

**NEURO-IMMUNOLOGICAL
INVESTIGATION OF
AUTO-IMMUNE THYROID FACTORS
IN BIPOLAR DISEASE**

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2017

DECLARATION

I, **Meleshni Naicker**, declare that:

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“As the candidate’s supervisor I **agree**/do not agree to the submission of this dissertation”

Supervisor: Dr Strinivasen Naidoo

Date

DEDICATION

**This dissertation is dedicated to my Parents, Mr and Mrs R.N. Naicker,
my Siblings, Yegeshni and Vinolin Naicker,
my Grandfather, Mr A.N. Naidoo,
my late Grandparents
& God**

PUBLICATIONS ARISING FROM THIS DISSERTATION

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For the full-text, refer to appendix, page 191 of dissertation

2. Manuscript in Preparation

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ABBREVIATIONS

AA, amino acid;

AITD, auto-immune thyroid disorder;

bp, base pairs;

BBB, blood-brain barrier;

BSA, bovine serum albumin;

cAMP, cyclic adenosine monophosphate;

cDNA, complementary DNA;

CHO, choline-containing compounds;

C_T, threshold cycle;

CSF, cerebrospinal fluid;

CNS, central nervous system;

DAB, diaminobenzidine;

dATP, deoxyadenosine triphosphate;

dCTP, deoxycytidine triphosphate;

DEPC, diethylpyrocarbonate;

dGTP, deoxydeoxyguanosine triphosphate;

dH₂O, distilled water;

DIG, digoxigenin;

DIT, di-iodothyrosine;

DNA, deoxyribonucleic acid;

dTTP, deoxythymidine triphosphate;

DTT, dithiothreitol;

etc, etcetera;

FFPE, formalin-fixed paraffin-embedded;

g, gram;

GAPDH, glyceraldehyde-3-phosphate dehydrogenase;

H₂O, water;

H₂O₂, hydrodgen peroxide;

HCl, hydrogen chloride;

HPT, hypothalamic-pituitary-thyroid;

IgG, immunoglobulin G;

IHC, immuno-histochemistry;

***in-situ* RT-PCR**, *in-situ* reverse-transcriptase polymerase chain reaction

iqr, inter-quartile range;

kb, kilobase;

kDa, kilodalton;

L, litre;

MeOH, methanol;

mg, milligram;

MIT, mono-iodotyrosine;

mRNA, messenger ribonucleic acid;

MRS, magnetic resonance spectroscopy

NAA, N-acetyl aspartate;

NaCl, sodium chloride;

NBT/BCIP, nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate;

ng, nanogram;

NIS, sodium-iodine symporter

PBS, phosphate buffered saline;

PCR, polymerase chain reaction;

RNA, ribonucleic acid;

ROI, region of interest;

ROS, reactive oxygen species

RT, room temperature;

RTqPCR, reverse-transcriptase quantitative polymerase chain reaction;

SSC, saline-sodium citrate buffer;

T₃, triiodothyronine;

T₄, thyroxine;

TBS, tris-buffered saline;

TG, thyroglobulin;

TPO, thyroid peroxidase;

TRH, thyrotropin-releasing hormone;

TSH, thyroid-stimulating hormone;

TSH-R, thyroid-stimulating hormone receptor;

μg, microgram;

μl, microliter;

μM, micromolar;

1°, primary;

2°, secondary;

°C, degrees celcius;

%, percent;

$\Delta C_T = C_{T(\text{Target})} - C_{T(\text{Reference})}$ i.e. $C_{T(\text{TSH-R})} - C_{T(\text{GAPDH})}$;

$\Delta\Delta C_T = \Delta C_{T\text{sample}} - \Delta C_{T\text{calibrator}}$;

$2^{-\Delta\Delta C_T}$, expression fold-change

ABSTRACT

Introduction:

The co-incidence of thyroid auto-immunity and neuro-psychiatric disorders is well-documented. However, the prevailing school of thought implicating auto-immune thyroid disease (AITD) in bipolar disorder is neither well-understood nor universally accepted. Thus, the lack of recent plausible data linking these two disorders provides the rationale for the present study and lends novelty to our results. We investigated the association between Hashimoto's disease (the most common cause of auto-immune hypo-thyroidism) and bipolar disorder through the extra-thyroidal localisation of thyroid-specific proteins, thyroid-stimulating hormone receptor (TSH-R) and thyroglobulin (TG) in major limbic regions of bipolar human brain. The limbic system represents the cerebral control centres for human emotional behaviour responses. Interestingly, the extra-thyroidal localisation of TSH-R and TG has been well-documented. Thyroid-stimulating hormone receptors have been identified in mammalian heart, kidneys, bone, lymphocytes, thymus, pituitary, adipose, skin, hair follicles, fish testes and astrocyte culture. Thyroglobulin has been identified in mammalian skin, hair follicles, thymus and kidney. With particular reference to central nervous system (CNS) localisation, the most relevant to the present study are reports of immuno-reactive TSH-R in human anterior pituitary tissue and cultured astrocytes, as well as in rat brain tissue and cultured astrocytes. However, no previous studies have specifically detected TSH-R and TG in other human cerebral cortical regions. Recently, in a pilot study to the present project, we immuno-localised TSH-R and TG to cortical neurons and cerebral vasculature, respectively, within various human non-limbic brain regions including occipital lobe, parieto-occipito-temporal, primary motor cortex and primary sensory cortex. The present study extends this investigation into the distribution of thyroid-specific proteins, specifically in limbic regions of normal and bipolar human brain at both protein and molecular levels. We postulated that changes in thyroid protein expression in bipolar limbic regions may contribute to mood dysregulation associated with bipolar disorder, thereby implying a significant role for the thyroid system in the pathophysiology of

bipolar disorder. Thus, we chose to compare the distribution of thyroid-specific mRNA and proteins in major limbic regions between normal and bipolar human adult brain and then use this data to infer whether any regulation of limbic thyroid protein expression could be related to the pathophysiology of bipolar disorder.

Methods:

Ethical approval for the present study was granted by the University of KwaZulu-Natal (UKZN), Biomedical Research Ethics Committee (Reference EXPO42/06). Forensic human brain limbic tissue samples (frontal cortices, amygdalae, cingulate gyri, thalamii, hippocampi and hypothalamii) were obtained from five individuals whom had succumbed to causes unrelated to head injury and who had demonstrated no evidence of brain disease or psychological abnormality. Bipolar brain limbic tissue samples of frontal cortices, amygdalae and cingulate gyri were obtained from five confirmed cases of bipolar disorder from a commercial brain bank. In addition, normal thyroid gland tissue was collected for use as a control tissue. Three experimental techniques were used to fulfil the objectives of this project: (i) Commercial polyclonal antibodies raised in rabbit against human thyroid-specific proteins, TSH-R and TG, were used to immuno-localise TSH-R and TG proteins in the major limbic regions of normal and bipolar human brain by standard immuno-histochemical techniques. Quantification of TSH-R and TG immuno-staining was determined by image analysis digitalisation followed by inter- and intra-group statistical comparisons. (ii) Using specific oligonucleotide primers, TSH-R mRNA in normal and bipolar limbic regions was detected by *in-situ* reverse-transcriptase polymerase chain reaction (*in-situ* RT-PCR). This cellular distribution of TSH-R mRNA was then graded according to visual intensity of labelling as well as the extent of cellular distribution. (iii) Solution-type reverse-transcriptase quantitative polymerase chain reaction (RTqPCR) was then used to determine fold-change in TSH-R gene expression in the bipolar limbic group, relative to normal controls, and normalised to the endogenous reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The comparative threshold cycle (C_T) method was used to calculate gene expression fold-differences between normal and bipolar groups.

Results:

There were a number of novel results that were demonstrated in this study:

(i) Immuno-reactive TSH-R and TG was demonstrated within the neurons and vasculature, respectively, of normal and bipolar limbic regions. Specifically, we immuno-localised TSH-R to large motor neurons whilst TG was evident in smooth muscle cells of the tunica media and tunica adventitia of limbic vasculature. Other results included the presence of TG in limbic neurons of cingulate gyrus and frontal cortex in both normal and bipolar limbic regions. Thyroglobulin was also evident in bipolar amygdala neurons but not in the normal neurons. Further, TSH-R and TG proteins were not detected in any limbic neuronal support cells examined, such as astrocytes, neuroglia, oligodendrocytes and satellite cells. Image analysis intra-group statistical comparisons indicated a significant reduction of TSH-R and TG proteins in the bipolar limbic groups when compared to matched normal groups. In addition, we obtained inter-group statistical comparisons for TSH-R and TG proteins in all limbic regions examined. Inter-regional comparisons for TSH-R in normal limbic regions showed significant differences in the hypothalamus and thalamus groups when compared to the other four limbic regions but not when compared to each other. No appreciable difference was noted amongst the remaining four normal limbic groups. Inter-regional comparisons in all bipolar limbic regions examined, indicated no appreciable difference between any of these categories. Inter-regional comparisons for TG in normal limbic vasculature demonstrated that when compared to the amygdala, there was a significant difference in the cingulate gyrus, frontal cortex and thalamus. None of the other inter-regional comparisons showed statistical differences in these normal groups. Inter-regional comparisons for TG in bipolar limbic vasculature demonstrated that when compared to the frontal cortex, significant differences were noted in the cingulate gyrus and amygdala.

(ii) The cellular localisation of TSH-R mRNA by *in-situ* RT-PCR demonstrated evident labelling for TSH-R mRNA in neurons within all matched normal and bipolar limbic regions. However, semi-quantitative comparisons indicated a down-regulated expression of TSH-R mRNA in all bipolar limbic regions when compared to normal controls. In other normal limbic regions examined, TSH-R mRNA was found exclusive to hypothalamic neurons, whilst the thalamus and hippocampus were

devoid of labelling. Further, TSH-R mRNA was not detected in cerebral vasculature or any neuronal support cells.

(iii) RTqPCR determinations of TSH-R gene expression between normal and bipolar groups confirmed the down-regulated expression of TSH-R mRNA in the bipolar limbic regions. Bipolar inter-group statistical comparisons demonstrated TSH-R expression to be significantly reduced in the cingulate gyrus when compared to the amygdala. None of the other comparisons showed appreciable statistical differences.

Discussion and Conclusion:

The main study findings that demonstrate the extra-thyroidal expression of TSH-R and TG in limbic regions of normal and bipolar human brain are unique, and are suggestive of the translocation of thyroid-like proteins via the seemingly-impervious blood-brain barrier (BBB). Interestingly, multiple modes of egress beyond the BBB have been previously described, whereby the integrity of the BBB may be altered during neuro-pathological diseases including HIV-associated dementia, multiple sclerosis, Alzheimer's disease and bipolar disorder. Alteration of the protective BBB increases its permeability to leukocytes and other circulating compounds and triggers signal transduction mechanisms that facilitate the loss of tight junction proteins that constitute the highly specialised cerebral endothelium. Thus, those reports indicating an altered BBB during neuro-pathological conditions, lends support to our findings of TSH-R and TG proteins in the bipolar brain.

Further to these novel discoveries is the demonstration of reduced thyroid protein expression in major limbic regions of the bipolar brain. In particular, our findings of reduced TSH-R expression in limbic regions correlate with previous neuro-imaging reports that describe reduced physiological cortico-limbic tissue volumes and neuro-physiological activity during bipolar disorder. In addition, the presence of TG-like proteins, exclusive to bipolar amygdala neurons, may be associated with other

neuro-imaging findings of amygdala neuro-physiological hyperactivity and enhanced emotional sensitivity in bipolar disorder.

The importance of the thyroid hormones, T₃ and T₄, in mammalian CNS during various life stages has been reported. It is therefore reasonable to speculate that our findings of thyroid-related proteins, TSH-R and TG, in normal limbic neurons may also exhibit an alternate neuro-physiological role, specifically related to mood control. Further, the reduced expression of TSH-R/TG will likely result in reduced localised thyroid function in limbic neurons with consequent altered neuronal functioning and this may predispose or exhibit some involvement in the pathophysiology of mood dysregulation often observed in bipolar disorder. Interestingly, in the present study, we report the reduced expression of TSH-R and TG in limbic neurons of bipolar brain. Alternatively, we may attribute symptoms of mood dysregulation due to limbic-derived TSH-R providing potential targets for thyroid auto-immunity during Hashimoto's disease. We speculate that inhibitory-type auto-antibodies associated with Hashimoto's disease, may agonise TSH-R expressed in limbic neurons. This abnormal interaction may lead to the inactivation or cell-mediated atrophy of limbic neurons with consequential reduction in the levels of expressed TSH-R. Thus, the loss of limbic neurons or diminished neuronal activity may contribute towards mood dysregulation.

The neuro-pathology of diminished neuronal functioning or neuronal atrophy, is suggestive of a neuro-degenerative aetiology in bipolar disorder. This is particularly controversial, since current evidence implicating neuronal structural and functional changes in the pathophysiology of bipolar disorder is limited and does not provide enough support to classify bipolar disorder as a neuro-degenerative type disorder. However, our study suggests that neuronal alterations may be due to changes in thyroidal status in bipolar disorder. Moreover, our correlation of reduced thyroid protein expression levels in bipolar limbic regions with previous neuro-imaging findings of reduced

cortico-limbic volumes and activity during bipolar disorder, provides further supporting evidence indicating neuro-degeneration of mood-controlling limbic structures in bipolar disorder.

CHAPTER 1

Literature Review

1.1 THYROID SYSTEM

The thyroid gland is responsible for producing the thyroid hormones, thyroxine (T₄) and triiodothyronine (T₃), both of which play significant roles in cell differentiation, growth and metabolism (Yen, 2001, Landek and Caturegli, 2009).

In order to fully understand the structures and functions of the thyroid system in relation to the research objective of the current study and to integrate the results later at an anatomical level, a short description of the physiology, structure and function of the thyroid axis participants is required.

1.1.1 Structure of the thyroid gland

The thyroid gland is one of the largest endocrine glands in the body, weighing ~2 g at birth and 15 g in adults, (Landek and Caturegli, 2009) and is situated on the anterior side of the neck, inferior to thyroid cartilage (Adam's apple) and in close proximity to the larynx and trachea (Figure. 1.1). It originates at the base of the tongue and continues downward along the midline before terminating at the trachea. It is a butterfly-shaped organ comprising two lobes connected by the isthmus (Landek and Caturegli, 2009). The thyroid gland has an extensive blood flow of approximately 5 ml/g/min, and is supplied by the superior, inferior and lowest accessory thyroid arteries (Landek and Caturegli, 2009).

The thyroid gland is encased by a fibrous capsule from which septae extend into the gland dividing it into lobules. Each lobule contains about thirty follicles which represents the functional units of the gland (Landek and Caturegli, 2009) (Figure 1.2). Thyroid follicles are filled with a secretory substance called colloid and lined with a single layer of simple cuboidal epithelial cells (Landek and Caturegli, 2009). The size of the follicles and their epithelial cells tends to vary with the activity of the gland. In the hypo-thyroid state, where activity is reduced, the follicles appear large, containing increased amounts of colloid and lined by thin, flattened epithelial cells (Young and Heath, 2000). In the hyper-thyroid state, where activity of the gland is increased, follicles appear smaller and are lined with tall, cuboidal/columnar epithelial cells (Young and Heath, 2000).

