit hyperactivity disorder, hearing defects, as well as variable features of hyper- and hypothyroidism (Kopp et al, 1996; Behr et al, 1997). The majority of RTH disorders are inherited in an autosomal dominant manner or have been de novo heterozygous mutations of the thyroid receptor β gene leading to the production of mutant β1 thyroid hormone receptors (Behr et al, 1997). The mutant receptors act in a dominant negative manner to block the activity of normal thyroid receptor α and thyroid receptor β receptors in the heterozygote (Kopp et al, 1996; Behr et al, 1997).

Structural studies have led to the conclusion that mutations in two “hot spot regions” in the carboxy terminal region of the receptor are responsible for the dominant negative effects of RTH receptors (Behr et al, 1997). Mutations in these regions have been shown to preserve critical receptor functions such as dimerization and DNA binding, while inactivating other activities such as T3 binding and transcriptional activation (Kopp et al, 1996; Behr et al, 1997). The degree of resistance has been shown to vary with organ type (Behr and Loos, 1996). The RTH disorder demonstrates the importance of thyroid hormone receptor β1 as a critical element in proper bodily development and function.

Thyroid Hormone Receptor β1 Plasticity

In the context of stress and depressive illness interest in the plasticity of the β1 thyroid hormone receptor derives from the fact that, unlike the α and β2 subtypes, β1 is thought to be the predominant type of thyroid hormone receptor in brain (Bradley et al, 1989; Puymirat 1992). Our own laboratory has examined the plasticity of the receptor in addition to examining the relationship between levels of circulating thyroid hormones and the β1 receptor in brain (Nobrega et al, 1997). This study used immunohistochemistry to map the distribution of β1 thyroid hormone receptor in normal and thyroidectomized adult rat brain. It was found that thyroidectomy resulted in increased immunolabeling in many of the brain regions sampled, demonstrating that the β1 thyroid hormone receptor in brain is capable of plastic changes in
response to adult-onset alterations in thyroid hormone levels. Additionally, this study suggested a relationship between the thyroid hormone receptors and the level of thyroid hormone. Specifically, decreases in thyroid hormones (via thyroidectomy) as determined by RIA were countered by an increase in the levels of thyroid hormone receptors within the rat brain as measured with radioimmunohistochemistry.

Summary and Objectives

Following an initial demonstration that brain thyroid hormone receptors can be altered in response to physiological manipulations, the objective of this project was to determine whether these receptors can also be altered in response to external stressful stimulation. Interest in stress responses derives from the association between stress and affective disorders, and the postulated involvement of brain thyroid hormones in the latter. The β1 subtype was chosen for these initial studies because of its abundance in brain, particularly in limbic regions (Bradley et al, 1989; Puymirat 1992), which are often associated with affective processes in humans. On the basis of preliminary findings (Friedman et al, 1999), it was hypothesized that stressful manipulations would result in an increase in thyroid hormone levels and this would cause a decrease in the β1 thyroid hormone receptor.
2. GENERAL METHODOLOGY – MEASUREMENT OF β1-LIKE IMMUNOREACTIVITY

2.1 Assessing Thyroid Hormone Receptor Changes

Possible methods for measuring changes in β1 thyroid hormone receptor levels include techniques such as binding assays, Western blots, in situ hybridization and immunohistochemistry. Binding assays, the most direct method, need to be performed on nuclear tissue fractions, which in turn require very large amounts of tissue. Western blots circumvent this difficulty but still require preselection of only a few brain areas for analyses. In situ hybridization and immunohistochemical approaches can in principle be used to map changes throughout the brain without the need to decide a priori which brain areas are important. In this initial study we chose to use radioimmunohistochemistry because it makes it possible to quantify levels of expressed receptor protein in small anatomical regions throughout the brain of individual animals. Data from Strait et al. (1990) suggest that there may be important differences between mRNA levels and binding activity of expressed thyroid hormone receptors.

Immunohistochemistry

Immunohistochemistry uses antibodies to localize antigens in tissues or cells for microscopic examination and is a powerful technique for identifying and mapping the distribution of characterized neuronal systems in the brain. Briefly, a primary antibody is added to tissue and subsequently binds a target epitope; then, a secondary antibody is added which binds the primary antibody and provides a visible label, often by amplifying the signal. The use of different types of secondary antibodies and methods for their visualization distinguishes different types of immunohistochemical techniques. Some common immunohistochemical techniques include horseradish peroxidase (HRP), enhanced chemiluminescence (ECL) and avidin biotin complex (ABC). While these techniques have been used to establish the presence and localization of specific molecules within the brain, quantitation of antigens has often been a significant problem. To address this issue this
laboratory developed a quantitative immunohistochemical approach using $^{125}\text{I}$-labeled secondary antibodies which complex to primary antibodies directed against the antigen in question, allowing high resolution imaging of the receptor on film. This technique, first described by Correa et al (1988) is termed "radioimmunohistochemistry". It has been used by other groups with either radiolabeled primary or secondary antibodies (Raisman-Vozari et al, 1991).

![Diagram of radioimmunohistochemical method](image)

Figure 9. Schematic representation of the radioimmunohistochemical method. First, a primary antibody directed against a particular epitope is added. Second, a $^{125}\text{I}$-labeled secondary antibody is added specific for the primary antibody. Finally, exposure to film for 14 days yields the radioactive signal. ▲ is the antigen. ◇ are other antigens.

Detection of antigens by this technique is considered to be more sensitive than that by classical immunohistochemical methods and allows quantification in discrete areas of rat brain (Raisman-Vozari et al, 1991). One of the advantages of the radioactive technique over other immunohistochemical techniques is the possibility of precise quantification of signal using radioactive standards. Sample results from β1 immunohistochemistry can be seen in Figure 10.
Fig. 10  Representative sections illustrating the distribution of rTHβ1 immunoreactivity in rat brain at various levels. The frame at the bottom show non-specific levels obtained in sections incubated with $^{125}$I secondary antibody in the absence of primary antibody.
2.2 Development of the Radioactive Protocol

This laboratory had previously measured β1-like immunoreactivity in adult rat brain using a well characterized monoclonal antibody supplied by Dr. J. Puymirat (Dept. Human Genetics, CHUL Research Centre, St. Foye Quebec) (Puymirat et al, 1991a,b). Dr. Puymirat's antibody had been well characterized for sensitivity and specificity in rat brain sections in this laboratory in experiments which included adsorption of sections which the synthetic peptide used for production of the antibody. However, the decreased availability of this antibody made it necessary to turn to commercial sources for the present study. Testing of commercial antibodies and development of ideal conditions took the better part of one year. During this time approximately 25 different manipulations on the laboratory's standard immunohistochemical procedure were made. Our data with Dr. Puymirat's antibody provided an important reference in evaluating new antibodies. Below is a chronicle of the attempts that were made to adapt the existing radioimmunohistochemical method to the new antibody supplies.

*Antibodies from Affinity Bioreagents*

Antibodies tested included Affinity Bioreagents (ABR, Golden, CO) monoclonal and polyclonal anti thyroid hormone receptor antibodies. Incubation times of primary antibodies with tissues varied in several trials between 24 hours and 6 days. Dilutions of antibodies varied between 1:250 and 1:4000. Additionally, incubation with primary antibody was performed in a cold room, humid chambers and in slide mailers. None of these manipulations involving the ABR antibodies ever produced satisfactory results. Fixatives that were used included a) 4 % paraformaldehyde (PF) in PBS, b) 3 % PF in PBS, c) Zamboni's fixative and d) several combinations of paraformaldehyde and glutaraldehyde. One trial utilized no fixation, but results did not differ significantly between fixation techniques with the ABR antibodies.
Manipulations of tissues included: a) processing fixing tissues before slicing and then using free floating sections; b) slicing fixed tissues and placing them on slides for processing; and c) slicing unfixed tissues, placing them on slides for fixation and then processing these slides. The presence and absence of detergents (Triton X-100) in the procedure were also systematically investigated as well as use of differing molarities of PBS and Tris buffers in the protocols. Although some of these manipulations proved satisfactory for HRP immunohistochemistry, they never yielded appropriate results for radioimmunohistochemistry. It was decided to abandon the ABR antibodies and try a different antibody source.

*The Santa Cruz J52 Antibody*

The J52 anti-thyroid hormone receptor β1 monoclonal antibody was generated by Dr. Sheue-yann Cheng's group (NCI,NIH) using human placental c-erbA protein (human thyroid hormone receptor β1) expressed in E. coli as immunogen (Lin et al, 1991). The c-erbA protein from E. coli has been known to bind T3 with affinity and specificity similar to that described for thyroid receptors isolated from tissues and cultured cells (Lin et al, 1990). The in-vitro expression of purified c-erbA cDNA in E. coli results in the production of three proteins of 55 KDa, 52 KDa, and 35 KDa (Lin et al, 1991). All of these were immunized into mice to produce the J52 antibody.