Figure 1.1: Gross anatomy of the human thyroid gland, anterior view Adapted from Antranik (2011), <http://antranik.org/the-endocrine-system/>

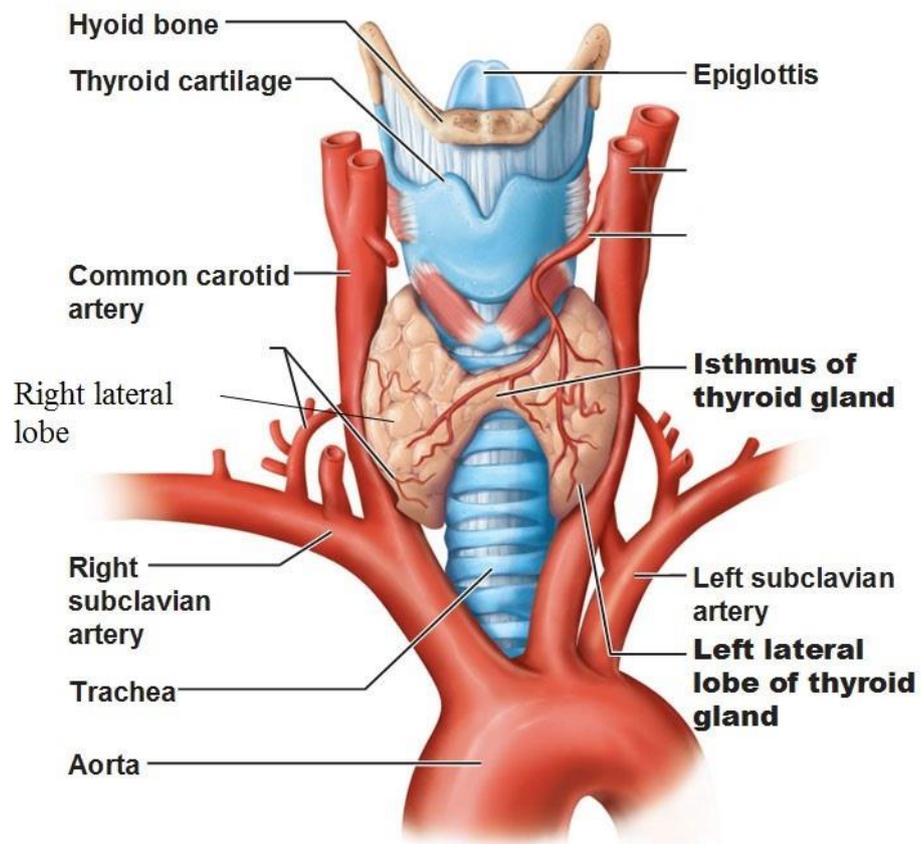


Figure 1.1 legend: Gross anatomy of the human thyroid gland, anterior view

Figure illustrates the anatomy of the human thyroid gland which is situated on the anterior side of the neck, inferior to thyroid cartilage and around the larynx and trachea. It is composed of two lobes that borders the right and left sides of the trachea and is connected by the isthmus. The thyroid gland has a rich blood flow and is supplied by the superior, inferior and lowest accessory thyroid arteries.

Figure 1.2: Histology of the human thyroid gland. Adapted from Barron (2010)

<http://jonbarron.org/article/endocrine-system-thyroid-and-parathyroid-gland>

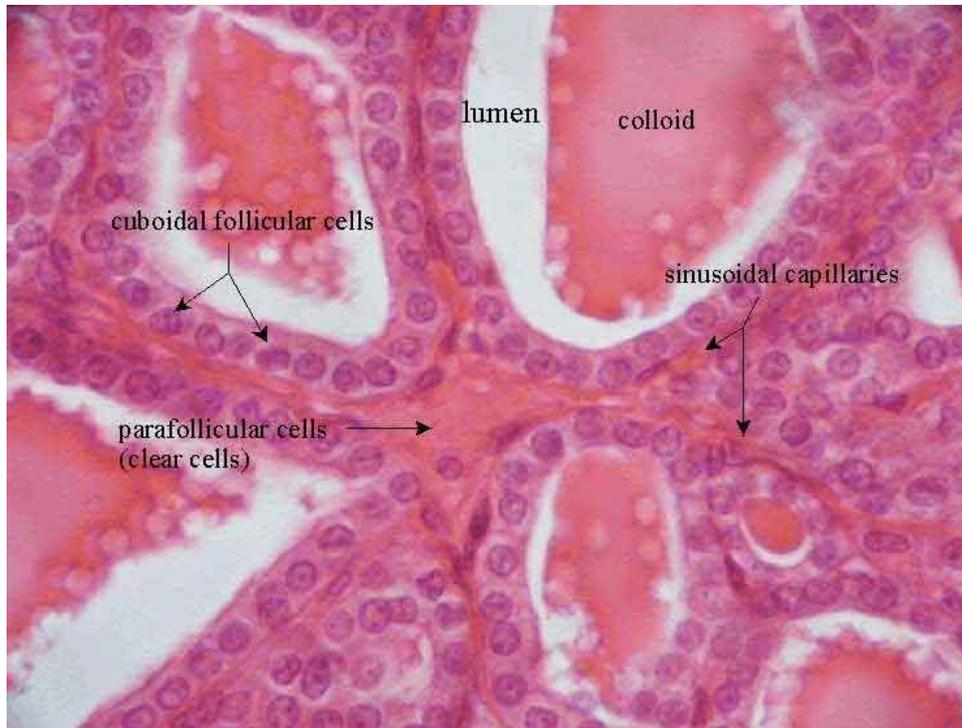


Figure 1.2 legend: Histology of the human thyroid gland

The thyroid gland is primarily composed of follicles that are lined by cuboidal epithelial cells which is responsible for the synthesis and secretion of thyroid hormones, T₃ and T₄. Thyroid follicles are filled with a secretory substance called colloid that contains TG which stores thyroid hormones prior to secretion. A surrounding lumen separates the colloid from the follicular epithelium. Parafollicular cells (clear cells) are single cells with extensive unstained cytoplasm that are scattered among follicular cells. These cells produce calcitonin in response to raised blood calcium levels.

In addition to the thyroid follicular cells, the thyroid gland also contains parafollicular cells also known as clear (C) cells, scattered around the follicular cells (Figure 1.2). Clear (C) cells synthesize and secrete the hormone calcitonin in response to increased blood calcium levels (Young and Health, 2000).

1.1.2 Synthesis of thyroid hormones

Thyroglobulin (TG) is a glycoprotein homodimer that is manufactured by the thyroid follicular cells and released into the colloid by exocytosis (Taurog, 2005, Landek and Caturegli, 2009), (Figure 1.3). Within the colloid, selected tyrosyl residues of the TG polypeptide become iodinated with the aid of the enzyme thyroid peroxidase (TPO), following active uptake of iodine from plasma. This results in the generation of mono-iodotyrosines (MITs) and di-iodotyrosines (DITs), which are inactive hormone precursors. A coupling reaction then leads to the formation of thyroxine (T_4) from two DIT residues, or triiodothyronine (T_3) from one DIT and one MIT (Taurog, 2005, Landek and Caturegli, 2009). Iodinated TG, carrying T_3 , T_4 , DIT and MIT, is stored as colloid in the lumen.

Following TSH stimulation, the thyroid follicular cells reabsorb TG by macro- and micropinocytosis (Figure 1.3), where hormone release occurs by enzymatic splitting of T_3 and T_4 from TG (Taurog, 2005, Landek and Caturegli, 2009). Within the follicular cell, TG gets digested in lysosomes, whilst T_4 and T_3 are released into the bloodstream through unidentified channels (Landek and Caturegli, 2009). Therefore, TG acts as a substrate for thyroid hormone synthesis as well as storage of the inactive forms of thyroid hormone.

The thyroid gland produces primarily T_4 and only 20% of T_3 (Davis et al., 2003, Kopp, 2005, Bauer et al., 2008). Additionally, site-specific T_3 is produced by deiodination of T_4 at peripheral tissues, by enzymes called deiodinases. The local conversion of T_4 to T_3 varies among tissues, with greater conversion occurring in the cerebral cortex (Larsen et al., 1981, Bauer et al., 2008).

Figure 1.3: Synthesis of thyroid hormones. Adapted from <http://www.slideshare.net/roger961/thyroid-gland-5514388>

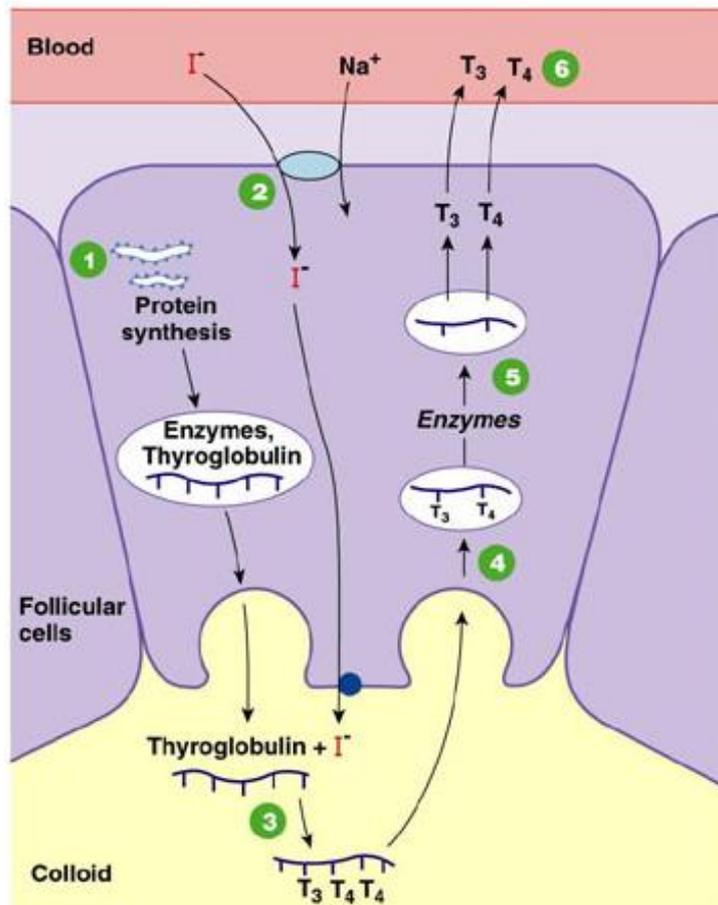


Figure 1.3 legend: Synthesis of thyroid hormones

(1) Synthesis of TG and enzymes within follicular cell and release into colloid, (2) active uptake of iodine from plasma along with Na^+ into cell, and colloid, (3) incorporation of iodine on selected tyrosyl residues of TG to produce T_3 and T_4 , (4) following TSH stimulation, the cell reabsorbs TG, mainly by micropinocytosis, (5) T_3 and T_4 release by enzymatic splitting from TG, (6) Free T_3 and T_4 enter the bloodstream.

1.1.3 Human thyroid-stimulating hormone receptor (TSH-R)

The structural and functional properties of human TSH-R as well as its location within the thyroid gland are listed in Table 1.1.

1.1.4 Human thyroglobulin (TG)

The structural and functional properties of human TG as well as its location within the thyroid gland are listed in Table 1.2.

1.1.5 Extra-thyroidal expression of TSH-R and TG

During auto-immune thyroid disorders (AITD), patients express immuno-reactivity directed towards thyroid-specific proteins including TSH-R and TG (Davies et al., 2002, Ai et al., 2003, Parvathaneni et al., 2012). However, anti-TSH-R and anti-TG auto-antibodies are not limited to binding thyroid proteins located in thyroid tissue only. Several studies have demonstrated the expression and localisation of functional TSH-R in mammalian extra-thyroidal tissues and cells (Table 1.3) including the heart, kidneys, bone, lymphocytes, thymus, pituitary, adipose, skin, hair follicles, astrocyte culture and fish testes (Chabaud and Lissitzky, 1977, Perkonen and Weintraub, 1978, Drvota et al., 1995, Endo et al., 1995, Murakami et al., 1996, Inoue et al., 1998, Valyasevi et al., 1999, Kumar et al., 2000, Prummel et al., 2000, Sellitti, 2000, Crisanti et al., 2001, Davies et al., 2002, Szkudlinski et al., 2002, Bodo et al., 2009, Cianfarani et al., 2010). Of these studies, the most relevant to our present study is that conducted by Prummel (2000) and Crisanti (2001). The Crisanti group demonstrated immuno-reactive TSH-R in cultured rat and human astrocytes and rat brain whilst the Prummel group reported TSH-R in human anterior pituitary tissue. However, TSH-R has not been reported in other human cerebral cortical regions. Thyroglobulin has also been identified in non-thyroid tissue (Table 1.3) such as human skin, hair follicles, thymus and kidney (Spitzweg et al., 1999, Sellitti, 2000, Bodo et al., 2009, Cianfarani et al., 2010).

Table 1.1: Biological properties of human TSH-R

Human TSH-R	
Structural properties of human TSH-R	Reference
<ul style="list-style-type: none"> • Belongs to family of guanine-nucleotide binding (G) protein-coupled receptor 	Patibandla and Prabhakar, 1996, Ando et al., 2005, de Lloyd et al., 2010,
<ul style="list-style-type: none"> • Encoded by the TSH-R gene located on chromosome 14q31 	Rousseau-Merck et al., 1990, Haraguchi et al., 1996,
<ul style="list-style-type: none"> • Full-length TSH-R gene is approximately 4 kb long, consists of 764 amino acid residues, including a 21 amino acid signal peptide • TSH-R protein comprises: <ul style="list-style-type: none"> - A large extracellular domain or ectodomain (A-subunit) - 7 transmembrane domains (B-subunit) - A short cytoplasmic tail in the C-terminus (B-subunit) 	Nagayama et al., 1989, Patibandla and Prabhakar, 1996, Porcellini et al., 1997
<ul style="list-style-type: none"> • Glycoprotein A-subunit is linked to the membrane-spanning B-subunit by disulphide bridges 	Chistiakov, 2003
<ul style="list-style-type: none"> • Has an unglycosylated molecular weight of approximately 84 kDa & a glycosylated molecular weight of 95-100 kDa 	Davies et al., 2002
Location of human TSH-R on thyroid gland	Reference
<ul style="list-style-type: none"> • Primarily expressed on the basolateral surface of thyroid follicular cells 	Ando et al., 2005
Biological functions of human TSH-R	Reference
<ul style="list-style-type: none"> • A-subunit represents TSH binding site on external surface of thyroid cell membrane • B-subunit is involved in signal transduction 	Ando et al., 2005
<ul style="list-style-type: none"> • Regulation of the synthesis and secretion of thyroid hormones from thyroid follicular cells • Controls growth and development of the thyroid gland 	Vassart and Dumont, 1992, de Lloyd et al., 2010,

Table 1.2: Biological properties of human TG

Human TG	
Structural properties of human TG	Reference
<ul style="list-style-type: none"> Encoded by human TG gene 8q24.2-8q24.3 	Baas et al., 1985, Berge-Lefranc et al., 1985, Rabin et al., 1985, Vono-Toniolo and Kopp, 2004
<ul style="list-style-type: none"> Full-length TG mRNA sequence is 8.5 kb long & contains an 8307 nucleotide segment 	Malthiery and Lissitzky, 1987, van de Graaf et al., 1997, Vono-Toniolo and Kopp, 2004
<ul style="list-style-type: none"> Mature TG exists as a 19S glycosylated dimer 	Dunn and Dunn, 2000, Vono-Toniolo and Kopp, 2004
<ul style="list-style-type: none"> Has a molecular weight of 660 kDa 	Dunn and Dunn, 2000, Vono-Toniolo and Kopp, 2004
<ul style="list-style-type: none"> Sites for thyroid hormone synthesis are clustered at both ends of the TG gene 	Malthiery and Lissitzky, 1987
<ul style="list-style-type: none"> TG constitutes up to 75% of protein content in the thyroid gland 	Malthiery et al., 1989
Location of human TG in thyroid gland	Reference
<ul style="list-style-type: none"> Synthesised in thyroid follicular cells & exported to the thyroid lumen Exported to the colloid space for iodination of its tyrosine residues 	Taurog, 2005, Landek and Caturegli, 2009
Biological functions of human TG	Reference
<ul style="list-style-type: none"> It serves as a backbone for the synthesis of thyroid hormones Acts as a storage vehicle for iodine in the form of tyrosyl residues, i.e. MIT and DIT, the inactive hormone precursors 	Taurog, 2005, Landek and Caturegli, 2009
<ul style="list-style-type: none"> A recognised auto-antigen in the implication of Hashimoto's thyroiditis 	Vali et al., 2000

Table 1.3: Tissue distribution of thyroid-specific genes. Adapted and modified from Davies et al (2002)

Tissue/Cells	Gene expressed	mRNA ^a	Protein ^b	References
Lymphocytes	TSH-R	+	+	Chabaud and Lissitzky, 1977, Davies et al., 1978a, Perkonen and Weintraub, 1978, Bagriacik and Klein, 2000,
Thymus	TSH-R, TG	+	+	Murakami et al., 1996, Spitzweg et al., 1999
Pituitary	TSH-R	+	+	Prummel et al., 2000
Testis	TSH-R	+	+	Davies et al., 1978b, Kumar et al., 2000
Kidney	TSH-R, TG	+	+	Sellitti et al., 2000
Brain	TSH-R	+	+	Crisanti et al., 2001
Adipose/ Fibroblast	TSH-R	+	+	Roselli-Rehfuss et al., 1992, Cornelius et al., 1994, Endo et al., 1995, Haraguchi et al., 1996, Bahn et al., 1998, Valyasevi et al., 1999
Heart	TSH-R	+	+	Drvota et al., 1995, Sellitti et al., 1997
Bone	TSH-R	+	NR	Inoue, et al., 1998, Marians, 2001
Skin	TSH-R, TG	+	+	Cianfarani et al., 2010
Hair Follicles	TSH-R, TG	+	+	Bodo et al., 2009

^aRT-PCR/in situ hybridization; ^bImmuno-histochemistry/ligand binding assay; NR, not reported

Upon initiation of the current project, our group conducted a pilot study to determine whether AITD antibodies may immuno-localise in normal, human adult cerebral cortex (Moodley et al., 2011). Brain tissues were obtained post mortem from five patients, all of whom had died of unnatural causes unrelated to head injury and had no indication of psychological abnormality. Various human non-limbic cortical regions were investigated in all five brains, viz. parieto-occipito-temporal lobe, primary motor cortex and primary sensory cortex and the occipital lobe. In that study, we reported the immuno-localisation of anti-TSH-R IgG in normal human cortical neurons of the above regions. Our group also investigated the presence of TG in normal human brain tissue, where we immuno-localised anti-TG IgG to the cerebral vasculature of all brain regions, in all five different human brains examined. Specifically, TG was detected in the smooth muscle cells of the tunica media and tunica adventitia of cerebral blood vessels as well as in some perivascular structures (Moodley et al., 2011).

1.1.6 Regulation of thyroid hormones

Thyroid hormone production is regulated by the hypothalamic-pituitary-thyroid (HPT) axis (Shupnik et al., 1989, Taurog, 2005, Landek and Caturegli, 2009). In response to low circulating thyroid hormone levels, the basal medial hypothalamus releases thyrotropin-releasing hormone (TRH) which then stimulates production of thyroid-stimulating hormone (TSH) from the thyrotropes of the anterior pituitary gland (Figure 1.4). Thyroid-stimulating hormone then binds to TSH-Rs located on the basolateral plasma membrane of the thyroid follicular cell, causing an increase in intracellular cyclic adenosine monophosphate (cAMP) and stimulation of second messenger systems, for the production of thyroid hormones, T₃ and T₄, in order to restore a euthyroid state. Thyroid hormones form long negative feedback loops at the hypothalamic and pituitary levels: TSH production is suppressed when the levels of T₃ and T₄ are high, and TSH is increased when thyroid hormone levels are low (Yen, 2001, Szkudlinski et al., 2002, Bauer et al., 2008). A short negative feedback loop refers to the negative action of TSH on the hypothalamic secretion of TRH.

Figure 1.4: Thyroid hormone production as regulated by the hypothalamic-pituitary-thyroid axis. Adapted from

<http://teachmephysiology.com/endocrine-system/pituitary-gland/anterior-pituitary-gland/>

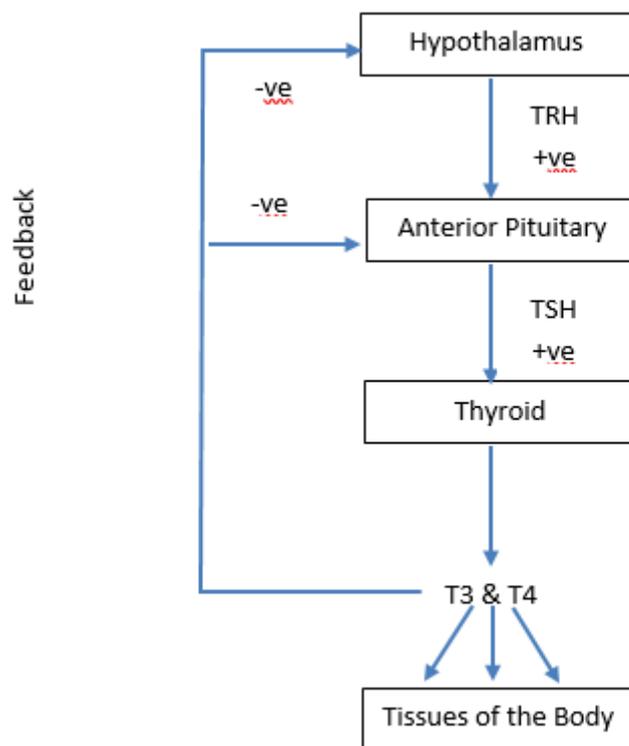


Figure 1.4 legend: Thyroid hormone production as regulated by the hypothalamic-pituitary-thyroid axis

This figure schematically illustrates thyroid hormone production as regulated by the HPT axis. Short and long negative feedback loops along the HPT axis are depicted. The rate of synthesis of thyroid hormones is regulated by anterior pituitary TSH, which is stimulated by hypothalamic TRH. Thyroid hormones exert a negative feedback on thyroid hormone release at the hypothalamic and pituitary levels in order to restore a euthyroid state. A short negative feedback loop refers to the negative action of TSH on the hypothalamic TRH, thus decreasing high levels of pituitary TSH and consequently overall thyroid hormone production from the thyroid gland.

Thus, following an increase in TSH secretion from the anterior pituitary, it causes a corresponding decrease in the amount of TRH released by the hypothalamus. This leads to consequential decreases in pituitary TSH and thyroid hormones from the thyroid gland, thus, restoring a euthyroid state (Yen, 2001, Szkudlinski et al., 2002, Bauer et al., 2008).

1.1.7 Function of thyroid hormones in cognitive development

Thyroid hormones play critical roles in cell differentiation, growth, metabolism, energy and oxygen consumption, and physiological functions of all human tissues (Yen, 2001, Landek and Caturegli, 2009). To achieve this, the thyroid hormones, T₃ and T₄, must bind to thyroid hormone receptors which are expressed in all tissues. There are two major isoforms of human thyroid hormone receptors viz. TR α 1 and TR β -1 (Yen, 2001, Landek and Caturegli, 2009). However, the relative expression of the thyroid receptor isoforms can vary among the tissues. Both T₃ and T₄ can bind to α and β thyroid hormone receptors. However, T₃ has higher binding affinity for both receptors when compared to T₄ (Jameson and Weetman, 2005). In humans, both TR α -1 and TR β -1 are present in most adult tissues. TR β -1 is also highly expressed in liver, whilst TR β -2 is mostly expressed in the anterior pituitary and hypothalamus brain regions. Thus, the expression of thyroid hormone receptor isoforms in different tissue types suggests additional specific functions of the thyroid hormones in the various tissues throughout the body, namely:

1.1.7.1 In the developing brain

Thyroid hormone receptors are expressed in oligodendrocytes, astrocytes and neurons (Mellstrom et al., 1991, Strait et al., 1991, Bradley et al., 1992, Carlson et al., 1994, Carlson et al., 1996, Strait et al., 1997, Carre et al., 1998, Ahmed et al., 2008). TR α is expressed at high levels during early brain development whilst expression of TR β dramatically increases during later brain development (Anderson, 2001). Thyroid hormones have critical roles in the developing brain *in utero* and during the

neonatal period. Thyroid hormone deficiency during this period could result in permanent brain dysfunction that consequently affects intellectual development as well. Providing thyroid hormone replacement therapy to the newborn infant during this period will result in recovery of intellectual development. However, treatment delay beyond 3 months of age can have permanent deficits in intellectual development where “thyroid hormone exerts most of its effects on the maturation of the developing mammalian brain” (Anderson, 2001).

1.1.7.2 In the mature brain

Thyroid hormones appear to have a critical role in the mental and psychological stability in adults where an association between low thyroid hormone levels in hypo-thyroid patients and depression/mood disorders has been reported (Henley and Koehnle, 1997, Anderson, 2001). Further, the mental status of the individual may be improved/reversed following administration of thyroid hormone replacement therapy either solely or as combination therapy with other drugs such as lithium whilst treating for bipolar disorder (Jackson and Asamoah, 1999). In addition, the cognitive deficits observed in hypo-thyroid patients, such as memory impairment, learning, attentiveness and psychomotor slowing can be treated with thyroid hormone (Boyages, 2000, Whybrow and Bauer, 2000). Neuro-psychiatric changes such as dysphoria, anxiety, restlessness, irritability, emotional lability, impaired concentration, intellectual dysfunction, insomnia and behaviour that may mimic mania, have also been reported in hyper-thyroidism (Bennett and Cambor, 1961, Whybrow et al., 1969, Whybrow and Bauer, 2005b, Hage and Azar, 2012).

1.2 THYROID-ASSOCIATED DISEASE

1.2.1 Auto-immune thyroid disease (AITD)

Dysregulation of thyroid hormone production may occur as a consequence of auto-immune thyroid disease (AITD). Patients with AITD have immuno-reactivity directed to thyroid factors such as the

TSH-R, TG, TPO and sodium-iodine symporter (NIS) and can present as either hyper-thyroidism or hypo-thyroidism (Davies et al., 2002, Ai et al., 2003, Parvathaneni et al., 2012).

1.2.1.1 Pathological basis for hyper-thyroidism

Hyper-thyroidism refers to increased circulating levels of thyroid hormones. Grave's disease, an auto-immune genetic abnormality is the most common cause of excess thyroid function (Jameson and Weetman, 2005). The hyper-thyroidism in Grave's disease is caused by thyroid-stimulating immunoglobulins (TSI) which are synthesized in the thyroid gland, bone marrow and lymph nodes (Jameson and Weetman, 2005). These auto-antibodies bind to and stimulate the TSH-R causing over-activity of the thyroid gland (Davies et al., 2002, Szkudlinski et al., 2002, Ai et al., 2003).

1.2.1.2 Pathological basis for hypo-thyroidism

Hypo-thyroidism refers to a deficiency in the circulating levels of thyroid hormones. Clinical features include fatigue, cold intolerance, poor appetite coupled with an increase in body weight, constipation, cool dry skin, hair loss, menstrual irregularities, bradycardia and numerous neuro-psychiatric manifestations (Jameson and Weetman, 2005). Hashimoto's disease is the most common cause of auto-immune hypo-thyroidism. It is caused by auto-antibodies to TG, TSH-R, TPO and follicular microsomes (Jameson and Weetman, 2005). These auto-immune anti-thyroid antibodies fix complement and cause cell-mediated destruction of thyroid follicular cells. The resulting outcome is lymphocytic infiltration, thyroid follicle atrophy, glandular fibrosis and hypofunction (Ai et al., 2003). This leads to reduced TG, TSH-R, TPO and thyroid hormone production and a compensatory rise in TSH, all of which serve as diagnostic markers for clinical hypo-thyroidism (Ai et al., 2003, Jameson and Weetman, 2005).

1.2.1.3 Anti-TG antibodies

Anti-TG antibodies were the first type of circulating thyroid antibodies to be recognised in patients with AITDs. These antibodies act against the protein TG, which is manufactured by the thyroid follicular cells of the thyroid gland and serves as a substrate for thyroid hormone synthesis (Landek and Caturegli, 2009). Hence, this auto-immune antigen-antibody binding may result in destruction of the thyroid tissue.

1.2.1.4 Anti-TSH-R antibodies

Anti-TSH-R antibodies are directed against epitopes on the ectodomain of the TSH-R, located on the thyroid gland. Two classes of TSH-R antibodies can be associated with auto-immune thyroid disorders: (a) thyroid-stimulating auto-antibodies that mimic the actions of TSH and causes Graves' hyper-thyroidism and (b) thyroid-inhibitory auto-antibodies which blocks the receptor binding of TSH, thus, preventing TSH stimulation of the thyroid gland to cause hypo-thyroidism (Hashimoto's disease), (Spencer, 2013).

1.2.1.5 Anti-TPO antibodies

Anti-TPO antibodies are directed against TPO, the enzyme that is manufactured within the thyroid gland and catalyses the oxidation of iodide to iodine on tyrosine residues of the TG polypeptide, for thyroid hormone synthesis (Brix et al., 2004). Hence, this auto-immune antigen-antibody binding will prevent the synthesis of thyroid hormones resulting in lower circulating levels of thyroid factors. Anti-TPO antibodies have also been recognised to be a heritable marker, and thus may serve as an endo-phenotype since family studies have demonstrated aggregation of this type of antibody in healthy first-degree relatives of those with auto-immune thyroiditis.

1.2.1.6 Anti-microsomal antibodies

Recently, the microsomal antigen which was previously mis-identified as TPO due to their antigenic similarities, was found to be expressed on the thyroid cell surface and within the cytoplasm and may represent the cell-surface antigen involved in complement-mediated cytotoxicity (Labodia, 2005). Thyroid microsomal antibodies are directed against the microsomal antigen. Anti-thyroid antibodies provide useful measures for identifying subclinical hypo-thyroidism and for investigating the association between mood and thyroid disorders (Gold et al., 1982, Nemeroff et al., 1985, Reus et al., 1986, Haggerty et al., 1990, Haggerty et al., 1997, Carta et al., 2004).

1.3 MAJOR COMPONENTS OF THE HUMAN LIMBIC SYSTEM

In 1878, Paul Broca applied the term “limbic” (meaning *border* in Latin) to describe the curved rim of the cortex (later referred to as the limbic lobe), which included the cingulate gyrus and parahippocampal gyri (Fuchs and Flugge, 2003). In 1937, James Papez associated emotions, including consciousness, to these specific brain areas (Papez, 1937). Later, in 1952, Paul D. MacLean was the first to coin the phrase “limbic system” in order to describe the limbic lobe along with associated subcortical nuclei as the collective neural substrate for emotion, thereby characterising a functional system (MacLean, 1952). Functionally, the limbic system is responsible for providing most of the emotional drives for setting the other areas of the brain into action. The activities of the body that are governed by the limbic system are those relating with self-preservation (e.g. the search for food or fighting), preservation of species (e.g. reproduction and nurturing the offspring), expression of rage, fear and pleasure as well as the establishment of memory patterns. In addition, it provides the motivational drive for the process of learning (Guyton and Hall, 2000). The major areas of the limbic system includes the amygdala, cingulate gyrus, frontal cortex, hippocampus, hypothalamus and thalamus (Figure 1.5). The limbic areas along with their numerous interconnections forms a complex network for the overall control of emotion. Table 1.4 outlines the location, structure and function of the major human limbic areas.