Studies evaluating the cross reactivity of the antibody have looked at its ability to immunoprecipitate the in-vitro translation products of human α1 and α2 and rat β1, α1 and α2 (Lin et al, 1991). The J52 monoclonal anti thyroid hormone receptor antibody was found not to react with human or rat α thyroid hormone receptors. The antibody reacts only with human and rat β thyroid hormone receptors and has a slightly greater affinity for the in-vitro translation product of 52 KDa as opposed to 55 KDa. The Lin et al (1991) study, using deletional analysis on the thyroid hormone receptor, found that the immunogenic area of the β1 receptor that the J52 recognizes is confined to the second half of the A/B region. Other groups have produced and characterized antibodies against the same region of the β1 receptor and reported similar results (Fukada et al, 1998; Macchia et al, 1990). For the purposes of this project, we placed particular emphasis on the exact match between brain distribution and regional levels of the J52 antibody in comparison to the well characterized antibody from Dr.
J. Puymirat (Puymirat, 1991 a, b) used in previous work from this laboratory (Nobrega et al 1997).

For the first attempt at visualizing the β1 thyroid hormone receptor with the SC J52 mouse monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), it was decided to use standard perfusion techniques of fixation (300 ml of 3 % PF) and to follow the procedure exactly as described by Santa Cruz for free floating HRP immunohistochemistry. The results were better than those results utilizing the ABR antibodies and provided a near perfect match with the quantitative results that have been seen using well characterized β1 antibodies (Puymirat et al, 1991; Nobrega et al, 1997).

A technical problem that existed with perfused tissue was an inconsistency in staining intensity between left and right sides of sections and this occurred frequently. This problem was finally corrected when the method of fixation was changed from perfusing to fixing sections on slides with the same 3 % PF fixative. A new secondary antibody was introduced, namely Amersham sheep anti-mouse 125I-IgG. Standard fixation and tissue slicing procedures were followed. Once tested, it was found the combination of J52 primary and Amersham secondary yielded an appropriate signal.

The histological protocol was perfected by testing different types of slides (gelatin coated vs. lysine coated), section thickness (50 versus 20 microns), fixation times (5 minutes versus 10 minutes) and film exposure time (5 days versus 10 days versus 15 days). The best results were obtained with poly-l-lysine coated slides, 20 micron section thickness, 5 minute fixation time and 15 days of film exposure time.

2.3 Brain Preparation

Rats were decapitated and brains were rapidly removed and placed in ice cold 0.9% saline/distilled water solution for approximately 20 to 30 seconds. Brains were then cut in half with a clean, sharp razor blade and placed, cut side down, on aluminum foil. The foil was placed on dry ice in an insulated container and left for 3 to 5 minutes until the brains were completely frozen. Once frozen, the brains were wrapped in foil and stored at −80°C until needed.
**Brain Slicing**

A random order of slicing was assigned to the brains such that the effects of factors such as blade sharpness, cryostat temperature, and other environmental conditions would be minimized. Brains were removed from -80°C storage and placed on dry ice. They were then imbedded in OCT in brain moulds such that OCT would be evenly distributed over the entire outer surface of the brain. Each brain was then removed from the mould and excess OCT was removed with a sharp scalpel blade. The brain was placed on a cold chuck (with cerebellum nearest the chuck base) and allowed to equilibrate to the temperature of the cryostat (-20 °C). 20 μm sections were taken at 300 μm intervals and placed on gelatin coated slides. Slides were then placed in slide mailers and placed into a vacuum container with Drierite™ and allowed to desiccate for a minimum of 3 days. Finally, the slides were sealed in the mailers, wrapped in foil, and stored at -80°C until processed.

**Immunohistochemistry Protocol**

On the day of the assay, slides were removed from cold storage and left at room temperature between 30 and 60 minutes while still sealed in their mailer (to prevent condensation forming on the sections). The slides were then placed in slide trays (18 slides per tray) and quickly entered step 1 of the protocol described below. All incubations were performed at room temperature on an orbital shaker at 30 RPM unless otherwise specified. The final protocol used was as follows:

1. Sections fixed in 4% paraformaldehyde/.01 M phosphate buffered Saline pH 7.4 solution for 5 minutes.
2.Sections rinsed 3 times with .01 M phosphate buffered saline (PBS) pH 7.4. Each rinse lasted approximately 10 minutes.
3. Sections incubated with .01 % saponin/distilled water for 30 minutes.
4. Sections rinsed 3 times with .01 M PBS pH 7.4. Each rinse lasted approximately 5 minutes.
5. Sections incubated with 1.5 % goat serum/distilled water (blocking serum) solution for 1 hour.
6. Sections incubated overnight with anti thyroid hormone receptor β1 mouse monoclonal antibody/ blocking serum at 10 μL / mL. This incubation was done in slide mailers (approximately 12 mL per mailer) at 4°C.

7. Sections rinsed 3 times with .01 M PBS pH 7.4. Each rinse lasted approximately 5 minutes.

8. Sections incubated for 30 minutes with Amersham sheep anti mouse 125I-conjugated antibody/.01 M PBS pH 7.4 at a concentration of approximately 0.20 μCi / mL.

9. Sections rinsed 3 times with .01 M PBS pH 7.4. Each rinse lasted approximately 5 minutes.

10. A set of non-specific sections was processed in exactly the same way, except that primary antibody was omitted (step 6).

Sections were then rinsed briefly with distilled water, allowed to dry completely, and then placed against film in metal cassettes in the presence of calibrated radioactive standards. In the dark, the gamma emission sensitive film was placed on top of the slides and the film cassette was shut tightly. The cassette was then placed in a black plastic bag, labeled, and placed in a refrigerator at 4 °C. Approximately 2 weeks exposure time was allowed before film development occurred.

Kodak developer, stopper and fixer were prepared according to manufacturer’s directions. The exposed film was removed in the dark and placed in developer, stopper and fixer for 5 minutes, 1 minute and 10 minutes respectively. Then the film was placed under cold running water to rinse completely. After drying, the film was subjected to densitometry.

2.5 Quantification of β1-Like Immunoreactivity on Film

An essential aspect of using radioactive labeling for antibodies is the possibility of precise quantitation on film. To this end, calibrated radioactive standards are normally exposed with every film, and the resulting calibration curve is used to derive concentrations of 125I-labeled anti mouse secondary antibody from computer-assisted optical density measurements in different brain regions. The quantification procedure is thus identical, and
has the same resolution, as that routinely used in quantitative receptor autoradiography. Although the unavailability of purified B1 receptor peptide made it impossible to demonstrate the sensitivity of the procedure for this particular antigen, the general ability of the radioimmunohistochemical approach to resolve small amounts of antigen on film may be illustrated in the case of anti-T3 antibodies. The dot blots in Figure 11 represent different amounts of T3, measured by RIA, and labeled with $^{125}$I-IgG.

![Graph showing T3 ROD Curve](image)

Figure 11: T3 Relative Optical Density (ROD) Curve. The overall sensitivity of the radioimmunohistochemical protocol is illustrated by dot blots containing various amounts of T3. Insert shows curve relating T3 amounts to Relative Optical Density on film.

**Densitometry**

Computer assisted densitometric analyses was done with the MCID system (Imaging Research, St. Catharines, Ont.). After reading calibrated standards, densitometric reading from brain areas were automatically expressed in $\mu$Ci/g tissue. Quantification of thyroid hormone B1-like immunoreactivity was done with no a priori selection of regions to be examined. Densitometry was performed on coded films. As in other comprehensive mapping
studies of this type, assessment of regional brain differences was done by one way analysis of variance, followed where warranted by pairwise comparisons using independent t tests.
3. EXPERIMENT I: THE EFFECTS OF ISOLATION STRESS ON THYROID HORMONE $\beta_1$ RECEPTOR IMMUNOREACTIVITY

3.1 Introduction

Social isolation is a stress model which has long been known to produce enduring alterations in adult mammals, primarily primates and rodents (Hall, 1998). Several behavioural and neurochemical variables have been shown to be affected by this stress paradigm (Harlow et al, 1971; Hennessey, 1997).

Social Isolation as a Model of Human Pathology

A variety of neurotransmitter systems have been shown to be affected by social isolation (Krech et al, 1960). In particular, several studies have described changes in central monoaminergic systems after this procedure (Thoa et al, 1976; Weinstock et al, 1978; Jones et al, 1990; Jones et al, 1992), leading investigators to suggest that socially isolated animals may model certain aspects of human psychopathologies thought to be associated with abnormal monoaminergic activity, including schizophrenia, depression and anxiety (Everitt and Keverne, 1979; Willner, 1984; Deakin and Graeff, 1991; Robbins, 1992).

Studies have shown that the specific effects of social isolation are dependent upon the age of the subjects, but this fact is commonly overlooked when implementing the stress paradigm (Hall, 1998). The terms used to differentiate various types of isolation are defined in terms of three developmental stages: neonatal, postweaning, and adult. The single housing of weanling animals is referred to as “isolation rearing”, whereas the single housing of adult animals is referred to as “isolation housing”. This is in contrast to the social housing of weanlings, known as “social rearing” and the social housing of adults, called “social housing”. This discussion will focus on adult animals or “isolation housing” vs. “social housing” as these are relevant to this experiment.

The effects of social isolation are thought to be attributable to two causes, namely: a) deprivation of stimuli critical to development or maintenance of particular behavioural and
neurobiological mechanisms; and b) chronic stress (Hall, 1998). In this investigation mature animals were used and social isolation was therefore used as a model of chronic stress.