Figure 1.5: Major limbic structures of human brain. Adapted from
<http://gabipsychology.blogspot.co.za/2014/01/regions-of-brain-involving-emotion.html>

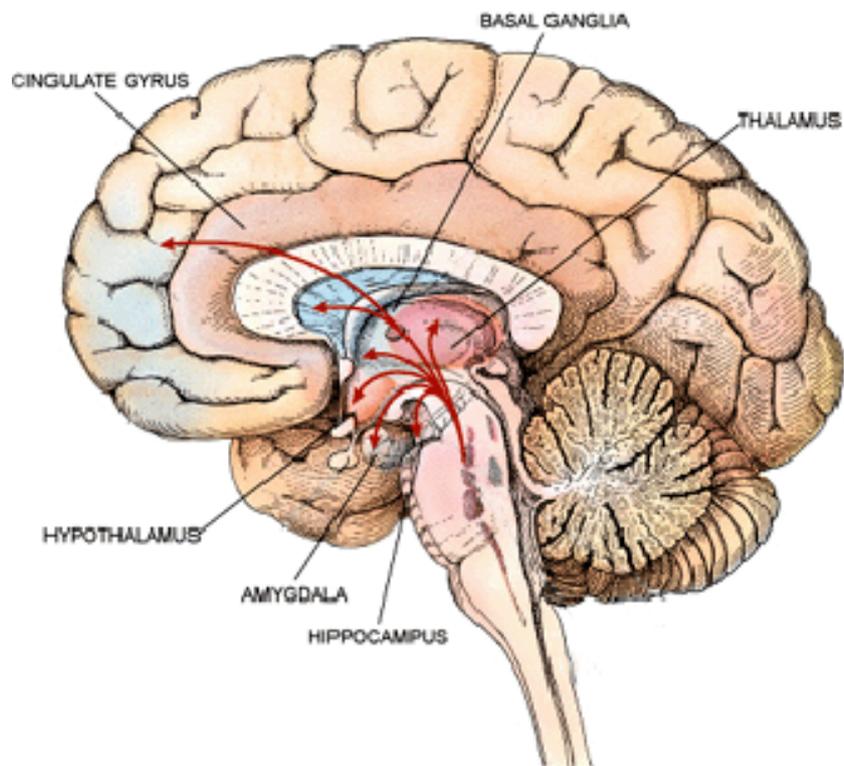


Figure 1.5 legend: Major limbic structures of human brain

Figure illustrates the major brain limbic structures namely: amygdala, cingulate gyrus, hippocampus, hypothalamus and thalamus. Together these structures support the limbic system to control human emotion, behaviour, motivation and memory.

Table 1.4: Major components of the limbic system

Brain Limbic Region	Location, Structure & Connections	Function	Reference
Amygdala	<ul style="list-style-type: none"> • Located near the temporal pole • Almond-shaped structure • Comprises multiple small nuclei • Has several bidirectional connections with the hypothalamus & other limbic areas 	<ul style="list-style-type: none"> • Several connections with hypothalamus & other limbic areas • Receives neuronal signals from limbic cortex • Assists the limbic system to pattern the individuals behavioural response • Emotional response to olfactory stimuli & smell • Choice of food & modulation of food intake • Role in consolidation & retrieval of memories • Fear responses & fear learning • Addiction, motivation & social cognition 	Guyton and Hall, 2000, Ganong, 2003a, Ganong, 2003b, LaBar and Cabeza, 2006, Rajmohan and Mohandas, 2007, Barr, 2009
Cingulate gyrus	<ul style="list-style-type: none"> • Connected to the parahippocampal gyrus by an isthmus around the splenium of the corpus callosum • Connected gyri forms the limbic cortex 	<ul style="list-style-type: none"> • Stimulation elicits autonomic responses that includes: • Regulation of heart rate & blood pressure • Cognitive, attentional & emotional processing 	Rajmohan and Mohandas, 2007, Barr, 2009
Hippocampus	<ul style="list-style-type: none"> • Trilaminar structure • Comprises 3 layers: (a) outer molecular layer; (b) middle pyramidal layer; (c) inner polymorphic layer • Layer of fibres adjacent to the polymorphic layer, coalesces to form the fornix. • Fornix is the main efferent pathway of the hippocampal formation • Hippocampus, dentate gyrus & subicular complex form the hippocampal formation 	<ul style="list-style-type: none"> • Most sensory experiences activate certain parts of hippocampus • Distributes out-going signals to the limbic areas • Stimulation of different areas of hippocampus, initiates various behavioural patterns of pleasure, rage, passivity & sex drive • Long-term declarative memory storage 	Mark et al., 1993, Guyton and Hall, 2000, Pierri and Lewis, 2004, LaBar and Cabeza, 2006, Rajmohan and Mohandas, 2007,
Hypothalamus	<ul style="list-style-type: none"> • Less than 1% of brain mass • Located at the centre of the limbic system; part of the diencephalon 	<ul style="list-style-type: none"> • Controls most vegetative & endocrine functions • Controls emotional behaviour 	Guyton and Hall, 2000, Pierri and Lewis, 2004,

	<ul style="list-style-type: none"> • Is at the confluence of many neural pathways • Divided into 3 regions: (a) supraoptic region; (b) tuberal region (c) mammillary region 	<ul style="list-style-type: none"> • Regulates arterial pressure, heart rate, body temperature & body water • Controls hunger & satiety • Endocrine hormone secretion by the anterior pituitary gland • Uterine contractility at termination of pregnancy & during milk ejection • Behavioural functions such as: (a) control of rage & tranquillity; (b) fear & punishment reactions; (c) sexual drive 	Rajmohan and Mohandas, 2007
Thalamus	<ul style="list-style-type: none"> • Egg-shaped structure • Largest component of diencephalon • Divided into 3 principle nuclear masses, by the internal medullary lamina: (a) anterior; (b) medial; (c) lateral 	<ul style="list-style-type: none"> • Sensory perception & regulation of motor functions 	Barr, 2009

1.4 LIMBIC-ASSOCIATED DISEASE

1.4.1 Bipolar disorder

Bipolar disorder, also known as manic-depressive illness, is a brain disorder that causes unusual shifts in mood, ranging from extreme elation and hyperactivity (mania) to severe depression, with periods of normal mood in between (Figure 1.6), (Emilien et al., 2007, NIMH, 2012). Sometimes during an episode, patients may experience symptoms of both mania and depression, referred to as “mixed state”. Further, the individual’s sleep pattern, energy, activity levels and overall ability to function is affected. The symptoms of bipolar disorder are much more severe than the normal highs and lows that people experience, and can result in damaged relationships, poor job or school performance, impulsive and reckless behaviour, substance abuse and, sadly, even suicide. Bipolar disorder typically develops during late adolescent or early adult years. Some individuals may experience initial symptoms during childhood, whilst others may develop symptoms later in life (Emilien et al., 2007, NIMH, 2012).

1.4.2 The limbic system and bipolar disorder

The limbic system plays a critical role in emotional and behavioural responses. This is supported by Jaak Panksepp’s theory of emotional systems, based on neuronal mechanisms of emotions, to describe “integrated neurobiology of affect, behavioural biology, evolutionary psychology and psychoanalysis” (Zechowski, 2017). Dysfunction of the emotional systems will result in different variants of excitation, which may then predispose to mental disorders (Panksepp, 2005, Panksepp and Biven, 2012).

The limbic system’s intricate functional neuro-anatomy along with its diverse circuits may explain some of the manifestations of neuro-psychiatric disorders (Rajmohan and Mohandas, 2007). Functional neuro-imaging studies have shown decreased prefrontal and anterior cingulate activity in affective disorders (Rajkowska, 2006, Rajmohan and Mohandas, 2007). The anterior cingulate represents a functional centre for the integration of attentional and emotional output and facilitates control of

Figure 1.6: Scale showing varying range of moods associated with bipolar disorder. Adapted and modified from NIMH (2012)

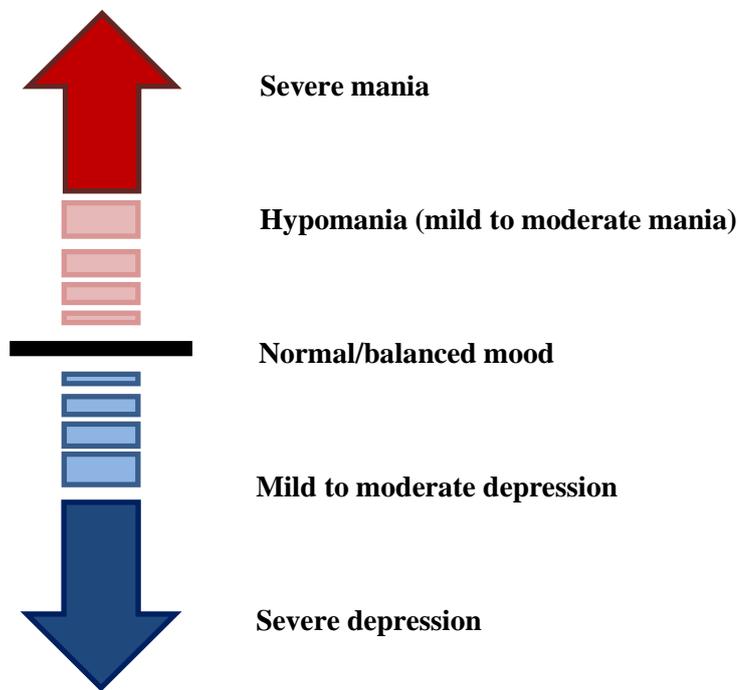


Figure 1.6 legend: Scale showing varying range of moods associated with bipolar disorder

Figure illustrates the various mood states reported in bipolar disorder. The upper half of the scale (pink-red) indicates hypomania and severe mania; whilst the bottom half of the scale (light blue-dark blue) indicates mild-to-moderate depression with severe depression at its extreme end. The centre of the scale (black bar) indicates a normal or balanced state of mood.

emotional arousal. It has been suggested that the spectrum of affective and cognitive symptoms is as a result of dysfunction within the anterior limbic network. This network includes the prefrontal regions and subcortical structures such as the thalamus, striatum, amygdala and the midline cerebellum. Neuro-imaging studies provide evidence that dysfunction of the anterior limbic system may be responsible for the symptoms of bipolar disorder (Strakowski et al., 1999, Strakowski et al., 2005). These studies have shown that neuro-anatomic abnormalities do exist in the network of bipolar patients and include decreased gray matter volumes of prefrontal sub-regions, enlargement of the amygdala and striatum and midline cerebellar atrophy (Strakowski et al., 2005). The neuro-anatomic abnormalities in the prefrontal cortex are consistent with prefrontal histological abnormalities that have also been reported in bipolar patients, and include decreased glial and neuronal density with glial hypertrophy in prefrontal regions of bipolar patients (Rajkowska et al., 2001, Strakowski et al., 2005). Interestingly, it was noted that some of these abnormalities, specifically the reduced volumes of gray matter in the left inferior prefrontal cortex, the reduced size of the cerebellar vermis as well as ventriculomegaly appear to influence the number of affective psychiatric episodes. This suggests that brain changes occur in response to illness course; however, the underlying mechanisms are unclear (Strakowski et al., 2005). Such brain changes may increase the vulnerability of the patient to further affective episodes or even disability (Strakowski et al., 2005). Enlargement of the amygdala and striatum, on the other hand, has not been associated with clinical course, but rather appears to be present at the clinical onset of affective disorders (Strakowski et al., 2005).

The amygdala and hippocampus project into the prefrontal-striatal-thalamic networks and have roles in modulation of emotional and cognitive behaviour (Strakowski et al., 2005). Interestingly, findings of normal hippocampal volumes together with increased amygdala volumes have been reported as a specific neuro-anatomic abnormality dyad in bipolar patients when compared to healthy individuals (Strakowski et al., 1999, Altshuler et al., 2000, Brambilla et al., 2003, Strakowski et al., 2005). In contrast, DelBello and co-workers (2004), reported decreased amygdala volumes in bipolar adolescents, (DelBello et al., 2004). “These findings suggest that amygdala enlargement may therefore be a

consequence of abnormal development of this structure during adolescence and early adulthood” (Strakowski et al., 2005).

Differences in thalamic volumes between bipolar versus healthy subjects have also been reported (Strakowski et al., 1999). Three groups have reported enlargement of the thalamus in bipolar patients compared with healthy subjects (Swayze et al., 1992, Dupont et al., 1995, Strakowski et al., 1999). However, Strakowski and colleagues (1999) found that the effects due to differences in thalamic volumes were considered minimal as opposed to the significant/larger effect exhibited by differences in amygdala volumes (Strakowski et al., 1999).

The midline cerebellum, also referred to as the cerebellar vermis, was initially thought to be solely involved in motor control (Strakowski et al., 2005); however, it has recently been recognised to have strong interconnections with limbic regions (Schmahmann and Sherman, 1998, Strakowski et al., 2005). Several past computed X-ray tomography (CT) studies have reported decreased cerebellar volumes in bipolar disorder (Nasrallah et al., 1981, Lippmann et al., 1982, Nasrallah et al., 1982, Yates et al., 1987). However, this work had not been extended for years until DelBello and co-workers (1999) conducted a study that aimed to determine the size of the cerebellar vermis in bipolar patients afflicted with repeated affective episodes in comparison to first-episode patients and healthy control subjects (DelBello et al., 1999). The DelBello group found that the multiple-episode patients displayed reduced vermis size, compared to the two control groups (i.e. first-episode patients and healthy subjects). Hence, there appears to be an association between vermal size and the number of repeated affective episodes (or recurrent mood episodes), where increasing number of episodes predicts a smaller vermis.

Lateral ventriculomegaly is another well-established neuro-anatomic finding in bipolar disorder (Strakowski et al., 2002a, Strakowski et al., 2005). However, it is unclear as to whether ventricular

enlargement is present at the onset of the disorder or develops during illness. To investigate this, Strakowski and co-workers compared the ventricular volumes among first- and multiple-episode bipolar patients, and healthy subjects (Strakowski et al., 2002b, Strakowski et al., 2005). They found that the lateral ventricles were significantly larger in the patients presenting with multiple-episode bipolar disorder than in the first-episode and healthy subjects. No differences were observed in the latter two groups. In addition, enlarged lateral ventricles correlated strongly with a higher number of prior manic episodes. This finding was supported by the work of Brambilla and co-workers (2001) who also found a correlation between ventricular volume and number of prior episodes in bipolar disorder (Brambilla et al., 2001). These results suggest that brain ventricles increase in size during the course of bipolar disorder and may present as a consequence of prior affective episodes.

1.5 ASSOCIATION BETWEEN AITD AND NEURO-PSYCHIATRIC DISEASES

Neurological conditions associated with thyroid auto-immunity have been widely reported, but not conclusively defined (Krishnan, 2005, Whybrow and Bauer, 2005a, Whybrow and Bauer, 2005b). Suggested potential mechanisms include: (a) A blunted TSH response to TRH, observed in some patients with depression (Jackson, IM, 1998, Larsen et al., 2004); (b) the interactions of the thyroid system with neurotransmitters: Norepinephrine (Whybrow and Bauer, 2005a), serotonin (Bauer et al., 2002) and dopamine (Mano et al., 1998); and (c) the presence of thyroid hormone receptors in limbic structures that influences mood (Whybrow and Bauer, 2005a).

1.5.1 Neuro-psychiatric manifestations in thyroid disorders

There appears to be a neuro-immunological link between AITD and psychiatric disorders, particularly bipolar disease. Hyper-thyroidism or thyrotoxicosis is also accompanied by psychiatric symptoms such as dysphoria, anxiety, restlessness, irritability, emotional lability, impaired concentration, intellectual dysfunction, insomnia and behaviour that may mimic mania (Whybrow and Bauer, 2005b,

Hage and Azar, 2012). In hyper-thyroid elderly patients, depressive symptoms such as apathy, lethargy, pseudo-dementia and depressed mood may also occur (Taylor, 1975, Hage and Azar, 2012).