**Neuronal Changes**

A considerable variety of neurochemical measures have been made in socially deprived rats, much of it based on post-mortem analyses of brain tissue. Many important neurotransmitters have been found to respond to this paradigm including dopamine (Thoa et al, 1977; Czyrak et al, 1992; Ahmed et al, 1993), serotonin (Gambardella et al, 1994; Segal et al, 1973; Angulo et al, 1991), norepinephrine (Morinan and Parker, 1985; Jaffe et al, 1993; Wilkinson et al, 1991), acetylcholine (Bickerdike et al, 1993; Fulford et al, 1994; Fulford and Marsden, 1997 (a, b), neuropeptides (Schenk et al, 1982; Katz and Steinberg, 1970; Schenk et al, 1987; Gentsch et al, 1988) and GABA (Naranjo and Fuentes, 1985; Angulo et al, 1991; Miachon et al, 1991; Michon et al, 1990). Social isolation has also been known to affect neuronal morphology in some cases (Ichikawa et al, 1993).

**Social Isolation And Thyroid Hormones**

A single study was found in the literature. Valenti et al (1981) reported that plasma levels of T3 and T4 were significantly increased in isolated animals in comparison with controls.

**Rationale**

This chronic stress paradigm was chosen as an initial, mild form of stress which nevertheless had been demonstrated to affect several neuronal systems in addition to peripheral thyroid hormone levels. Also, its ease of implementation and design simplicity make it an ideal paradigm with which to investigate the effects of chronic stress on brain β1 receptors.

3.2 Method

**Animal Testing**

Experiments were carried out on male Sprague-Dawley rats (Charles River Laboratories, Montreal, Quebec) weighing 450 to 550 g at sacrifice. A total of 16 animals
were used in this experiment. Initially, all rats were housed in groups of 4 for a total of 4 weeks. Half of the animals were then separated into isolated housing for a total of 3 weeks prior to sacrifice. Single and grouped animals were kept in a controlled environment maintained at a constant temperature (21 °C ± 1 °C) and humidity on a 12 hour light / dark cycle (lights on between 07:00 and 19:00 hours), with free access to food and water. Rats were handled periodically for cage cleaning and body weight measurements.

At the end of the deprivation procedure, rats were sacrificed and brain processed for immunohistochemistry as described in Section 2 above. Brain sections from both groups were processed together.

3.3 Results

Table 1 shows hormone receptor β1-like immunoreactivity in separate brain regions as determined by densitometry. Separate one-way analyses of variance indicated that exposure to isolation stress did not affect levels of β1-like thyroid hormone receptor immunoreactivity when compared to controls. There were no apparent trends in receptor change for isolates versus controls.
Table 1: β1-like Immunoreactivity under Social Isolation

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Isolates (n=8)</th>
<th>Control (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus accumbens</td>
<td>120.2 ± 6.9</td>
<td>120.3 ± 5.0</td>
</tr>
<tr>
<td>Amygdala</td>
<td>111.2 ± 3.7</td>
<td>112.8 ± 3.1</td>
</tr>
<tr>
<td>Cerebellar nuclei</td>
<td>55.6 ± 6.7</td>
<td>53.7 ± 6.9</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>104.9 ± 4.8</td>
<td>102.4 ± 6.2</td>
</tr>
<tr>
<td>Central gray</td>
<td>90.1 ± 13.1</td>
<td>84.4 ± 8.7</td>
</tr>
<tr>
<td>Caudatum</td>
<td>104.9 ± 6.1</td>
<td>100.2 ± 6.4</td>
</tr>
<tr>
<td>Cingulate cortex</td>
<td>117.0 ± 4.3</td>
<td>124.0 ± 4.2</td>
</tr>
<tr>
<td>Infrahilimbic cortex</td>
<td>106.0 ± 9.9</td>
<td>114.1 ± 7.5</td>
</tr>
<tr>
<td>Piriform cortex</td>
<td>150.0 ± 4.3</td>
<td>156.0 ± 4.3</td>
</tr>
<tr>
<td>Temporal cortex</td>
<td>89.9 ± 9.7</td>
<td>108.7 ± 4.6</td>
</tr>
<tr>
<td>Dorsal tegmentum</td>
<td>107.7 ± 5.8</td>
<td>96.2 ± 8.0</td>
</tr>
<tr>
<td>Hippocampus CA1</td>
<td>133.8 ± 3.7</td>
<td>135.8 ± 3.7</td>
</tr>
<tr>
<td>Hippocampus CA3</td>
<td>127.4 ± 3.5</td>
<td>127.8 ± 3.1</td>
</tr>
<tr>
<td>Hippocampal dentate gyrus</td>
<td>134.8 ± 3.3</td>
<td>135.3 ± 3.0</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>104.0 ± 2.8</td>
<td>104.7 ± 2.6</td>
</tr>
<tr>
<td>Interpeduncular nucleus</td>
<td>101.0 ± 25.7</td>
<td>96.5 ± 0.7</td>
</tr>
<tr>
<td>Lateral septum</td>
<td>98.5 ± 5.1</td>
<td>109.4 ± 4.8</td>
</tr>
<tr>
<td>Locus coeruleus</td>
<td>122.7 ± 2.7</td>
<td>114.4 ± 5.4</td>
</tr>
<tr>
<td>Substantia nigra</td>
<td>80.5 ± 8.6</td>
<td>90.4 ± 6.1</td>
</tr>
<tr>
<td>Superior colliculus</td>
<td>103.7 ± 6.2</td>
<td>92.5 ± 7.4</td>
</tr>
<tr>
<td>Superior olivary nucleus</td>
<td>118.8 ± 0.9</td>
<td>112.2 ± 11.6</td>
</tr>
<tr>
<td>Thalamus</td>
<td>90.7 ± 4.2</td>
<td>96.0 ± 1.6</td>
</tr>
<tr>
<td>Ventral tegmental area</td>
<td>60.9 ± 8.4</td>
<td>61.1 ± 9.0</td>
</tr>
</tbody>
</table>

*Values are means of ^125^I-IgG ± s.e.m. in μg / g tissue. N = 8 per group.

3.4 Discussion

Exposure to isolation did not produce changes in brain thyroid hormone receptor β1-like immunoreactivity. The absence of changes is interesting as isolation stress has been shown to affect many different neuronal systems as well as levels of peripheral thyroid hormones (Hall, 1998).

The conclusion that isolation stress does not affect β1 receptors should be balanced against two other possibilities. One possibility is that the stressor may affect not the abundance of the receptor, but its ability to bind thyroid hormones – perhaps by changing the
availability of coactivators, corepressors or even the heterodimerization partners involved in transcriptional activity. The radioimmunohistochemical technique is able to detect changes in the amount of β1 thyroid hormone receptor, but it is unable to detect changes in the binding affinity of the receptor for thyroid hormones. Affinity changes have been demonstrated in other contexts. For example, in patients with RTH a decreased affinity of thyroid hormone receptors for the thyroid hormones is mediated by the introduction of a 1 or 2 base point mutation in the 52 KDa protein. Conceivably, isolation stress could play some role in the post translational modification or expression of the receptors such that the affinity of the receptor for the hormones is decreased.

Another possible explanation for the absence of changes in the receptor is that the chosen duration of the chronic isolation, three weeks, although widely utilized in the paradigms in the literature, may not have been ideal. Some neuropeptide systems, including met-enkephalin and preproenkephalin, appear to be very sensitive to time factors in that changes are seen in 2 to 3 weeks, but not after 6 weeks (Hall, 1998). It may be the case that the changes in β1 receptors are only seen much later (i.e. 6 weeks). Another possibility is that changes occur but compensatory mechanisms return many of the variables to near normal levels with time.

It is also possible that the isolation experience, although an established stressor according to the literature, may not have been a stressful enough event to cause changes in the β1 receptor. The possibility that chronic isolation may not be a harsh stress is suggested by the lack of corticosterone response at the end of the study (Hall, 1998; Fagin et al, 1983). Corticosterone changes are considered to be the most important endocrine component of the response to stress and the one which is necessary for successful adaptation (Checkley, 1996). It is a much more consistent finding in uncontrollable footshock studies (Jessop et al, 1989; Swenson and Vogel, 1983; Irwin et al, 1990) thus demonstrating the greater effectiveness of footshock as a stressor. The second study addressed this possibility.
4. EXPERIMENT II: EFFECTS OF ACUTE INESCAPABLE FOOTSHOCK ON THYROID HORMONE RECEPTOR β1 IMMUNOREACTIVITY

4.1 Uncontrollable Stress

Uncontrollable stressors provoke a wide array of behavioural changes, including disruption of shuttle escape performance (Anisman et al, 1979; Maier and Seligman, 1976), responding in appetitive tasks (Rosellini et al, 1982), exploration (Bruto and Anisman, 1983), dominance hierarchies (Williams, 1982) and perseveration (Anisman et al, 1984).

A widely used uncontrollable stressor is inescapable or uncontrollable footshock (Tsuda et al, 1983; Weiss, 1968, 1971). Uncontrollable stress conditions have been widely proposed to increase vulnerability to depression in humans (Anisman and Zacharko, 1983; Frank and Stewart, 1983; Leff et al, 1970; Lloyd C, 1980). Equal amounts of controllable shock have not been shown to produce similar effects on animals (Corum and Thurmond, 1977; Redmond et al, 1973; Seligman and Maier, 1967; Weiss, 1968; Weiss et al, 1981). Additionally, as previously noted, depressed individuals have reported feelings of helplessness, hopelessness or being unable to control events and consequently exposure to uncontrollable stressful events has been hypothesized in bringing about depression (Seligman, 1974, 1975). Thus the uncontrollable shock model appears to share etiological factors with depression observed in humans.

Uncontrollable footshock induces a number of behavioural changes that resemble symptoms seen in human depression. According to Koob (1989), these include a) weight loss and decreased intake of food and water; b) decreased ability to produce active behaviour; c) decreased ability to compete with other animals and loss of normal aggressiveness; d) decreased grooming and play activity; e) decreased responding for appetitive rewards; f) decreased responding for rewarding brain stimulation; g) deficits in the ability to make correct choices in an attentional situations; and h) decreased sleep. The effects of uncontrollable shock correspond well with DSM IV symptomatology for depression.
Furthering the parallels between uncontrollable shock and depression are the responses to treatments. Changes in behaviour produced by exposure to uncontrollable shock are reversed by electroconvulsive therapy, MAO inhibitors, tricyclic antidepressants, and atypical antidepressants (Glazer et al, 1975; Leonard, 1984; Petty and Sherman, 1979; Sherman et al, 1982; Telner and Singhal, 1981). The behaviours are typically not affected by acute applications of drugs, but instead require several days of drug administration (Leonard, 1984; Petty and Sherman, 1979; Telner and Singhal, 1981), similar to what is seen in human depression. Additionally, the behavioural depression has been found to be unaffected by anxiolytics, phenothiazine, stimulants and tranquilizers (Sherman et al, 1982).

Studies which have examined the uncontrollable shock model of depression have utilized two different types of shock, namely short and severe, or long and mild. These seem to produce slightly different results (Weiss et al, 1985) with higher intensity shock producing symptoms more similar to those of human depression. The depression-like symptoms produced by uncontrollable shock are acute, with most symptoms dissipating in two or three days depending on the shock procedure, although learning deficits have been shown to persist for much longer periods of time (Koob, 1989).

Uncontrollable footshock has been shown to alter the immune system, plasma levels of corticosterone, norepinephrine, epinephrine, and dihydroxyphenylglycol (DHPG), novelty-induced ACTH, serotonin, opioids and GABA, among others (Razin et al, 1998; Shibasaki et al, 1998; Koob, 1989).

A study by Josko (Josko, 1996) has shown that inescapable footshock stress causes serum TSH, T4 and T3 concentrations to significantly decrease, indicating inhibition of the pituitary-thyroid axis. More recent work from this laboratory found differences in brain, but not plasma levels of thyroid hormones three hours after an uncontrollable footshock session (Friedman et al, 1999). In the present experiment we examined the effects of different amounts of uncontrollable footshock on β1-like immunoreactivity in brain.
4.2 Method

*Animals*

Experiments were carried out on male Sprague-Dawley rats (Charles River Laboratories, Montreal, Quebec) weighing 450 to 550 g. Animals were housed singly in wire cages and were kept in a controlled environment maintained at a constant temperature (21 °C ± 1 °C) and humidity on a 12 hour light cycle (lights on at 07:00 h), with free access to food and water.

*Shock Procedure*

Rats were divided into 3 groups of 8 matched for body weight, and tested 4 at a time in sound attenuated operant boxes (Model ENV-008CT, Med Associates, St. Albans, VT). Group 1 rats received a total of 40 minutes shock; Group 2 received 20 minutes of shock; and Group 3 received no shock (Control rats). Footshocks were given at random time intervals (between 5 and 60 seconds) and at random durations (between 5 and 60 seconds). The shock stimulus consisted of scrambled pulsed 0.8 mA shock delivered to the metal bar floor of the operant chamber by a software controlled shock generator (Med Associates, St. Albans, VT). The shock session ended when the desired total shock times were reached. To equate apparatus time for all groups, all rats were left in the operant boxes for the same time (approximately 80 minutes). Rats were sacrificed by decapitation 24 hours after the footshock session. Brains were collected and processed as described previously. In addition, brain and plasma levels of T3 and T4, brain activity of the type II deiodinase enzyme (which converts T3 to T4), and plasma levels of corticosteroids, were also measured. Assay procedures for each are described in Friedman et al (1999).

4.3 Results

As shown in Table 2, 20 or 40 min of footshock uncontrollable stress produced no significant changes in brain or plasma levels of T3 or T4, or brain activity of type II deiodinase. Corticosteroid levels were elevated in plasma but the difference did not achieve statistical significance. As shown in Table 3, the stress procedures did not affect brain β1-like immunoreactivity in any of the regions examined.
Table 2: Effects of 0, 20 and 40 minutes of uncontrollable shock

<table>
<thead>
<tr>
<th></th>
<th>0 min (n=8)</th>
<th>20 min (n=8)</th>
<th>40 min (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Whole-brain levels (pg/g T)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>2168.7 ± 67.3</td>
<td>2112.2 ± 154.3</td>
<td>2112.2 ± 85.9</td>
</tr>
<tr>
<td>T4</td>
<td>5095.9 ± 195.4</td>
<td>4697.8 ± 397.8</td>
<td>5538.4 ± 647.5</td>
</tr>
<tr>
<td><strong>Type II deiodinase activity in whole-brain brain (fmol/mg/hr)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>79.31 ± 11.63</td>
<td>67.14 ± 4.02</td>
<td>77.06 ± 7.19</td>
</tr>
<tr>
<td><strong>Plasma levels</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3 (ng/dL)</td>
<td>82.0 ± 8.2</td>
<td>80.5 ± 3.3</td>
<td>81.4 ± 5.6</td>
</tr>
<tr>
<td>T4 (μg/dL)</td>
<td>4.5 ± 0.3</td>
<td>4.7 ± 0.4</td>
<td>5.1 ± 0.2</td>
</tr>
<tr>
<td>Corticosteroids (ng/mL)</td>
<td>18.4 ± 6.7</td>
<td>36.3 ± 12.2</td>
<td>29.8 ± 8.6</td>
</tr>
</tbody>
</table>
Table 3: Brain Levels of Thyroid Hormone Receptor β1 Immunoreactivity after Uncontrollable Footshock Stress *

<table>
<thead>
<tr>
<th>Brain Level</th>
<th>40 min</th>
<th>20 min</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus accumbens</td>
<td>164.1 ± 8.3</td>
<td>165.1 ± 9.3</td>
<td>163.9 ± 9.0</td>
</tr>
<tr>
<td>Amygdala</td>
<td>75.3 ± 11.7</td>
<td>81.3 ± 10.5</td>
<td>95.0 ± 10.5</td>
</tr>
<tr>
<td>Cerebellar nuclei</td>
<td>105.6 ± 8.1</td>
<td>115.0 ± 9.8</td>
<td>115.5 ± 6.5</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>122.1 ± 7.5</td>
<td>139.3 ± 7.0</td>
<td>131.6 ± 4.5</td>
</tr>
<tr>
<td>Central gray</td>
<td>138.8 ± 12.8</td>
<td>150.9 ± 6.0</td>
<td>139.0 ± 16.3</td>
</tr>
<tr>
<td>Caudate-putamen</td>
<td>147.4 ± 9.6</td>
<td>151.5 ± 10.4</td>
<td>143.9 ± 5.5</td>
</tr>
<tr>
<td>Cingulate cortex</td>
<td>153.9 ± 10.4</td>
<td>155.9 ± 12.1</td>
<td>145.5 ± 6.1</td>
</tr>
<tr>
<td>Infrafimbic cortex</td>
<td>157.1 ± 9.6</td>
<td>152.6 ± 8.7</td>
<td>159.9 ± 9.0</td>
</tr>
<tr>
<td>Piriform cortex</td>
<td>202.9 ± 7.4</td>
<td>203.3 ± 11.8</td>
<td>188.2 ± 6.9</td>
</tr>
<tr>
<td>Temporal cortex</td>
<td>77.3 ± 13.2</td>
<td>101.5 ± 14.9</td>
<td>112.5 ± 10.6</td>
</tr>
<tr>
<td>Dorsal tegmentum</td>
<td>122.5 ± 8.0</td>
<td>134.4 ± 6.4</td>
<td>117.8 ± 6.3</td>
</tr>
<tr>
<td>Hippocampus CA1</td>
<td>90.3 ± 10.5</td>
<td>95.8 ± 14.0</td>
<td>130.5 ± 20.3</td>
</tr>
<tr>
<td>Hippocampus CA3</td>
<td>87.9 ± 10.1</td>
<td>91.5 ± 16.7</td>
<td>123.7 ± 17.5</td>
</tr>
<tr>
<td>Hippocampal dentate gyrus</td>
<td>90.4 ± 10.1</td>
<td>90.3 ± 13.0</td>
<td>121.4 ± 20.5</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>63.8 ± 11.7</td>
<td>77.9 ± 9.7</td>
<td>89.2 ± 11.6</td>
</tr>
<tr>
<td>Interpeduncular nucleus</td>
<td>165.9 ± 10.8</td>
<td>165.1 ± 10.9</td>
<td>175.3 ± 11.3</td>
</tr>
<tr>
<td>Mammillary nucleus</td>
<td>164.1 ± 8.7</td>
<td>191.8 ± 13.0</td>
<td>147.3 ± 26.3</td>
</tr>
<tr>
<td>Lateral septum</td>
<td>144.2 ± 10.2</td>
<td>157.1 ± 9.0</td>
<td>136.4 ± 7.9</td>
</tr>
<tr>
<td>Locus coeruieus</td>
<td>124.8 ± 3.6</td>
<td>117.4 ± 2.5</td>
<td>118.7 ± 2.22</td>
</tr>
<tr>
<td>Substantia nigra</td>
<td>156.5 ± 8.4</td>
<td>159.4 ± 6.5</td>
<td>156.2 ± 14.7</td>
</tr>
<tr>
<td>Superior colliculus</td>
<td>169.4 ± 9.5</td>
<td>153.4 ± 7.4</td>
<td>155.3 ± 13.1</td>
</tr>
<tr>
<td>Superior olivary nucleus</td>
<td>139.2 ± 13.5</td>
<td>147.4 ± 9.0</td>
<td>154.0 ± 12.3</td>
</tr>
<tr>
<td>Thalamus</td>
<td>66.6 ± 10.9</td>
<td>74.1 ± 9.2</td>
<td>90.5 ± 12.2</td>
</tr>
<tr>
<td>Ventral tegmental area</td>
<td>133.2 ± 7.2</td>
<td>141.5 ± 9.8</td>
<td>131.7 ± 17.3</td>
</tr>
</tbody>
</table>

* Values are means of $^{125}$I-IgG ± s.e.m. in μg/g tissue. N = 8 per group.