In hypo-thyroid patients, depression-like symptoms include, psychomotor retardation, insomnia, decreased appetite, irritability, anger, fatigue and lethargy (Whybrow and Bauer, 2005a). This may be accompanied by neuro-cognitive dysfunction and depression as well as impaired perception with paranoia and visual hallucinations (Whybrow and Bauer, 2005a). It appears that severe hypo-thyroidism may mimic melancholic depression and dementia (Whybrow et al., 1969, Hage and Azar, 2012).

1.5.2 Relationship between mood changes and TSH release in response to TRH stimulation

Mood disorders have often been associated with Hashimoto's disease, following a closely-established link between the mood changes (depression and mania), characteristic of bipolar disorder and TSH release in response to TRH. It is well known that the TSH response to TRH stimulation is reduced in overt depression or mania (Kirkegaard, 1981, Loosen, 1985), but this has not been previously demonstrated in milder mood disturbances. In order to determine whether these mild fluctuations in mood are also accompanied by changes in the HPT axis, Larsen and co-workers (2004) conducted a study to investigate a possible relationship between mild mood changes and the TSH response to TRH stimulation in patients with bipolar disorder (Larsen et al., 2004). Their findings were that minor fluctuations in mood, as assessed using mania and depression rating scales, in bipolar patients, actually did correlate with changes in the TSH response to TRH stimulation and, hence, changes in the HPT axis. As mood symptoms become more severe, the TSH release in response to TRH stimulation is reduced. Therefore, these changes mimic those observed in overt depression and mania (Kirkegaard, 1981, Loosen, 1985). Further, observations made in major depressed patients are consistently normal or increased serum T₄ levels, both total and non-protein-bound (free) whereas

serum T₃ levels are normal (Kirkegaard, 1981, Baumgartner et al., 1988). In addition, tracer studies showed a 30% increase in daily production of T₄ and normal production of T₃ in depressed patients (Kirkegaard et al., 1990). These findings suggest some degree of centrally-mediated hyperactivity of the HPT axis during depression. In contrast, Larsen and co-workers (2004) demonstrated a negative correlation between serum T₃ and mood symptom rating scores i.e. the greater the severity of the symptoms, the lower their serum T₃ concentration (Larsen et al., 2004). However, it has long been known that levels of T₃ are reduced in somatic illness and during fasting (Doctor et al., 1993). Therefore, Larsen's findings could be attributed to the possibility that increasing symptom severity is associated with a state that is similar to stress caused by somatic disease, as well as reduced caloric and carbohydrate intake (Doctor et al., 1993, Larsen et al., 2004).

1.5.3 Thyroid disorders as a risk factor for rapid-cycling bipolar disorder

Hypo-thyroidism has been reported to be a probable risk factor for the rapid-cycling type of bipolar disease (Heinrich and Grahm, 2003). Rapid-cycling bipolar disorder occurs when patients experience four or more episodes within a twelve-month period (Heinrich and Grahm, 2003). An episode can consist of depression, mania, hypomania or even a mixed state. Anti-thyroid antibody titres were found in 50% of rapid-cycling bipolar patients (Hennesey and Jackson, 1996, Heinrich and Grahm, 2003). In a study using lithium concurrently, those authors reported overt hypo-thyroidism in 50% of the rapid-cycling bipolar patients and zero in the non-rapid-cycling patients (Heinrich and Grahm, 2003).

1.5.4 Cerebral perfusion abnormalities in thyroid and mood disorders

Functional imaging studies have shown an association between thyroid dysfunction and cerebral hypo-perfusion. These studies included hypo-thyroid patients of varying levels of severity from auto-immunity or thyroid cancer (Bauer et al., 2008). Cerebral hypo-perfusion has been notably observed in the posterior brain regions (Krausz et al., 2004, Nagamachi et al., 2004,

Krausz et al, 2007, Bauer et al., 2008), the parietal lobe (Constant et al., 2001, Bauer et al., 2008) as well as in limbic regions such as the amygdala and hippocampus (Bauer et al., 2006, Bauer et al., 2008). Nevertheless, several studies indicate that perfusion abnormalities are reversible following treatment or when patients achieved a euthyroid state; however, the mechanism remains unclear (Forchetti et al., 1997, Kinuya et al., 1999, Bauer et al., 2006, Schraml et al., 2006, Bauer et al., 2008). A study involving depressed bipolar patients, revealed that receiving supra-physiological dosages of T₄ (300µg/day), resulted in significant improvement in mood which was accompanied by normalisation of cerebral metabolic perfusion in frontal, limbic and subcortical regions (Bauer et al., 2005, Bauer et al., 2008). Associations between thyroid and neuro-psychiatric disorders have, therefore, long been established. This work later prompted investigators including our group to investigate further the link between the thyroid disorders, particularly hypo-thyroidism and bipolar disorder.

1.5.5 Thyroid factors and the acute phases of bipolar disorder

1.5.5.1 Depression

The following alterations in the HPT axis have been reported in patients having experienced depressive episodes:

- (a) an altered (blunted) TSH response to TRH,
- (b) abnormally elevated levels of anti-thyroid antibodies,
- (c) elevated levels of TRH in cerebrospinal fluid (CSF),
- (d) increased serum T₄ levels

Cole and colleagues (2002) investigated pre-treatment thyroid values in bipolar depressed patients, as a predictor for response to anti-depressant treatment (Cole et al., 2002). They reported that low levels of free T₄ and high levels of TSH, both within the normal ranges, were significantly associated with a slower treatment response during early phase treatment for bipolar depression. These patients had a median time to remission of nearly a year. Conversely, high levels of free T₄ and low levels of TSH

were associated with more rapid remission of depression. “Patients with this optimal thyroid profile experienced remission an average of at least 4 months earlier than the remainder of the study group” (Cole et al., 2002). Two earlier studies by Prange (1990) and Frye (1999) also found a similar association between pre-treatment thyroid values and poorer treatment response to anti-depressants (Prange et al., 1990, Frye et al., 1999). In addition, Frye (1999) associated low levels of free T₄ with an increase in the number of affective episodes and greater severity of depression during lithium treatment for bipolar disorder. Similar results were obtained for T₃ levels by Hatterer and colleagues (Hatterer et al., 1988). In addition, it has been shown conclusively for T₃ to augment the efficacy of a variety of anti-depressants (Musselman and Nemeroff, 1996). Some studies have suggested that local hypo-thyroidism in the brain may contribute to depression, especially bipolar depression. Interestingly, this occurs despite the presence of a euthyroid state as indicated by routine peripheral blood thyroid hormone results (Bauer et al., 1990a, Sullivan et al., 1999, Fountoulakis et al., 2004). Lastly, treatment for alleviating symptoms of depression has been shown to reverse alterations in thyroid function in most depressed patients (Jackson, 1998, Konig et al., 2000, Sagud et al., 2002, Fountoulakis et al., 2004).

1.5.5.2 Manic and mixed states

Studies conducted by Zarate (1997) and Chang (1998) compared two states of bipolar disorder: Manic versus mixed states, but lacking a control group (Zarate et al., 1997, Chang et al., 1998). In both those studies, the authors reported significantly elevated TSH levels in their mixed groups compared to the pure manic group. However, they identified differences in the T₄ levels, where Chang (1998) reported a significantly lower T₄ concentration in the mixed group compared to the manics. No significant differences were noted with regards to the T₃ concentration or with previous lithium exposure. Due to their findings, Chang and colleagues, concluded that thyroid axis dysfunction was a common occurrence in the bipolar mixed state rather than the bipolar manic state. In contrast, Cassidy and colleagues (Cassidy et al., 2002) reported no differences in the thyroid profiles between their mixed and

manic groups. It is possible that the differences between the Chang (1998) and Cassidy (2002) studies were due to study design and were influenced by sample number, age, gender or even ethnicity.

1.5.5.3 Rapid-cycling variant of bipolar disorder

Considered as the more severe form of the illness, rapid-cycling occurs when a patient experiences four or more episodes of major depression, mania, hypomania, or mixed symptoms within a twelve-month period. Rapid-cycling usually affects about 9% to 20% of all bipolar patients, with women being more susceptible than men (Ahmed and Morriss, 1997, Kilzieh and Akiskal, 1999, Schneck, 2006, Azorin et al., 2008).

All categories of HPT axis dysfunctions have been reported in rapid-cycling bipolar disorder. These include overt hypo-thyroidism (Wehr and Goodwin, 1979, Cowdry et al., 1983, Khouzam et al., 1991, Szabadi, 1991, Chakrabarti, 2011), increased TSH levels (O'Shancik and Ellinwood, 1982, Cowdry et al., 1983, Bauer et al., 1990, Kusalic, 1992, Chakrabarti, 2011), an exaggerated TSH response to TRH stimulation (Kusalic, 1992), and increased antibody titres (Oomen, et al., 1996).

However, there have also been inconsistent findings with some studies suggesting no association between thyroid dysfunctions and rapid-cycling (Joffe et al., 1988, Wehr et al., 1988, Bartalena et al., 1990, Maj et al., 1994, Post et al., 1997, Valle et al., 1999, Kupka et al., 2002). This could be attributed to “methodological problems such as retrospective designs, lack of controls, predominance of female subjects and varying definitions of hypo-thyroidism” (Chakrabarti, 2011). More importantly, it should be noted that the studies that suggest a positive association, recruited rapid-cycling bipolar patients who were being treated with long-term lithium prophylaxis. Lithium is an agent that has demonstrated anti-thyroid properties (Lazarus, 1998), and thereby contributing to hypo-thyroidism in rapid-cyclers (Cho et al., 1979, Gyulai et al., 2003).

1.5.6 Thyroid auto-immunity as a genetic vulnerability for bipolar disorder

Bipolar disorder and genetics:

It is long known that bipolar disorder occurs within families. Results from family-studies and, in particular, twin-studies suggest that there is a genetic basis for bipolar disorder (Craddock and Jones, 1999). That study reported that the approximate lifetime risk of bipolar disorder in relatives of a bipolar patient is 40-70% for a monozygotic co-twin; first-degree relative 5-10%; and 0.5-1.5% for unrelated individual.

Studies investigating monozygotic (identical) twins who share common genetic makeup have shown that if one of the twins is bipolar, the other twin is more vulnerable (60 to 80%) to develop the illness as compared to another sibling (NIMH, 1998). In contrast, a dizygotic twin of one affected stands only an 8% chance of getting the disorder. Therefore, bipolar disorder is not the result of abnormal genes only (NIMH, 1998). Instead, bipolar disorder is the result of the roles, of both, genes along with other factors including environmental factors. Similarly to other mental illnesses, bipolar disorder is not necessarily the result of a single gene but of multiple genes acting together, and in combination with other factors of the individual or the individual's environment to produce the illness (Hyman, 1999).

Some studies have also considered a genetic relationship between bipolar disorder and biological factors relating to thyroid auto-immunity (Hillegers et al., 2007, Vonk et al., 2007, Holtmann et al., 2010). Thyroid factors have been suggested as state and/or trait related to the development of bipolar disorder and as mentioned earlier, changes in the HPT axis are evident in patients with mood disorders (Kirkegaard and Faber, 1998).

Auto-immune thyroiditis with accompanying increased levels of TPO antibodies has been linked with bipolar disorder. Vonk and co-workers (2007) “compared the prevalence of TPO antibodies among 22 monozygotic twins and 29 dizygotic twins with bipolar disorder, with 35 healthy control twins.” (Vonk et al., 2007). They found that TPO antibody titres were positive in 27% of the bipolar twins and only 16% in the control twins. Those authors, therefore, suggest that auto-immune thyroiditis with increased TPO antibody titres as markers, could serve as an endo-phenotype for bipolar disorder and could be related to the genetic vulnerability to develop the disease as well (Vonk et al., 2007).

Another study by Hillegers and co-workers (2007) investigated the prevalence of auto-immune thyroiditis in adolescent offspring of parents with bipolar disorder (Hillegers et al., 2007). Those authors examined serum TSH levels and the presence of TPO antibodies in the investigative group. They found that female offspring, in particular, exhibited a higher prevalence of auto-immune thyroiditis as indicated by increased positive TPO antibody titres as compared to control groups. Further, in TPO antibody positive offspring, an increased prevalence of thyroid failure was evident by the raised serum TSH levels or T₄ treatment. Therefore, children of bipolar parents appear more vulnerable to develop thyroid auto-immunity independently from their vulnerability to develop psychiatric disorders (Hillegers et al., 2007). A more recent study suggests that altered thyroid function among children presenting with severe affective, behavioural and cognitive impairments may form part of a broad behavioural phenotypic expression of bipolar disorder (Holtmann et al., 2010).

1.5.7 The influence of lithium on the thyroid status of bipolar patients

Lithium continues to remain the most effective long-term preventative treatment for manic - depressive bipolar disorder, as well as unipolar depression in some cases. Further, it is the only drug approved by FDA for treating acute mania and bipolar disorder in adolescents between the ages of 12 to 18 years (Emilien et al., 2007). However, a negative aspect to patients receiving lithium treatment, is that their renal and thyroid function and lithium levels require regular monitoring since lithium therapy is

associated with hypo-thyroidism, goitre and increased anti-thyroid antibody titres, as discussed in greater detail below (Bocchetta and Loviselli, 2006, Emilien et al., 2007, Chakrabarti, 2011).

Interestingly, significantly higher rates of overt and subclinical hypo-thyroidism, goitre and a rise in anti-thyroid antibody titres have been reported among patients receiving lithium treatment when compared to the general population and non-bipolar controls (Bocchetta and Loviselli, 2006, Chakrabarti, 2011). This has been attributed to the known anti-thyroid effects of lithium therapy. Lithium has inhibitory actions on normal cell functioning. It inhibits adenosine triphosphatase (ATPase) activity, cAMP and intracellular enzymes. It was shown to alter the response of cultured cells to TRH and to stimulate DNA synthesis (Lazarus, 1998). In the thyroid gland, where lithium is concentrated, it inhibits iodine uptake, iodothyrosine coupling and alters TG structure, thus, further inhibiting thyroid hormone secretion by the thyroid gland. In addition, it inhibits the conversion of T₄ to T₃. The resulting effect is, therefore, hypo-thyroidism as well as goiter due to subsequent increase in TSH levels following the decreased release of T₃ and T₄ (Lazarus, 1998). Lithium therapy is also associated with the exaggerated TSH response to TRH in 50% to 100% of bipolar depressive patients (Singer et al., 1979, Lazarus, 1998). Lithium has been reported to affect some aspects of cellular and humoral immunity, both, *in vitro* and *in vivo* (Lazarus, 1998). The effects of lithium on the brain may include deiodinase enzymes and alterations in the concentration of thyroid hormone receptor. However further work is required in this area (Lazarus, 1998).

1.5.8 The influence of thyroid hormones on the neurotransmitter systems that regulate mood disorders

1.5.8.1 Thyroid-serotonin system

Studies have provided evidence linking the serotonergic system in the pathogenesis of depression. A central serotonergic deficiency in association with local hypo-thyroidism, may provide a pathological

factor in the development of depression (Cleare et al., 1995, Heinrich and Grahm, 2003). Brain serotonin levels were found to correlate positively with T₃ levels in the rat, suggesting that serotonin synthesis is reduced in the hypo-thyroid state (Cleare et al., 1995, Heinrich and Grahm, 2003). Serotonin has a constant inhibitory effect on TRH, which further suppresses secretion of TSH from the pituitary gland and subsequent production of thyroid hormones. This inhibitory effect on the CNS, “calms, soothes and generates feelings of general contentment and satiation” (Ho, 1999). It is not surprising then, that a deficiency in cerebral serotonin can alter mood and may provide a contributing factor for obsessive compulsive disorders and schizophrenia (Ho, 1999).

1.5.8.2 Thyroid-catecholamine system

In 1981, Whybrow and Prange (1981) suggested that thyroid hormones enhanced the beta-adrenergic receptor response to catecholamine stimulation thus promoting neurotransmission in central noradrenergic pathways and accelerating the recovery process from depression (Whybrow and Prange, 1981). They further suggested that thyroid dysfunction may be associated with abnormalities in central noradrenergic neurotransmission. Puymirat (1985) further reported that alterations in thyroidal status, affects catecholaminergic neurons in the developing and adult brain (Puymirat, 1985). Mano and co-workers (1998) reported that the alterations in catecholamine and catecholamine metabolites are responsible, in part, for the central nervous system symptoms that are observed in hyper-thyroidism and hypo-thyroidism (Mano et al., 1998). The noradrenergic receptor system appears to respond to alterations in the HPT axis function as was demonstrated in the rat brain. The Tejani-Butt group (1994) showed that, following a thyroidectomy, region- and receptor-specific pre- and postsynaptic noradrenergic system alterations were noted (Tejani-Butt et al., 1994). A thyroidectomy showed decreased ligand binding to β - and α_2 - adrenergic receptors in the cortex as well as the limbic regions of the rat brain. However, these changes were reversed upon l-thyroxine administration and this suggests a neuro-modulatory link between thyroid hormones and the central noradrenergic systems (Tejani-Butt et al., 2000).

Thyroid hormones also regulate levels of dopamine receptors (Crocker and Overstreet, 1984, Crocker et al., 1986). Dopamine is thought to inhibit TSH secretion (Rao et al., 1990), since treatment with dopamine blockers have resulted in elevated serum TSH levels (subclinical hypothyroidism) (Magliozzi et al., 1989). Moreover, increased serum levels of dopamine were reported in schizophrenic patients, whilst the levels of other pituitary parameters, including TSH, T₄ and prolactin were reduced (Rao et al., 1984). “The increased dopaminergic activity was hypothesised to affect the pituitary secretory function, and decreased beta-adrenergic activity was thought to be a consequence of decreased serum TSH concentration” (Santos et al., 2012). This is interesting since α 1- and β - adrenergic catecholamines are critical in deiodinase activity maintenance, and thus brain thyroid status (Kundu et al., 2009).

1.5.9 AITD antibodies in bipolar disorder

There have been earlier studies, but not all, that have reported high prevalences of anti-thyroid antibodies during psychiatric disorders, mainly depression (Gold et al., 1982, Nemeroff et al., 1985, Reus et al., 1986, Joffe, 1987, Haggerty et al., 1990). However, the generalisability of available data is limited because these studies failed to include control sample groups, used different antibody assay methods and varying means of addressing the demographic factors responsible for creating variability in the rate of occurrence of thyroid auto-immunity. However, one particular study conducted by Haggerty and colleagues (1997), the authors investigated the prevalence of anti-thyroid antibodies (anti-microsomal and anti-TG antibodies) in unipolar and subtypes of bipolar affective disorder in comparison with two control groups: psychiatric inpatients with adjustment disorder and family medicine outpatients without current psychiatric illness (Haggerty et al., 1997). Those authors reported the highest occurrence of anti-thyroid antibodies in the bipolar depressed and bipolar mixed groups, but not in the bipolar manic and unipolar depressed groups, when compared to the control groups. In that study, they also found that older women with these diagnoses had high rates of antibodies: 40% in bipolar depressed group and 45.5% in bipolar mixed group. Further, the authors report that positive

antibody occurrence was unrelated to lithium exposure. In contrast to the Haggerty study, Joffe et al (1987) reported their findings of the presence of anti-thyroid antibodies (anti-microsomal and anti-TG antibodies) in 5 of 58 patients with unipolar major depressive disorder and prior to lithium treatment (Joffe et al., 1987). However, the prevalence rate of detectable titers of antibodies was similar to that observed in healthy subjects.

1.6 RATIONALE

A pilot study that had been previously conducted by our group was the first to demonstrate the presence of TSH-R and TG in cortical neurons and cerebral vasculature, respectively, within specific regions of normal human adult cerebral cortex (Moodley et al., 2011). In order to fully explore this novel possibility, in the present study, we extend this investigation of the distribution of thyroid-specific proteins associated with Hashimoto's disease, in major limbic regions of normal and bipolar human adult cerebral cortex. The limbic system is comprised of several functionally- and anatomically-interconnected cortical and subcortical structures. These limbic structures are responsible for our emotions and emotional learning, behaviour, memory, motivation and reward (Krebs et al., 2012). We postulate that thyroid-like antigens may be expressed in human limbic structures and where anti-thyroid antibodies may recognise and agonise limbic-associated thyroid-specific receptors. This abnormal association, should it exist, may either have a stimulatory or inhibitory effect on the regulation of limbic thyroid-specific receptors. Consequently, this could determine an up- or down-regulation in the expression levels of limbic-associated, thyroid-specific receptors. We further postulate that any differences identified in the thyroid protein expression levels between the two experimental groups (normal and bipolar limbic regions), may contribute to mood dysregulation symptoms often observed in bipolar disorder (a brain disease that is associated with unusual shifts in mood), thereby suggesting a significant role of the thyroid system in the implication of bipolar disorder. Our findings will therefore contribute to the extensive school of thought that suggests an association between AITDs and neuropsychiatric disorders, with particular focus on bipolar disorder, an area where research is currently

lacking. Further, this prevailing school of thought implicating hypo-thyroidism in bipolar disorder is neither well-understood nor universally-accepted. Thus this lack of plausible data linking these two disorders provided the rationale for the present study.

Curiously, our postulation of anti-thyroid antibodies binding limbic components together with the findings of our previous pilot study that demonstrated TG-like proteins in non-limbic areas of human brain, suggests a migratory potential of thyroid-specific proteins across the seemingly-impervious blood-brain barrier (BBB). Interestingly, there does appear to be multiple modes of egress beyond the protective BBB. Studies have reported that during neurological diseases such as HIV-associated dementia, multiple sclerosis, Alzheimer's disease and bipolar disorder, the integrity of the BBB can be altered causing it to increase permeability to leukocytes and various other circulating compounds into the brain (Lou et al., 1997, Minagar et al., 2002, Patel and Frey, 2015). This influx of leukocytes was reported to trigger signal transduction cascades that would cause the loss of tight junction proteins within the highly specialised cerebral endothelial cells of the BBB. Consequently, this would result in the disruption of the protective BBB during various neuro-pathological conditions including bipolar disorder (Bolton et al., 1998). Thus, those reports indicating an altered BBB during neuro-pathological conditions, lends support to our present postulate that circulating TG-like proteins as well as anti-thyroid antibodies, may gain ingress into the CNS to reach limbic targets.

There exists no previous evidence, until now, of any quantitative comparison of these thyroid-specific proteins in limbic regions. Whereas our previous pilot study had used semi-quantitative analysis to demonstrate TSH-R and TG-like protein localisation in various human brain regions, the present study focuses our attention specifically on limbic structures and utilises image analysis digitilisation and quantification. In addition to quantitative comparisons, we will also obtain qualitative comparisons of thyroid proteins in limbic regions of normal and bipolar brain by molecular biological methods.

1.7 AIMS

- To compare the normal protein and gene expression of thyroid-specific proteins, thyroid-stimulating hormone receptor (TSH-R) and thyroglobulin (TG), with that of bipolar brain limbic tissue.
- To infer whether any change in limbic thyroid protein and gene expression could be related to the pathophysiology of bipolar disorder.

1.8 OBJECTIVES

- To determine the binding sites of antibodies directed against human TSH-R and TG in six primary limbic regions of adult human brain, viz. amygdala, cingulate gyrus, frontal cortex, hippocampus, hypothalamus and thalamus, in both normal and bipolar brain tissue using standard immuno-histochemical methods. This will provide a limbic map of TSH-R and TG protein synthesis.
- To obtain semi-quantitative determinations of the cellular localisation of TSH-R mRNA in normal and bipolar brain limbic tissue by *in-situ* reverse-transcriptase polymerase chain reaction (*in-situ* RT-PCR)
- To determine the gene expression of TSH-R in normal and bipolar brain limbic tissue by reverse-transcriptase quantitative polymerase chain reaction (RTqPCR) in order to determine the fold-change in TSH-R mRNA transcription.

1.9 HYPOTHESIS

Cross-reactivity between anti-thyroid antibodies and human neurons (in human adult brain limbic regions) accounts for mood dysregulation (depression or mania) observed in patients with thyroid disorders (Hashimoto's disease).

CHAPTER 2

Materials and Methods

2.1 SAMPLE COLLECTION

2.1.1 Ethical approval and Patient/Guardian Consent

Ethical approval was granted by the University of KwaZulu-Natal (UKZN), Biomedical Research Ethics Committee (Reference EXPO42/06). Consent for collection of post-mortem normal brain tissue samples was obtained from the families of the deceased. Normal brain tissue was collected at autopsy by an attending forensic surgeon.

Diseased (bipolar) brain tissue samples were obtained from the Netherlands Brain Bank (NBB) (Netherlands Institute for Neuroscience, Amsterdam, The Netherlands) which donates post-mortem specimens from clinically well-documented and neuro-pathologically confirmed cases. Autopsies were performed on donors from whom written informed consent had been previously obtained, or obtained from next of kin. The work of the NBB abides by the Ethical code of conduct approved by the ethics committee.

2.1.2 Post-mortem normal brain and thyroid tissue

Normal brain tissue (n=5) was collected at autopsy, within 24 hours from individuals whom had died in or soon after arrival at hospital, or were declared dead on arrival at hospitals in the Durban area. These individuals had succumbed to unnatural causes other than head injury. There was no evidence of trauma to the head or brain disease and there was no apparent indication of psychological or physical abnormality. In addition to collection of brain tissue, normal human thyroid gland tissue was also collected from these patients. This served as control tissue. Table 2.1 provides a summary of the demographic details of these patients.

Table 2.1 Demographics of individuals from whom tissue samples of normal human brains were obtained at autopsy

Subject No.	Gender	Ethnic Origin	Age
9	female	Indian/Coloured	25
10	female	Indian	40
11	male	Caucasian	24
12	male	Indian	46
14	female	Asian	31

2.1.3 Bipolar brain tissue

Tissue from the amygdala, cingulate gyrus and frontal cortex were obtained from five subjects with bipolar disorder. Table 2.2 provides a summary of the demographic details of the bipolar patients.

Table 2.2 Demographics of individuals from whom tissue samples of bipolar human brains were obtained at autopsy (Netherlands Institute for Neuroscience, Amsterdam, The Netherlands)

Subject No.	Gender	Ethnic Origin	Age
2004-040	female	unknown	76
2006-075	female	unknown	80
2012-048	male	unknown	81
2012-127	male	unknown	68
2014-070	male	unknown	66

2.2 SAMPLE PROCESSING

2.2.1 Tissue fixation

Post-mortem tissue samples were immediately immersed in 5% formal saline fixative [41% formaldehyde (Saarchem, Midrand, SA), 0.9% NaCl, 1:8 v/v (Sabax, Durban)], for 24 hours at room temperature (RT).

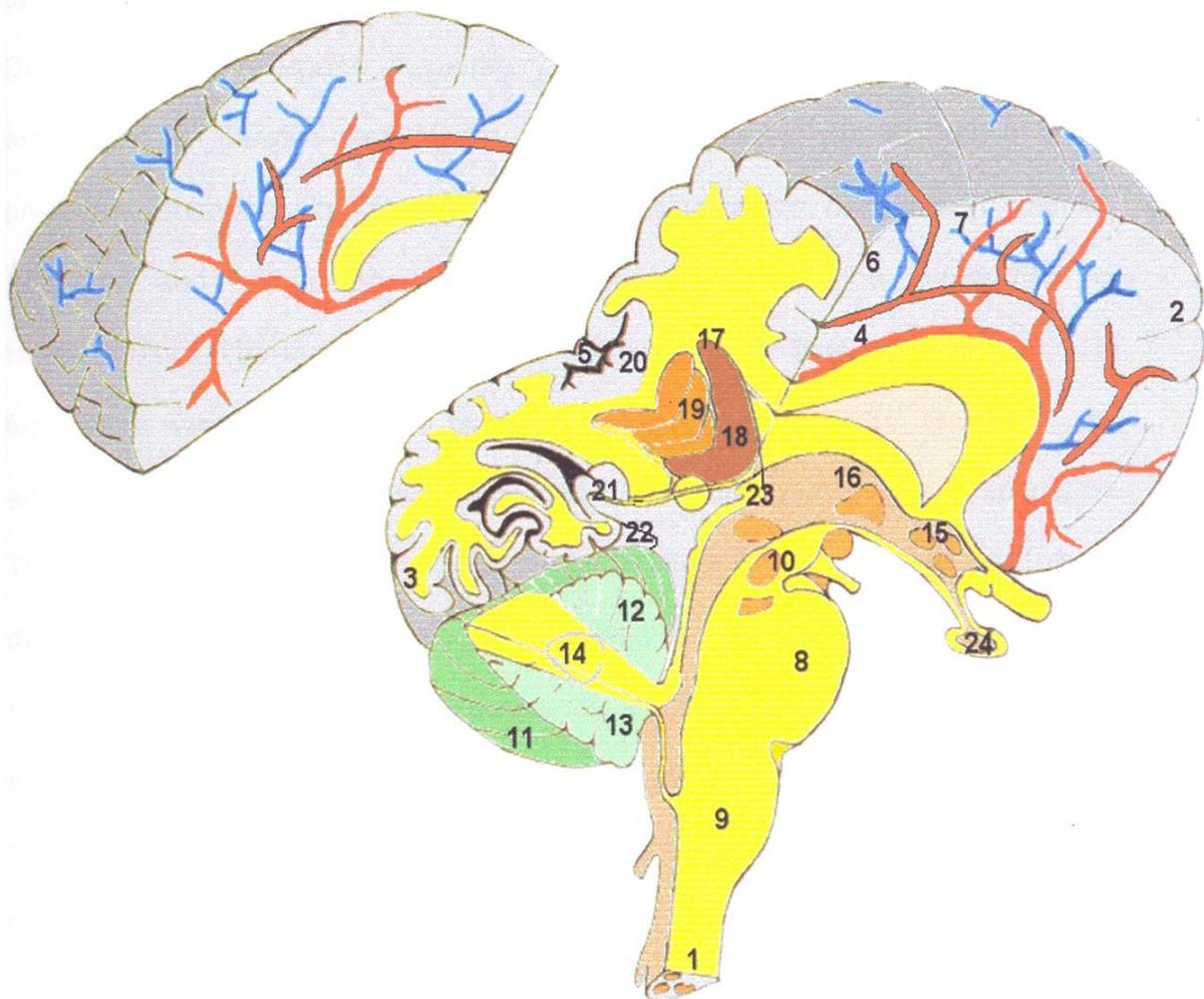
2.2.2 Dissection and orientation of brain sections for microscopy

The topographical distribution of the various brain regions (Figure 2.1) was established from neuro-anatomical texts with structural orientation maintained by the staining of histological landmarks with India ink (Reeves, London) and 1% Alcian blue (ph 2.5; Sigma Chemicals, St. Louis, USA), as described by Raidoo (Raidoo, 1999). For the purposes of this study, cortical tissue obtained from the major limbic regions (from each of the 5 brains collected) were prepared for immuno-histochemistry (IHC), *in-situ* reverse-transcriptase polymerase chain reaction (*in-situ* RT-PCR) and reverse-transcriptase quantitative polymerase chain reaction (RTqPCR). The limbic regions that were investigated included: amygdala; cingulate gyrus; frontal cortex; hippocampus; hypothalamus and thalamus.