4.4 Discussion

As was the case with Experiment I, exposure to electrical footshock produced no changes in brain thyroid hormone receptor β1 immunoreactivity. These results were unexpected, as uncontrollable footshock has been shown to affect many different neuronal systems including central thyroid hormone levels (Shibasaki et al, 1998; Razin et al, 1998; Josko, 1996; Friedman et al 1999).
A possible explanation for the lack of change in receptor density might be the time elapsed post stress. Twenty-four hours was chosen as a starting point to observe changes in the receptor, but this may have been insufficient for changes to occur in β1 thyroid hormone receptor expression.

A second possibility is that uncontrollable stress does not affect all animals equally. The literature has shown that symptom profiles vary considerably in depression (Rush, 1986) and that depression itself appears to be a biochemically heterogeneous disorder (Janowsky and Risch, 1987; Jimmerson, 1987; Siever, 1987). This is also the case in animals, where there is much variability in the impact of stressors on central neurochemical measures and in the profile of behavioural alterations (Zacharko and Anisman, 1989). In uncontrollable stress paradigm used here group comparisons were made. The individual response to stress by each rat was not assessed, and rats which may have reacted either very poorly or very well to stress were combined into the same group. To address the possibility that individual differences in response to stress may be reflected in differential β1 thyroid hormone receptor changes a final experiment was conducted in which the learned helplessness paradigm was utilized.
5. EXPERIMENT III: LEARNED HELPlessness AND THE β1 THYROID HORMONE RECEPTOR

5.1 Introduction: Learned Helplessness and Depression

Since originally conceptualized in the 1960s by Seligman and co-workers, learned helplessness has been accepted as a useful model of animal depression (Seligman et al, 1972; Gurgius et al, 1996). An attractive feature of this paradigm is the breadth of symptomatic parallels to depression (Willner, 1991).

The term “learned helplessness” designates instances in which some phenomenon is produced by the uncontrollability of events experienced by the organism rather than by the events per se (Maier, 1984). In other words, it is a condition in which exposure to an uncontrolled aversive stimulus leads to a decreased ability to escape future aversive situations (Brochet et al, 1987). The exposure to uncontrollable stress provides the basis in animals as well as in people for learning that stress is uncontrollable and it is thought that this learning is responsible for the production of depression in humans (Willner, 1991).

A typical learned helplessness experiment uses two groups of animals over two experimental sessions and it involves a variation of the degree to which subjects are able to control the event administered. During the first session, one group is subjected to uncontrollable stress, while the control group is not. Later, both groups are screened to assess their ability to escape in a controllable shock situation and rats are then divided into subgroups showing high or low escape impairment. Although shock is a commonly utilized stressor in the learned helplessness paradigm, the induction of learned helplessness in rats is not specific to shock. Escape deficits can also be produced by other types of stress such as tumbling, defeat in fighting and restraint (Seligman, 1972).

Learned helplessness was originally described in dogs, but the effects have since been noted in several species including rats, cats, fish, mice and men (Seligman, 1972). The majority of recent experiments have utilized rats as test subjects because learned helplessness in this species of animal has proven to be a robust finding (Willner, 1994; Willner, 1985).
The Leamed Helplessness Theory of Depression

The Leamed helplessness theory proposes that the cause of depression is the belief that a person is helpless and was based largely on the number of parallels between depression and the laboratory phenomena of learned helplessness. They include passiveness and a belief in the futility of action, loss of appetite and weight, a decrease in aggression and in social and sexual interest, and the fact that both dissipate in time if untreated. Similarities in treatment outcome and biochemistry should also be noted (Maier, 1984).

According to Maier (1984), there are two mechanisms which are responsible for the behaviours associated with learned helplessness, namely: a) motivational changes; and b) associated perceptual changes. First, it is thought that the idea of no control should reduce the organism's incentive to attempt to escape, thus resulting in a motivational response initiation deficit. Second, having learned response-outcome independence should interfere with the organism's propensity to associate or perceive the relationship between the new escape response and shock termination should a successful escape response occur. Having learned that behaviour and shock termination are uncorrelated should interfere with learning that they are now related.

5.2 Behavioural Defects in Learned Helplessness

A number of behavioural changes have been documented as a result of learned helplessness (Maier and Jackson, 1979). According to Maier (Maier, 1984), these changes may be divided into three classes: a) cognitive-associative-perceptual deficits; b) motivational response initiation/maintenance deficits; and c) affective deficits.

Cognitive Associated Perceptual Deficits

Inescapable shock produces a true cognitive deficit that cannot be explained as simply reduced motor activity. Subjects that receive shock over which they have no control are later less likely to associate their behaviour with relief when they are exposed to the escapable situation. This demonstration of cognitive retardation does not follow exposure to escapable shock and is therefore attributable to the uncontrollability of the inescapable shock (Maier, 1984).
Motivational Response Initiation/Maintenance Deficit

Inescapably shocked subjects are later less active in the presence of shock, even under conditions in which the test shocks can be terminated by responding. Also, this activity reduction is present from the very first test shock and appears to represent a true reduction in unconditioned activity elicited by shock (Maier, 1984).

Affective Deficits

Studies show that inescapably shocked rats are less aggressive in shock-elicited aggression tests, while escapable shock has no effect on aggression. Another consequence of uncontrollable shock is the induction of stress symptomatology over that seen with escapable shock. Rats subjected to inescapable shock tend to eat less, lose more weight and develop a greater number of gastric lesions as compared to rats subject to escapable shock (Weiss et al., 1981). Also, rats subjected to escapable shock show more similarity in stress profile to the non-shocked controls rather than to the inescapably shocked rats (Weiss et al., 1981). Finally, inescapable shock has been shown to produce immunosuppression. This is demonstrated by the finding that uncontrollable shock facilitates the growth of implanted tumors (Sklar and Anisman, 1979, Visintainer et al., 1982) and by evidence of immunosuppression as measured by mitogen induced lymphocyte proliferation (Laudenslager et al., 1983). Escapable shock has not been shown to produce any of the above effects.

Behavioural Prevention

If helplessness is learned, then it is understandable that immunization to inescapable shock should occur by learning that the effects of shock can be overcome via prior exposure to escapable shock. Experiments which have examined immunization via escapable shock have found that rats which have been exposed to escapable shock prior to inescapable shock have a more difficult time learning to be helpless (Seligman and Maier, 1967). This demonstrates that the behaviour is learned and provides strong evidence that helplessness is not due to stress per se, since prior exposure to stress, although increasing total stress, decreased helplessness.
Pharmacology of Learned Helplessness

Studies have shown that some drugs are able to reverse and even block the learned helplessness effect. One study (Sherman et al., 1982) found the inescapable behaviour to be reversed by chronic administration of tricyclic antidepressants (amitriptyline, desipramine, doxepin, imipramine, nortriptyline), atypical antidepressants (iprindole, nisanserin), monoamine oxidase inhibitors (iponiazid, pargyline) and electroconvulsive shock. Acute treatment with antidepressants proved unsuccessful. Additionally, this study also showed that chronic treatments with a host of anxiolytics and stimulants were ineffective.

Many neurochemicals are affected by the learned helplessness state. Studies have shown that brain levels of norepinephrine, dopamine, serotonin, and GABA are modified relative to non-shocked controls and to escapable shocked rats (Brochet et al., 1987; Massol et al., 1990; Gurguis et al., 1996; Martin et al., 1985; Maier, 1985).

Learned Helplessness and Thyroid Hormones

One study (Massol et al., 1996) found that inducing helplessness in rats did not result in any change in peripheral thyroid hormone levels, but TCA therapy dose-dependently decreased plasma T3 levels.

Validity of the Model

The validity of a model is an important consideration when determining which animal model should be used as a simulation for investigating the psychobiology of depression. The procedures for validating a model include evaluation of predictive validity (the success of predictions made from the model), face validity (phenomenological similarities between the model and the disorder) and construct validity (a sound theoretical rationale) (Willner, 1991).

Predictive Validity

A model has predictive validity if performance on the test predicts performance in the situation being modeled (Russel, 1964). In practice the primary application of predictive validation is to assess the effects of potential therapeutic treatments - primarily the correspondence between drug actions in the model and in the clinic. Thus a model has
predictive validity if it successfully discriminates between effective and ineffective treatments. Additionally, the validity of a model is increased if the drug potency seen in the model correlates with that seen in humans.