2.2.3 Tissue processing and wax embedding for light microscopy

The formalin-fixed tissue sections were trimmed to 5 µm in thickness, orientated and set in plastic tissue cassettes. These tissue samples were then processed by routine fixation and embedding techniques. This included fixation in 5% formalin for 24 hours, dehydration with absolute ethanol (Merck Chemicals, SA) and xylene (Merck Chemicals, SA), before embedding in paraffin-wax. All steps were carried out at the Department of Histopathology, UKZN, under sterile conditions in an automatic tissue processor (Shandon, UK).

Figure 2.1: Orientation of the dissected brain areas. Adapted from Naidoo (2002)



Blue = veins
Red = capillaries

Figure 2.1 legend: Orientation of the dissected brain areas

The numbered regions correspond to the following human brain areas: (1) spinal cord, (2) frontal cortex, (3) occipital cortex, (4) cingulate gyrus, (5) parieto-occipito-temporal association cortex, (6) primary sensory cortex (post-central gyrus), (7) primary motor cortex (pre-central gyrus), (8) pons, (9) medulla, (10) midbrain, (11) lateral lobe of the cerebellum, (12) vermis, (13) flocculo-nodular lobule, (14) dentate nucleus, (15) hypothalamus, (16) thalamus, (17) caudate nucleus, (18) choroid plexus, (19) lentiform nucleus, (20) insula and claustrum, (21) hippocampus, (22) amygdaloid, (23) pineal gland, and (24) pituitary gland.

2.3 LOCALISATION OF TSH-R AND TG BY IMMUNO-HISTOCHEMISTRY

Background

Immuno-histochemistry (IHC) is a combination of anatomical, immunological and biochemical methods performed for the localisation of discrete tissue components. To achieve this, it requires the use of specific labelled monoclonal or polyclonal antibodies to detect specific antigens within tissue sections (Duraiyan et al., 2012). The use of a labelled secondary antibody serves to link the antigen of interest to a stain that renders the antigen-antibody interaction visible under light or fluorescence microscopy (Figure 2.2). Immuno-histochemistry allows determination of tissue distribution and localisation of specific cellular antigens and can therefore be employed for diagnostic and research purposes to determine the up-regulation or down-regulation of specific antigens in disease states compared to healthy controls.

Immuno-localisation of thyroid-specific proteins, TSH-R and TG

Formalin-fixed paraffin-embedded (FFPE) brain and thyroid tissue sections (section 2.2.3), adhered onto silane-coated glass slides, were used for the immuno-detection of the thyroid-stimulating hormone receptor (TSH-R) and thyroglobulin (TG) at the protein level. Standard immuno-histochemistry techniques were employed using polyclonal rabbit anti-human TSH-R and polyclonal rabbit anti-human TG serum as the primary antibodies, respectively. This was followed by the application of conjugated antibodies containing streptavidin-horse radish peroxidase immuno-enzyme complex and diaminobenzidine (DAB) as the chromogen visualised by light microscopy (Figure 2.2).

2.3.1 Antibodies and Reagents

Polyclonal rabbit anti-human TSH-R (200 µg/mL, Santa Cruz Biotechnology, USA) and polyclonal rabbit anti-human TG (8.1 µg/mL, Dako, UK) IgGs were purchased for use with an avidin-biotin

Figure 2.2: Localisation of TSH-R and TG by Immuno-histochemistry

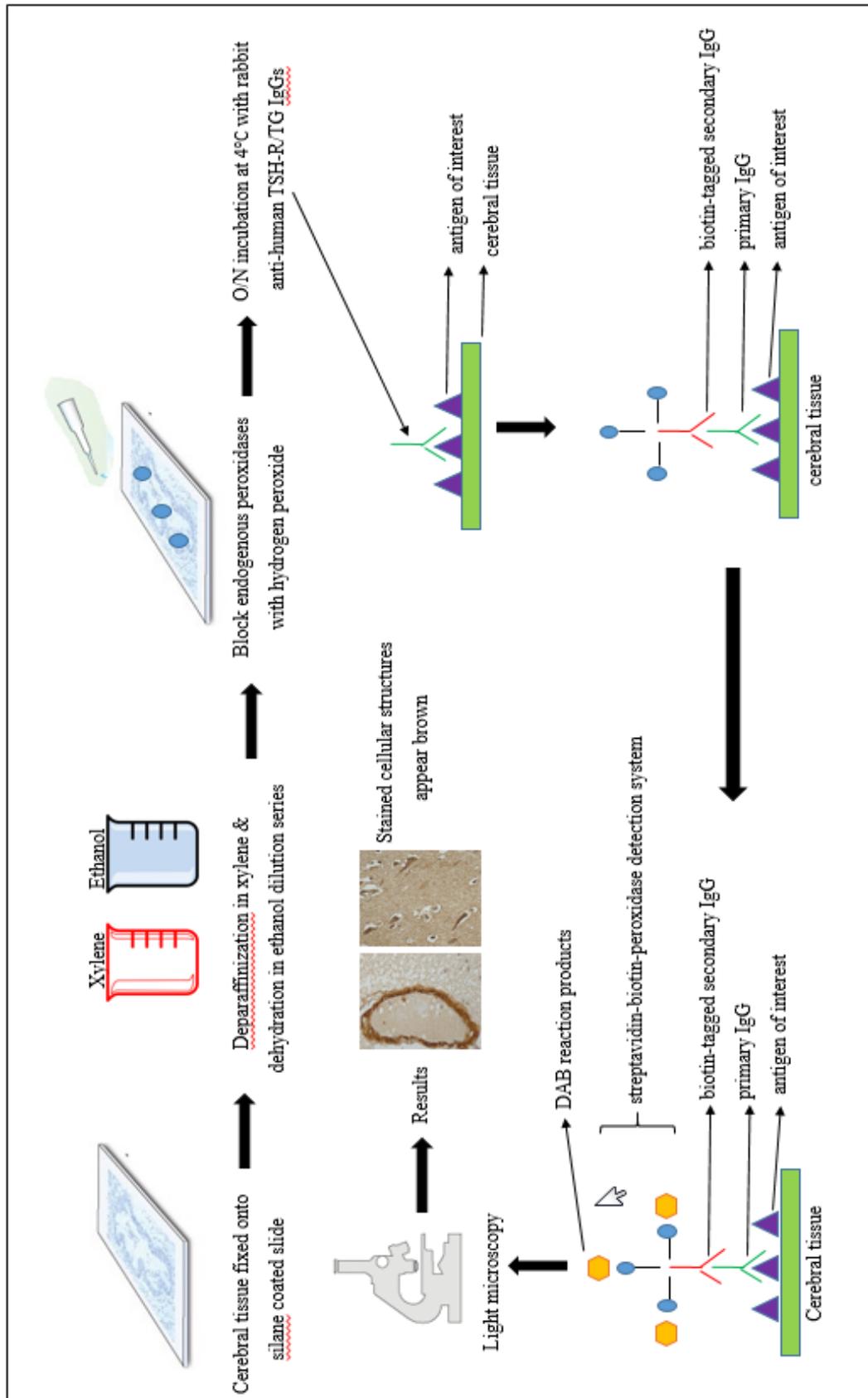


Figure 2.2 legend: Localisation of TSH-R and TG by Immuno-histochemistry

An avidin-biotin immuno-complexation technique was used for the immuno-histochemical localisation of TSH-R and TG. FFPE tissue sections were deparaffinised in xylene and rehydrated through a graded series of ethanols. For antigen retrieval, the tissue sections were boiled in 0.1 M sodium citrate buffer, cooled and incubated with 20% hydrogen peroxide/80% methanol to quench endogenous peroxidases. The sections were then incubated with primary antibody followed by incubations with the streptavidin-biotin peroxidase system. Bound peroxidase was rendered visible with DAB chromogen substrate application which was used to immuno-precipitate and allow visualisation with light microscopy.

immuno-staining kit, (LSAB[®], Dako) and chromogen, 3, 3'-diaminobenzidine (DAB), (Dako, UK). All other bio-reagents were purchased from Roche Molecular (USA), unless otherwise indicated. All chemicals were purchased from Merck Chemicals (SA).

2.3.2 Silanation of glass slides

Glass slides were silanated, in order to enhance the adherence of tissues, by immersion in a 2% 3-aminopropyltriethoxysilane (Sigma) solution with acetone (Merck Chemicals, SA) as the solvent, for 10 minutes. The silane solution was made up fresh for immediate use. Excess silane was washed off in acetone for 30 seconds. The slides were then immersed in distilled water (dH₂O) for 30 seconds, to wash off excess acetone. All the above steps were carried out at room temperature (RT). Slides were dried overnight in an oven at 50°C. The following morning, the slides were removed from the oven and stored in dust-free boxes at RT.

2.3.3 Sectioning of control tissue

Five µm tissue sections of formalin-fixed paraffin-embedded (FFPE) human brain and thyroid (control) were prepared using a rotary microtome system from Thermo Scientific (Shandon Finesse 325). Tissue sections were immersed into a pre-warmed water-bath to facilitate adherence onto silanised glass slides and then left to dry overnight in an oven at 50°C. The following day, all slides were stored in dust-free boxes at RT.

2.3.4 Immuno-histochemistry (IHC)

Formalin-fixed paraffin-embedded tissue sections (5 µm) were deparaffinised in xylene twice for 10 minutes each at RT and then rehydrated through a graded series of ethanol for 5 minutes each at RT [100% (twice), 90%, 70% and 50% ethanol] and finally into dH₂O. For antigen retrieval, the tissues

were boiled in 0.1 M sodium citrate buffer (pH 6.0) for 5 minutes and allowed to cool to RT for approximately 20 minutes. The sections were then allowed to reach equilibrium in dH₂O for 5 minutes at RT. Endogenous tissue peroxidases were quenched with methanol (20 minutes during rehydration) and 20% hydrogen peroxide/80% methanol (4x for 20 minutes each at RT). The sections were then washed twice in tris-buffered saline (TBS) for 5 minutes each. A hydrophobic pen (Dako) was used to create a temporary 'incubation well' around each tissue section. Tissue sections were blocked for non-specific protein binding with a 150 µl volume of 3% bovine serum albumin (BSA)/tris-buffered saline (TBS) blocking buffer each and incubated for 30 minutes at RT, with one change of buffer for a further 30 minutes. The buffer was then carefully aspirated and replaced with a 150 µl volume of primary antibody. Commercial anti-TSH-R (diluted 1:200 in 3% BSA/TBS) and anti-TG (diluted 1:1500 in 3% BSA/TBS) IgGs were used as primary antibodies for these experiments. Incubation with primary antibody was performed at 4°C for 18 hours within a humidified chamber. Following incubation, the sections were allowed to reach RT for approximately 20 minutes and then washed with TBS for 5 minutes. Tissues were then incubated with the streptavidin-biotin system (biotinylated link, LSAB Plus, Dako, UK) to enhance specificity of localisation, and used according to the manufacturer's instructions. Bound peroxidase was then rendered visible with diaminobenzidine (DAB) chromogen substrate application which was used to immuno-precipitate and allow visualisation under light microscopy. Following the first indication of a colour change, the sections were immediately immersed in TBS to stop the reaction. Sections were then counter-stained with Mayer's haematoxylin (Sigma) for 1 minute at RT, rinsed under streaming water for 5 minutes, followed by dH₂O for 5 minutes, then dehydrated through a series of ethanol for 5 minutes, each at RT [50%; 70%; 90%; 100% (twice ethanol)]. The slides were allowed to dry in an incubator for 15 minutes before immersing twice in xylene for 5 minutes each at RT. Finally the sections were mounted with DPX permanent mountant (Merck Chemicals, SA) and air-dried [See addendum A.1 for list of reagents (A.1.1) and for detailed procedure (A.1.2)].

Normal human thyroid gland tissue served as method controls for these experiments and were incubated with and without primary antibody. Negative controls were prepared by substituting the primary antibody with blocking/dilution buffer. These controls served to validate the immuno-localisation methodology and demonstrate specificity of the antibody used.

2.3.5 Image Analysis

Immuno-stained tissue samples were captured as digital TIFF images using a Leica digital camera (DCF300X, Leica, Heidelberg, Germany) coupled to a Leica microscope (DMLB) and interfaced with Leica IM50 software. Regions of interest (ROI) were obtained by using the following considerations: tissue integrity; presence of neurons; the least amount of white matter and minimal background staining. Immuno-localisation on the control and brain regions was determined by image analysis (AnalySIS v5, Soft Imaging Systems, Germany). Briefly, the DAB intensity range was converted to an 8-bit grey-scale with 255 phases. This grey-scale intensity of specific label in the digitised images was quantified and pre-determined, threshold range-limited, $\text{pixel} \cdot \mu\text{m}^{-2}$ values were calculated for the mean intensity of immuno-labelling. On the grey scale, we considered the upper range (160-255) as an indication of positive labelling, in order to exclude background and artifactual labelling of the tissue. For each limbic region (n=6) from each of 5 normal human brains (n=5), images were analysed from 5 fields of view (n=5) at 40x magnification (Wright et al., 2008). The average of *mean integral intensities* ($\times 10^2 \text{ pixels} \cdot \mu\text{m}^{-2}$) was calculated for each limbic structure. For our determinations, the *mean integral intensity* was considered to represent the mean value of the integral intensity of a specific ROI. The *integral intensity* is the sum of all the labelling intensities within a ROI multiplied by the pixel area, and was obtained as an output of the analysis software.

2.3.6 Statistical Methods

All statistical analyses were performed using STATA Software, v 13.1 (StatCorp, USA). It appeared that some of the data analysis for the categories, TSH-R in cerebral neurons and TG in cerebral vasculature, did not meet the statistical assumption of normality; therefore, these categories were transformed from linear-linear data to natural log (Ln) \pm SEM. Parametric assessments were determined for both categories. Firstly, an assessment of thyroid protein intensity levels for TSH-R and TG, across the three different limbic regions (amygdala, cingulate gyrus and frontal cortex) were determined in the normal control group. A similar assessment was performed for limbic regions within the bipolar group. Statistical methods employed for both assessments included an *F-test* and *Tukey's multiple comparisons for significance test* whereby significance was tested at $p < 0.05$. Intra-group comparisons of thyroid protein intensity levels between matched normal and bipolar limbic groups were determined using a *T-test* followed by *Tukey's multiple comparisons for significance test*. Significance was tested at $p < 0.05$.

For TG neuronal labelling, the data did not appear to follow a normal distribution. As a result, non-parametric assessments were employed for statistical comparisons in this category. The median values were obtained and transformed using the natural log (Ln) with accompanying *inter-quartile range-error* calculations. A *two-sample Wilcoxon rank-sum (Mann-Whitney) test* along with *Bonferonni's multiple comparisons for significance test* was used to correct for distribution in the normal group. Thus, inter-group statistical comparisons for the normal groups was tested at $p < 0.003$. Inter-group statistical comparisons for the bipolar groups were determined using the *Kruskal- Wallis rank test* followed by *Dunn's Pairwise comparisons with Sidák adjustment* and significance was tested at $p < 0.05$. Finally, a *T-test* followed by *Tukey's multiple comparisons for significance test* was used to obtain intra-group comparisons of neuronal-labelled TG intensity levels between matched normal and bipolar limbic groups. Significance was tested at $p < 0.05$.

When analysing the data obtained, the statistical comparisons were categorised as follows: (a) inter-group (comparison within the same category, i.e. comparing various limbic regions within either the control group or the bipolar group), and (b) intra-group (comparing various limbic regions between the control and bipolar groups).