Studies have shown that several common antidepressant treatments can reverse learned helplessness behaviour. Successful treatments include tricyclics (imipramine, amitriptyline, DMI, nortriptyline, doxepin), MAOI’s (iponiazid, nialamide), atypical antidepressants (iprindole, maintain) and also ECT (Willner, 1986). Treatment with drugs not known for their clinical efficacy against depression such as neuroleptics (chlorpromazine, haloperidol), stimulants (amphetamine, caffeine), sedatives (phenobarbitol, ethanol) and anxiolytics (lorazepam) have proven ineffective in reversing learned helplessness (Willner, 1986).

Hence, it is accepted that the model has good predictive validity in that subjects are able to respond to a wide range of clinically effective treatments and discriminate between effective and ineffective treatments.

**Face Validity**

In order to satisfy the requirements of face validity, a model should resemble the condition being modeled in etiology, biochemistry, symptomatology and treatment. Due to the unknowns in etiology and biochemistry of the disease, symptomatology and treatment are the usable criteria for establishing face validity (McKinney and Bunney, 1969). Two other criteria which are commonly used to add to face validity include the requirement that there should be no major dissimilarities between the model and the condition it models, and that the specificity of the model should be examined to establish whether the symptoms addressed by the model are specific to the condition modeled, or whether they are general features of a number of different psychopathological conditions.

There are a large number of symptoms seen in rats that are associated with learned helplessness. The defining feature of learned helplessness in rats is a lowered voluntary response initiation which produces passivity and psychomotor retardation. Other key features of learned helplessness include loss of appetite and weight. These are commonly seen in
variouss forms of depression, although it is not clear to which specific subgroup of the disease the symptoms of learned helplessness correspond.

Although, further clarification of depression may, in the future, cast a doubt upon the model, the many symptoms of learned helplessness are commonly found in the clinical course of depression. Additionally, given that no behaviours have been found (to date) which completely disagree with the clinical picture, there is good evidence that the stress model does satisfy the basic requirements of face validity.

Construct Validity

The criterion for establishing construct validity is that the model should be based on a sound theoretical rationale. In order to show this, two requirements must be satisfied: a) it must be established that homologous constructs are being studied in animals and people; and b) it must be shown that a change at the level of the construct being modeled is central to the disorder.

The construct validity of the learned helplessness paradigm lies on three assumptions (Maier, 1984): 1) animals exposed to uncontrollable aversive events do become helpless; 2) a similar state is induced in people by uncontrollability; and 3) helplessness in people is the central symptom underlying depression.

With regards to assumption 1, proponents of learned helplessness assert that subjects learn that they have no control thus decreasing their motivation to perform in subsequent tasks. However, other explanations for this apparent lack of motivation to escape include: a) inescapable shock produces a learning difficulty and activity deficit which results from a decrease in pain sensitivity; and b) inescapable shock is more stressful than escapable shock, and therefore depletes the brain of neurochemicals essential to initiate responding. While the first explanation remains uninvestigated, the second can be critically addressed. Consider immunization - the process of making an animal more resistant to the effects of learned helplessness (Maier, 1984). Immunization demonstrates that prior experience with escapable shock makes rats more resistant to the effects of inescapable shock (as tested with a further escapable shock session) as compared to rats that were first exposed to inescapable shock and then tested with escapable shock. The group made more resistant to the effects of inescapable
shock has actually had more monoamine depletion (via shock) than the group non immunized group, but are more motivated to escape the shock, thus disproving the second claim.

Taking into account the evidence that cognitive changes do occur when animals are exposed to inescapable shock, it is not outlandish to suggest that the lack of motivation also has a cognitive origin. However, this remains unproven as does the hypothesis that inactivity is responsible for performance deficits.

With regards to assumption 2, opponents of the model have claimed that studies subjecting humans to uncontrollable aversive events such as noise or insoluble anagrams are frequently found to produce subsequent performance deficits. However, it is often the case that there are no impairments or performance may actually be facilitated (Willner, 1986). However, this should not be unexpected as the learned helplessness model employs a screening to identify those rats made helpless by inescapable shock, that is, there is an endogenous component to becoming helplessness – perhaps genetic in nature - and it should not be expected that every animal exposed to helplessness should become "depressed". This is also the case with depression, in that some people are more susceptible than others depending on many complex and different variables.

Finally, with regards to assumption 3, studies have found that non-depressed volunteers subjected to a helplessness inducing procedure show performance deficits similar to those seen in severely depressed patients. Additionally, many of the specific circumstances under which learned helplessness is to be expected have been verified.

Hence, it appears that learned helplessness meets the criteria for predictive, face and construct validity and it is one of the very few models of depression to fulfill these requirements. It thus appears to be a reasonably good model of depression. While debate still exists about the fundamental nature of the deficits observed (motivational, motoric, cognitive, or combinations), this does not detract from the usefulness of the model as a marker for a general physiologic state that is closely analogous to what is seen in human depression. A feature of the paradigm which was of particular interest in the present study was the ability to distinguish rats according to their individual responses to stress.
5.3 Methods

Animals

Experiments were carried out on male Sprague-Dawley rats (Charles River Laboratories, Montreal, Quebec) weighing between 250 g and 300 g. Animals were housed singly in wire cage housing and were kept in a controlled environment maintained at a constant temperature (21 °C ± 1 °C) and humidity on a 12 hour light cycle (lights on at 07:00 h), with free access to food and water.

Experimental Procedure

The procedure lasted two days. Rats were divided into 3 groups matched for body weight, and tested 4 at a time in sound-attenuated operant boxes (24 cm X 30.5 cm X 21 cm; model ENV-008CT, Med Associates, St Albans, VT) containing retractable levers. Group 1 rats received uncontrollable shock on day 1 and controllable shock on day 2. Group 2 rats received no shock on day 1 and controllable shock on day 2; Group 3 rats were normal controls which received no shock on day 1 or 2. The shock stimulus consisted of scrambled pulsed 0.8 mA shock delivered to the metal bar floor of the operant chamber by a software controlled shock generator, as described in Experiment II.

On day 1, Group 1 rats were subjected to one session of intermittent inescapable shock established by a random probability generator resulting in a total of 40 minutes total shock, with an intershock interval between 1.5 and 60 seconds. Groups 2 and 3 received no shock. On day 2, Group 1 and 2 rats were exposed to a session of controllable shock (controlled with a bar press) where trials began with shock and a light cue. If no bar press occurred within 60 seconds of shock onset, the shock was automatically terminated such that no rat could receive more than 60 seconds of shock in any one trial. Intertrial intervals of 24 seconds were used and exactly 15 trails of escapable shock were given. Failure to escape was defined as any latency over 20 seconds in pressing the pressing the bar on any trial. After the session, animals with 11-15 failures to escape were defined as helpless while those with 0-4
failures in the 15 trials were defined as non-helpless. We chose to follow procedures and definitions that had been described previously by other groups (Edwards et al., 1986, 1991; Sherman et al., 1982) and which had been successfully used to study a number of different neurochemical changes (Edwards et al., 1991, Sherman and Petty, 1982). In this paradigm, animals exposed only to the escape session (Group 2) provide baseline behavioural responding, whereas normal unshocked controls (Group 3) provide controls for neurochemical measures. Since only a fixed percentage of animals were expected to meet the criteria for helplessness, enough animals were used to yield a final N of at least 8 animals per group.

Rats were sacrificed by decapitation 24 hours after the trials. Plasma samples were obtained from trunk blood and brains were rapidly removed and processed as described in Section 2 above.

5.4 Results

Behavioural Results

As a group, rats in Group 1 (previously exposed to inescapable shock) had significantly longer latencies to escape than Group 2 rats (no previous exposure to shock) (34.7 ± 0.99 sec vs. 28.9 ± 1.14, t= 3.8, p < 0.001) over the 15 trials given on day 2. When Group 1 rats were divided according to number of failures, mean latencies for the helpless (>10 failures) subgroup was significantly higher than those for the non-helpless (<5 failures) subgroup (53.8 ± 1.29 vs. 15.04 ± 1.37, t= 20.4, p< 0.0001). Brain analyses concentrated on the helpless and non-helpless subgroups. Table 4 shows the average number of failures in 15 trials of escapable shock in these groups.
Table 4: Number of failures during the Controllable Shock Session

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Number of failures *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1: LH subgroup</td>
<td>9</td>
<td>13.8 ± 0.8</td>
</tr>
<tr>
<td>Group 1: non-LH subgroup</td>
<td>7</td>
<td>2.1 ± 1.2</td>
</tr>
<tr>
<td>Group 2: (No previous shock)</td>
<td>16</td>
<td>7.5 ± 5.9</td>
</tr>
</tbody>
</table>

* Failure is defined as a latency of 20 seconds or more in escaping controllable shock.

β1 immunoreactivity

Table 5 shows levels of β1-like immunoreactivity in separate brain regions as determined by densitometry. Immunoreactivity levels in rats in the non-LH group did not differ from those in home cage controls in any of the brain regions examined. In contrast, β1-like immunoreactivity levels in LH rats were significantly higher than those in the normal control group in cerebellum (+9%, p<0.02), locus coeruleus (+12%, p<0.02) and superior olive (+17%, p<0.004). In the latter region, levels in the LH group were also significantly higher than those on the non-LH group (+18%, p<0.02).
Table 5: β1-like Thyroid Hormone Receptor Immunoreactivity in Learned Helpless Rats *

<table>
<thead>
<tr>
<th></th>
<th>LH</th>
<th>non-LH</th>
<th>CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus accumbens</td>
<td>104.0 ± 5.5</td>
<td>109.4 ± 6.9</td>
<td>107.4 ± 5.4</td>
</tr>
<tr>
<td>Amygdala</td>
<td>101.4 ± 8.0</td>
<td>110.2 ± 1.7</td>
<td>108.7 ± 2.8</td>
</tr>
<tr>
<td>Cerebellar nuclei</td>
<td>94.4 ± 4.4</td>
<td>78.5 ± 8.5</td>
<td>90.3 ± 8.9</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>124.3 ± 3.3**</td>
<td>112.2 ± 2.8</td>
<td>113.8 ± 5.2</td>
</tr>
<tr>
<td>Central gray</td>
<td>112.7 ± 4.7</td>
<td>115.4 ± 3.0</td>
<td>114.3 ± 5.1</td>
</tr>
<tr>
<td>Claustrum</td>
<td>98.9 ± 5.2</td>
<td>105.3 ± 11.0</td>
<td>97.4 ± 3.4</td>
</tr>
<tr>
<td>Caudate putamen</td>
<td>91.0 ± 3.7</td>
<td>94.6 ± 3.9</td>
<td>92.1 ± 2.4</td>
</tr>
<tr>
<td>Cingulate cortex</td>
<td>105.1 ± 3.5</td>
<td>109.4 ± 3.7</td>
<td>105.3 ± 3.7</td>
</tr>
<tr>
<td>Infrahilaric cortex</td>
<td>99.9 ± 4.4</td>
<td>110.2 ± 10.8</td>
<td>103.2 ± 4.2</td>
</tr>
<tr>
<td>Piriform cortex</td>
<td>131.9 ± 3.8</td>
<td>135.0 ± 3.8</td>
<td>129.2 ± 3.4</td>
</tr>
<tr>
<td>Temporal cortex</td>
<td>109.1 ± 8.2</td>
<td>114.4 ± 3.8</td>
<td>114.6 ± 4.0</td>
</tr>
<tr>
<td>Dorsal tegmentum</td>
<td>120.7 ± 6.1</td>
<td>113.3 ± 6.8</td>
<td>118.7 ± 6.9</td>
</tr>
<tr>
<td>Hippocampus, CA1</td>
<td>137.9 ± 5.6</td>
<td>133.1 ± 2.9</td>
<td>131.5 ± 2.9</td>
</tr>
<tr>
<td>Hippocampus, CA3</td>
<td>125.9 ± 4.1</td>
<td>119.8 ± 5.4</td>
<td>123.3 ± 1.9</td>
</tr>
<tr>
<td>Hippocampal dentate gyrus</td>
<td>133.1 ± 4.3</td>
<td>132.1 ± 1.9</td>
<td>130.6 ± 2.1</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>92.9 ± 9.6</td>
<td>108.7 ± 2.9</td>
<td>103.2 ± 5.4</td>
</tr>
<tr>
<td>Interpeduncular nucleus</td>
<td>114.6 ± 6.0</td>
<td>118.4 ± 3.3</td>
<td>119.0 ± 9.8</td>
</tr>
<tr>
<td>Locus coeruleus</td>
<td>120.1 ± 5.8**</td>
<td>102.4 ± 3.8</td>
<td>106.7 ± 5.7</td>
</tr>
<tr>
<td>Mammillary nucleus</td>
<td>119.8 ± 5.8</td>
<td>112.4 ± 12.2</td>
<td>126.8 ± 5.7</td>
</tr>
<tr>
<td>Lateral septum</td>
<td>91.6 ± 4.0</td>
<td>94.4 ± 4.3</td>
<td>86.3 ± 2.4</td>
</tr>
<tr>
<td>Substantia nigra</td>
<td>118.8 ± 2.5</td>
<td>117.9 ± 3.5</td>
<td>120.1 ± 3.3</td>
</tr>
<tr>
<td>Superior colliculus</td>
<td>127.3 ± 3.4</td>
<td>131.6 ± 3.5</td>
<td>123.1 ± 5.0</td>
</tr>
<tr>
<td>Superior olivary nucleus</td>
<td>130.1 ± 4.9***</td>
<td>109.7 ± 2.7</td>
<td>110.8 ± 4.6</td>
</tr>
<tr>
<td>Thalamus</td>
<td>87.6 ± 4.0</td>
<td>91.2 ± 2.2</td>
<td>91.4 ± 2.9</td>
</tr>
<tr>
<td>Ventral tegmental area</td>
<td>88.7 ± 4.2</td>
<td>93.3 ± 2.6</td>
<td>97.6 ± 7.6</td>
</tr>
</tbody>
</table>

* Values are means ± s.e.m. in μg/g tissue. N = 7-9 per group. ** P < 0.02, ***p < 0.001 compared to control group.

Other measures

Thyroid hormone levels in three brain regions and other indices are presented in Table 6. Neither plasma nor brain levels showed any significant differences between any groups.
Table 6: Effects of Learned Helplessness on other Biochemical Measures

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NLH</th>
<th>LH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amygdala</td>
<td>2637.9 ± 167.7</td>
<td>3186.7 ± 228.7</td>
<td>3093.2 ± 281.9</td>
</tr>
<tr>
<td>(n=13)</td>
<td>(n=11)</td>
<td>(n=10)</td>
<td></td>
</tr>
<tr>
<td>Hippocampus</td>
<td>2271.0 ± 271.3</td>
<td>1922.5 ± 248.8</td>
<td>1850.5 ± 173.6</td>
</tr>
<tr>
<td>(n=12)</td>
<td>(n=10)</td>
<td>(n=8)</td>
<td></td>
</tr>
<tr>
<td>Frontal Cortex</td>
<td>2494.0 ± 306.2</td>
<td>2229.9 ± 271.2</td>
<td>2432.7 ± 322.6</td>
</tr>
<tr>
<td>(n=13)</td>
<td>(n=9)</td>
<td>(n=10)</td>
<td></td>
</tr>
<tr>
<td><strong>T4</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amygdala</td>
<td>7761.6 ± 1869.1</td>
<td>9765.1 ± 2532.6</td>
<td>8012.5 ± 2007.2</td>
</tr>
<tr>
<td>(n=7)</td>
<td>(n=5)</td>
<td>(n=6)</td>
<td></td>
</tr>
<tr>
<td>Hippocampus</td>
<td>11376.5 ± 1647.8</td>
<td>8725.8 ± 1075.4</td>
<td>9217.4 ± 1484.9</td>
</tr>
<tr>
<td>(n=7)</td>
<td>(n=8)</td>
<td>(n=7)</td>
<td></td>
</tr>
<tr>
<td>Frontal Cortex</td>
<td>10544.3 ± 1560.9</td>
<td>8624.3 ± 1663.5</td>
<td>10891.7 ± 1718.1</td>
</tr>
<tr>
<td>(n=9)</td>
<td>(n=9)</td>
<td>(n=9)</td>
<td></td>
</tr>
<tr>
<td>Type II deiodinase activity in whole-brain brain (fmol/mg/hr)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>44.0 ± 7.8</td>
<td>23.20 ± 10.1</td>
<td>27.70 ± 10.1</td>
</tr>
<tr>
<td></td>
<td>(n=12)</td>
<td>(n=6)</td>
<td>(n=6)</td>
</tr>
<tr>
<td>Plasma levels</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3 (ng/dL)</td>
<td>86.4 ± 3.2</td>
<td>81.56 ± 4.3</td>
<td>81.06 ± 4.4</td>
</tr>
<tr>
<td>(n=13)</td>
<td>(n=11)</td>
<td>(n=11)</td>
<td></td>
</tr>
<tr>
<td>T4 (µg/dL)</td>
<td>5.8 ± 0.2</td>
<td>5.51 ± 0.2</td>
<td>6.20 ± 0.3</td>
</tr>
<tr>
<td>(n=11)</td>
<td>(n=11)</td>
<td>(n=11)</td>
<td></td>
</tr>
</tbody>
</table>

5.5 Discussion

Rats exposed to the learned helplessness paradigm showed significantly higher levels of β1-like immunoreactivity in three brain areas. The results of the previous experiment, where different amounts of shock failed to elicit changes in β1-like immunoreactivity, suggest that the changes observed in the LH experiment are not due to different amount of shock received by the groups. These results suggest that the receptor is amenable to changes in response to uncontrollable stress, but that these changes occur only in a subgroup of animals showing pronounced escape deficits.
The locus coeruleus (LC) is a central noradrenergic structure in brain and the possibility that β1 receptor may be upregulated in this region may be of interest for several reasons. It has been shown that stress-induced behavioural depression is highly correlated with a large fall in NE levels in the locus coeruleus (Weiss et al, 1981). The literature indicates that animals exposed to uncontrollable shock evidenced behavioural depression when tested 1.5 or 48 hours after the shock and also showed a large depletion of NE in the LC at those times. Changes in NE or other amines in other brain regions were not nearly as pronounced as the change in NE in the LC. Moreover, animals that were tested 72 to 96 hours after shock exposure showed neither behavioural depression nor depletion of NE in the LC region. Thus behavioural depression was accompanied by a large depletion of NE in the LC region, being present when NE was greatly depleted in the LC and absent when large depletion of the amine in the LC was no longer present suggesting the importance of the LC region as an important component representing the behavioural response to depression. Part of the antidepressant efficacy of tricyclic antidepressant drugs, ECT, lithium, and MAO inhibitors is believed to involve their ability to increase NE levels in synapses

Several studies have addressed relationships between the adrenergic and thyroid systems in brains of adult rats. One of the earlier findings was that thyroid hormone excess or deficiency altered brain norepinephrine levels and metabolism, in addition to altering plasma levels of the hormone (Claustre et al, 1996). More recent studies (Dratman and Gordon, 1996; Rozanov and Dratman, 1996), focusing on central structures, have found that T3 may play a neuromodulatory or neurotransmitter role in the adrenergic nervous system. Thus, the data suggest a very close relationship between the thyroid and adrenergic systems in normal animals. It is possible that insufficient thyroid hormones in this area interfere with adrenergic signaling systems involved in depressive disorders. Antidepressant treatments might then correct thyroid hormone concentration abnormalities and lead to downstream normalization of the adrenergic centres in time.

The possible significance of changes observed in the other two brain areas is more difficult to ascertain. Literature regarding the superior olivary complex has shown it to play major roles in the processing of auditory signals (Harrison and Feldman, 1970) especially with regards to spatial localization of sounds in the environment (Lohrke et al, 1998). The finding
of decreased thyroid hormone receptor β1 in this brain region is unexpected in that this organ is not known to be involved in any way in depressive disorders. However, there are human studies which suggest that the deaf have a significantly higher incidence of depression than those with hearing (Watt and Davis, 1991) and that hearing impairment in the elderly correlates with the lack of willingness to live (Jorm et al, 1995). Likewise, the possible significance of downregulation of thyroid hormone β1 receptors in the cerebellum is obscure. The cerebellum has been well documented in humans to be associated with motor and coordination activities in addition to problem-solving, error detection, and language in humans (Mulller et al, 1998). In rats, there have been studies showing that the cerebellum is involved in certain types of memory functions in addition to movement (Guillaumin, 1991; Dahhaoui et al, 1992; Dahhaoui et al, 1990). As with the olivary nucleus, there is no prior literature suggesting an involvement of the cerebellum in depressive states.

A previous study which examined the relationship between thyroidectomy and β1 thyroid hormone receptor immunoreactivity (Nobrega et al, 1997) found that receptor upregulation occurred following thyroidectomy. Since receptor upregulation was also observed in the present study, it is possible that learned helplessness is associated with decreased levels of thyroid hormones in these particular areas. This would be consistent with the relative hypothyroidism hypothesis of Whybrow and Bauer (Bauer, M. S. and Whybrow PC, 1988) hypothesis regarding the involvement thyroid hormones in depression. However, preliminary measurements of T3 and T4 in brains of these animals (Table 6) do not indicate consistent changes in T3 or T4 levels in the areas examined so far.

If hormone assays indicate that T3 and T4 levels are not altered in LH brains, the observed receptor upregulation would imply an increase in receptor activity in specific brain areas, the functional equivalent of increased T3 activity in brain, as suggested by Joffe (Joffe et al 1994). It would be technically difficult to dissect the locus coeruleus for RIA determinations of T3 or T4 levels. It might be possible, however, to obtain some information in this regard by using antibodies against T3 and T4, and following a quantitative radioimmunohistochemical procedure such as used here for the β1 receptor.
6. GENERAL DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

The main question addressed in this series of experiments was whether the β1 thyroid hormone receptor would show changes in different stress paradigms. Receptor changes were not seen under chronic isolation stress nor uncontrollable footshock stress paradigms, but were detected in the learned helplessness study. These results suggest that the receptor is not equally affected by all types of stress but may undergo alterations only in animals that show depressive type responses to stressful stimulation. These findings are novel and may help clarify the role of thyroid hormones and the β1 thyroid hormone receptor in stress and depression.

It should be noted however that the present design does not allow a determination of whether the observed receptor changes are a consequence of the exposure to uncontrollable shock in susceptible animals, or whether the changes represent a preexisting state in these animals. One approach to distinguishing brain changes that are immediate effects of the shock experience from those that may be permanent states might involve sacrificing LH animals for brain analyses several weeks after the test rather than 24 hr after the test.

The finding of β1 thyroid hormone plasticity in the locus coeruleus under the learned helplessness model of depression may have important implications. Plasticity in this region is in line with findings by other groups that this structure seems to be very important in the development of depressive behaviour. For instance, it has been shown that depression (modeled by uncontrollable stress in animals) is associated with depletion of NE in this region and restoration of NE function follows relief from the depressive state (Weiss et al, 1981). The present results suggest that depressive behaviours may also be associated with decreases in thyroid hormone levels in the locus coeruleus. Hence these experiments suggest a close relationship between the noradrenergic and thyroid hormone systems within this structure of the brain.

Limitations of the Immunohistochemical Approach

Although the radioimmunohistochemical technique used in these studies has been shown to have excellent sensitivity, it is constrained by a number of limitations. In choosing an antibody and the conditions for its use in brain we were guided by the previous quantitative
data previously obtained with another antibody whose specificity in brain had been directly verified. Ideally however, we would have repeated the verification for the new antibody and this was not done because lack of access to the synthetic peptide used for the production of the J52 antibody from Santa Cruz. Until such direct demonstrations are performed, the results obtained here should be conservatively labeled β1-like immunoreactivity.

A second limitation, common to immunohistochemical approaches in general, is the inability of the technique to detect possible changes in binding affinity of the receptor for its endogenous ligand. These at present can only be addressed by direct binding studies using nuclear fractions dissected from the regions of interest. This would be technically difficult in the case of small structures such as the locus coeruleus.

Receptor Changes in Learned Helplessness: Cause or Consequence?

One issue to be further investigated is whether the observed receptor changes are a cause or an effect of learned helplessness procedures. As suggested above, one way to gain information on this point might involve a stress-free period of several weeks between testing and sacrifice of learned helpless animals. If receptor changes are not seen in this delayed sacrifice group, this would be evidence they were induced by the stress procedure rather than being a state marker. However, if receptor changes are still seen after several weeks, it would not be possible to reach a conclusion about whether the changes are a cause or consequence of the learned helpless behaviour.

An interesting possibility in this regard would be to examine receptors in animals that have been selectively bred for learned helpless behaviour using the same paradigm described here. Such animals have been bred by Dr. E. Edwards (Univ. Maryland) for more than 40 generations; 100% of the rats from the congenital LH line have now been meeting the criteria for learned helplessness for several generations. This makes it possible to examine receptors in the absence of any shock stimulation. These experiments are currently under way.

Functional Significance of Receptor Changes in Learned Helplessness

Another issue to be examined in future work is whether the observed receptor changes are functionally relevant for the behavioural state. If they are, one might expect the changes to be absent in animals treated with antidepressants. In particular, future studies should...
evaluate the effect of antidepressant therapies on levels of β1 thyroid hormone receptor in the LC to establish the response of the receptor. Additionally, this information could be used to elucidate the relationship between the β1 thyroid hormone receptors and the NE receptors in the LC in the depressive state. This could provide further insights into the mechanisms of depression.

Receptor Changes and Thyroid Hormone Levels in Brain

In terms of the two current hypotheses relating thyroid hormones to depression, it should also be important to determine whether the observed receptor changes are a consequence of diminished thyroid hormone levels or whether they occur independently of changes in hormone levels in brain. As noted earlier, this could in principle be addressed by RIA assays of hormone levels. However, it would be technically very difficult to accurately dissect regions as small as the locus coeruleus for such analyses. The development of a radioimmunohistochemical technique for assays of T3 and T4 in tissue sections might constitute a significant step in this direction.

Time Factors and Other Receptor Subtypes

Although we have shown that 24 hours was sufficient time to observe changes in the β1 thyroid hormone receptor levels, it is uncertain as to whether this is the optimal time to harvest the brains. Studies of the receptor's kinetics are sparse and it is possible that the receptor's changes are more pronounced at time points other than 24 hour post stress. Additionally, it may be possible that different brain regions have different β1 thyroid hormone receptor kinetics and changes in other regions may be evident at other times. Hence, experiments should be performed to titrate the time of sacrifice post stress and the level of change in the receptor in various brain regions. Such a study will not only provide information of the kinetics of the receptor and its regulation, but may provide a profile of the complexity of β1 thyroid hormone receptor changes to stressors with time.

Finally, future studies should examine other subtypes of thyroid hormone receptors in uncontrollable stress models. The β1 thyroid hormone receptor was chosen for these initial studies as this receptor is thought to be predominantly neuronal and this receptor might be a
good starting point for thyroid hormone receptor analysis. This choice was not meant to imply that this receptor would be the only thyroid hormone receptor of interest. Future studies should examine at the responses of the α1 and β2 thyroid hormone receptors. Measurements of the latter may provide further insights into the relationships between these receptors and stressors, in addition to the information regarding how the receptors are related to each other and to the β1 thyroid hormone receptor.

**Human Studies**

In Experiment III three areas of the rat brain have been linked to changes in β1-like immunoreactivity under the learned helplessness model of depression. However, learned helplessness is merely a model of a more serious illness found in humans. The findings of changing β1-like immunoreactivity in rat brains may provide new and essential information in human post-mortem brains, in terms of selection of brain areas for analysis, and also in terms of distinguishing brain effects related to the depressive condition itself from effects induced by antidepressant treatments. Additionally, parallels in the changes of β1-like immunoreactivity between the human and rat brain could further the understanding of depression in humans.
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