2.4 DETECTION OF TSH-R mRNA BY *in-situ* REVERSE-TRANSCRIPTASE POLYMERASE CHAIN REACTION (*in-situ* RT-PCR)

Background

In-situ reverse-transcriptase polymerase chain reaction (*in-situ* RT-PCR) is a histological technique that combines the methods of PCR and *in-situ* hybridization for the detection of minute copies of specific mRNA directly from freshly frozen or FFPE tissue sections and intact cells (Lossi et al., 2011). Following reverse-transcription, digoxigenin (DIG)-labelled nucleotides that are included in the PCR mix, become incorporated within the resulting amplification products which is then detected using an anti-digoxigenin antibody that is conjugated to alkaline phosphatase (Lossi et al., 2011), (Figure 2.3).

2.4.1 Silanation of glass slides

Glass slides used to capture tissue sections were thoroughly cleaned of RNAses and other contaminants by immersion in chloroform for 30 minutes at RT followed by absolute ethanol for 30 minutes at RT. Slides were next immersed in a 2% 3-aminopropyltriethoxysilane (Sigma) solution with acetone (Merck) as the solvent, for 5 minutes. The silane solution was made up fresh for immediate use. Excess silane was washed off by dipping twice in acetone and then diethylpyrocarbonate (DEPC)-treated H₂O. All the above steps were carried out at RT under RNase-free conditions. Slides were dried overnight in an oven at 50°C. The following morning, the slides were removed from the oven and placed into dust-free storage boxes at RT.

Figure 2.3: Detection of TSH-R mRNA by *in-situ* RT-PCR

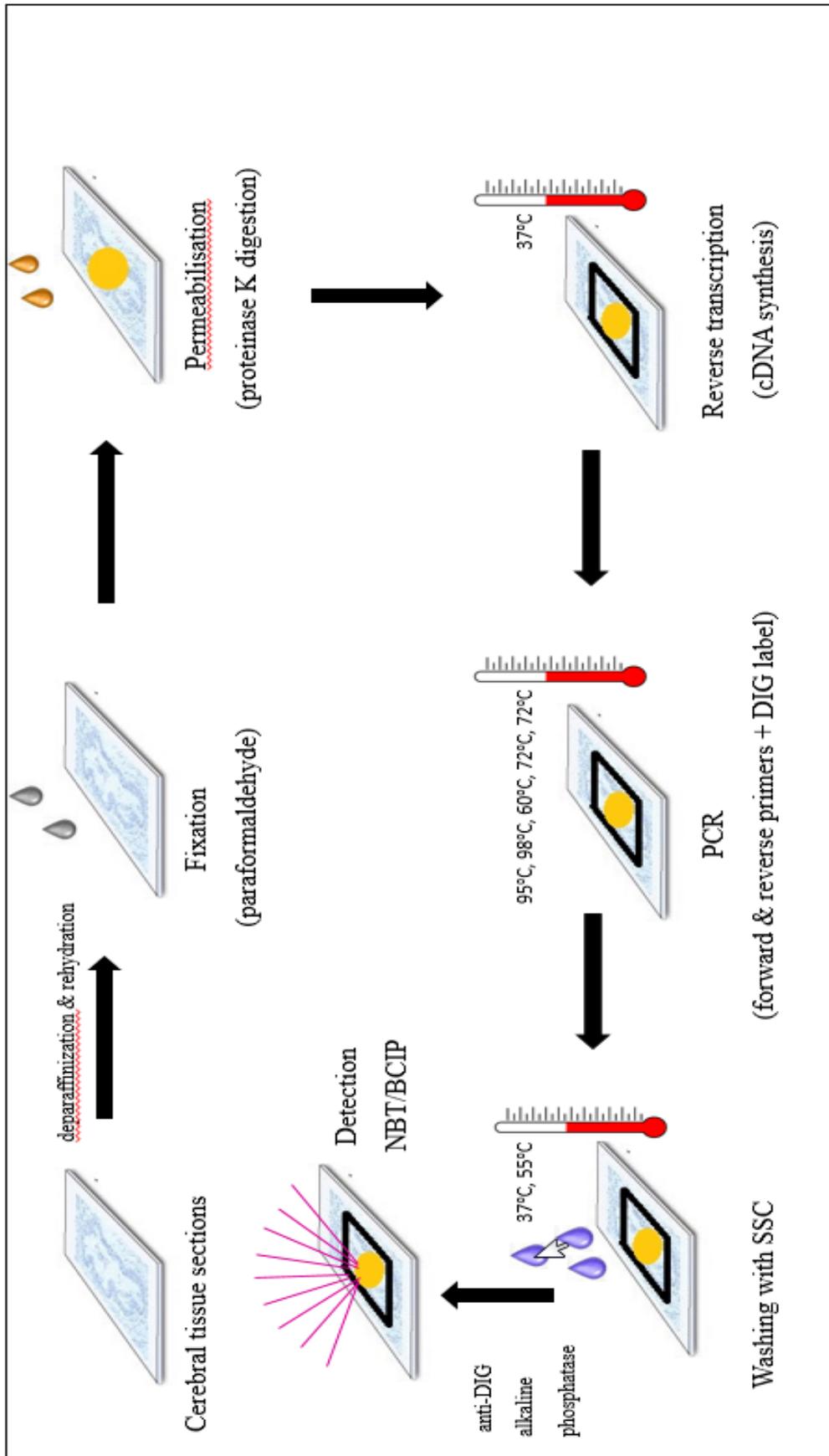


Figure 2.3 legend: Detection of TSH-R mRNA by *in-situ* RT-PCR

In-situ RT-PCR was used for the immuno-detection of TSH-R mRNA from FFPE tissue sections. Tissue sections were deparaffinised, rehydrated and permeabilised to make tissue cells accessible to bio-reagents. Using specific primers, TSH-R mRNA sequences were reverse-transcribed to form resulting cDNA templates for use in the amplification reactions whereby digoxigenin (DIG)-labelled nucleotides were incorporated within amplification products. All unbound reagents were removed following temperature-controlled SSC washes. The DIG marker was immuno-probed with an anti-DIG antibody that was conjugated to alkaline phosphatase and detected by NBT/BCIP chromogen precipitation.

2.4.2 Sectioning of wax-embedded tissue

Formalin-fixed, paraffin-embedded tissue blocks were frozen at -20°C overnight. All surfaces of the microtome including bench top, water-bath, blades holder, handles etc, were cleaned with RNase Zap (Sigma) before tissue was sectioned. A water-bath was filled with DEPC-treated H₂O and maintained at 55°C. Briefly, 5 µm tissue sections of wax-embedded control thyroid and brain tissue were prepared using a rotary microtome system from Thermo Scientific (Shandon Finesse® 325). Sliced tissue sections were immersed into the pre-warmed water-bath to allow easy adherence onto silanated glass slides and then left to dry overnight in an oven at 50°C. The following day, all slides were stored in dust-free boxes that were cleaned with RNase Zap.

2.4.3 Pre-treatment of tissue sections

Tissue sections were dewaxed in xylene twice for 10 minutes each, and then rehydrated through a graded series of ethanol [100% (twice), 90%, 70%, and 50%] and finally into DEPC-treated H₂O. All these steps were carried out at RT in a laminar flow cabinet. The tissues were then fixed in freshly-prepared 4% paraformaldehyde (Merck) in 1 x phosphate buffered saline, pH 7.4 (PBS) for 1 hour and rinsed in 1 x TBS, pH 7.5. The tissues were then partially digested in 200 mM HCl (Merck) for 10 minutes and rinsed in 1 x TBS. A 65 µl Gene Frame incubation chamber (Advanced Biotechnologies Ltd, UK) was carefully inserted around each tissue section to create an incubation compartment, and this was followed by proteinase K digestion (Sigma, USA) at optimal concentration (See addendum A.2.1.2 for table showing optimal proteinase K concentration) for 30 minutes at 37°C in a thermocycler (Peltier Thermal Cycler-200, MJ Research). The tissues were then rinsed extensively in cold TBS three times for 10 minutes each at 4°C, in order to inactivate the enzyme. The tissues were then immersed in DEPC-treated H₂O, followed by dehydration through a graded series of ethanol [50%, 70%, 90%, 100% (2x)], all at 5 minutes each at RT, in a laminar flow cabinet. A drop of chloroform (Merck) was added to each section for 1 minute to remove all traces of ethanol, dried in a laminar flow cabinet for 10 minutes and finally stored in a dust-free slide box that

had been cleaned with RNase Zap [see addendum A.2.1 for list of reagents (A.2.1.1) and detailed procedure (A.2.1.3)].

2.4.4 Reverse-transcriptase (RTase) and polymerase chain reaction for first-strand cDNA synthesis of TSH-R mRNA

2.4.4.1 RTase reaction

For the optimisation of *in-situ* RT-PCR experiments, we used the mRNA expression of the ubiquitous *housekeeping* gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in various normal human tissues including kidney, thyroid gland and brain (cingulate gyrus).

The RTase reaction mixture was prepared using the First-Strand cDNA Synthesis Kit (Amersham, UK). All reagents for this procedure with the exception of the reverse primer (Table 2.3) were included in this kit. A 60 μ l RT reaction mix containing 2 μ l of external (GAPDH or TSH-R) reverse primer, 2 μ l of 200 mM DTT and 8 μ l of bulk first strand cDNA reaction mix [cloned, FPLCpure murine reverse transcriptase, RNAGuard (porcine), RNase/DNase-free BSA, dATP, dCTP, dGTP, and dTTP in aqueous buffer] and 48 μ l of RNase-free H₂O was placed into each incubation chamber on a tissue section and covered with a plastic coverslip. The slides were placed into the thermocycler and incubated at 37°C for 2 hours (See addendum A.2.2 for detailed description). Negative control reactions were also carried out in which the primers were omitted from the RT reactions.

Table 2.3: List of primers used for RT-PCR

Gene	Primer sequences (5'-3')	PCR product (bp)
GAPDH	Forward: TGC ACC ACC AAC TGC TTA GC Reverse: GGC ATG GAC TGT GGT CAT GAG	87
TSH-R	Forward: GGG TGC AAC ACG GCT GGT TT Reverse: CTG GGT TGT ACT GCG GAT TTC GG	367

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

TSH-R: thyroid-stimulating hormone receptor

bp: base pairs

2.4.4.2 *in-situ* PCR

After incubation, the RTase mixture was carefully aspirated from each incubation chamber and replaced with 50 µl of PCR mix that was prepared using the Kapa Hifi reaction kit (Kapa Biosystems, Inqaba Biotech). The reaction mix contained 10 µl of 5 x Kapa Hifi Fidelity buffer, 2 µl of appropriate 10 µM forward and reverse primers (Table 2.3), 5 µl of 10 x PCR digoxigenin (DIG) labelling mix (Roche), 1 µl Kapa Hifi (Hotstart) DNA polymerase (1 U/µl) and 30 µl of PCR-grade H₂O. A plastic coverslip was used to seal the incubation chamber to prevent evaporation of the PCR mixture during thermal cycling. The following cycles were used: initial denaturation at 95°C for 3 minutes, followed by 30 cycles of denaturation of 98°C for 30 seconds, annealing at 60°C (GAPDH and TSH-R) for 30 seconds and extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. Negative control reactions were also carried out in which the primers were omitted from the PCR mix [see addendum A.2.3 for list of reagents (A.2.3.1) and detailed procedure (A.2.3.2)]. After thermal cycling, the coverslip was carefully removed and the tissue washed (Wash Module, Hybaid, UK) in 2 x saline-sodium citrate (SSC) at 37°C for 30 minutes, 1 x SSC at 55°C for 20 minutes, 0.5 x SSC at 55°C for 20 minutes and 0.1 x SSC at 55°C for 20 minutes (see addendum A.2.4.1 for detailed description).

2.4.4.3 Immuno-detection of DIG-labelled amplificants

Following the SSC washes, the slides were washed in TBS (pH 7.5) at RT and then treated with 1 x Blocking Solution (DIG Wash and Block Buffer Set, Roche) for 15 minutes at RT. Thereafter, the blocking solution was gently aspirated and the tissue was incubated for 1 hour at RT with polyclonal sheep anti-DIG (Fab fragments, Roche) conjugated to alkaline phosphatase (1:500 in 10% blocking solution). The slides were then washed with TBS at RT and the tissue was covered with a freshly prepared colour substrate solution consisting of a 1:50 dilution of nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolylphosphate (NBT/BCIP), (Roche) in 1 x detection buffer (1 mM Tris, 1 mM NaCl, 0.5 mM MgCl₂, pH 9.5). The slides were incubated in the dark at RT until a reddish-purple precipitate became clearly visible. Thereafter, the slides were washed with tap water, counter-stained with Mayer's haematoxylin (Sigma) for 1 minute at RT and then rinsed under streaming tap water. The slides were then immersed in dH₂O, dehydrated through a graded series of ethanol and then xylene, all at 5 minutes each at RT, and then mounted with DPX permanent mountant (Merck) [see addendum A.2.4 for list of reagents (A.2.4.1) and detailed procedure (A.2.4.2)].

2.4.4.4 Documentation of *in-situ* RT-PCR results

DIG-labelled tissue sections were viewed with a phase contrast, bright field microscope (DMLB, Leica). A DCF300x digital camera (Leica) was attached to the microscope and interfaced with AnalySIS ProTM image analysis software (Soft Imaging Systems, Germany). Images were digitised and recorded as 24-bit tagged image format files (TIFF). These images were properly scaled (according to the magnification of the microscope objective and digital camera) and archived. Positive labelling for *in-situ* PCR experiments were identified as blue-black colour indicating the precipitation of the chromogen, NBT/BCIP.

2.4.4.5 Semi-quantitative grading system for the distribution of *in-situ* PCR mRNA

A grading system for the *in-situ* PCR mRNA labelling studies was performed that comprised a two-step approach. For each field of view observed under light microscopy, the cellular labelling of TSH-R was quantified according to (i) the visual intensity and (ii) extent of labelling. A “0” was assigned when no cells were observed as stained, “1” indicated low labelling, “2” for moderate labelling and “3” for intense labelling. Further, these scores were correlated with the extent of cellular labelling. A “0” was assigned when no cells stained, “1” indicated that < 25% of cells per field of view were observed for labelling, a “2” indicated labelling between 25 and 75% of cells and “3” for labelling in > 75% of cells. Semi-quantitative analysis was performed on multiple fields of view for each of normal and bipolar brain limbic regions. Further, semi-quantitative analysis was performed by two independent observers and the mean of the semi-quantitative scores collated.

2.5 DETECTION OF TSH-R mRNA FROM FFPE TISSUE BY REVERSE-TRANSCRIPTION, QUANTITATIVE POLYMERASE CHAIN REACTION (RTqPCR)

Background

Reverse-transcription quantitative polymerase chain reaction (RTqPCR) is reported to be the most accurate, sensitive and simple method for quantifying mRNA transcripts in biological specimens (Pfaffl et al., 2004, Bustin, et al., 2009). In addition, previous studies have shown that FFPE tissue specimens could also be subjected to this method (Kuramochi et al., 2006a, Kuramochi et al., 2006b, Shimizu et al., 2006, Vallbohmer et al., 2006a, Vallbohmer et al., 2006b, Soes et al., 2013). Reverse-transcription quantitative polymerase chain reaction is based on the detection and quantification of fluorescence that is emitted from a reporter molecule that gets incorporated into the PCR products during real-time reactions (Figure 2.4). For the present study, the fluorescent chemistry that was employed to monitor the amplification of the target sequence, was SYBR Green 1, a

Figure 2.4: Quantitative detection of TSH-R mRNA by RTqPCR

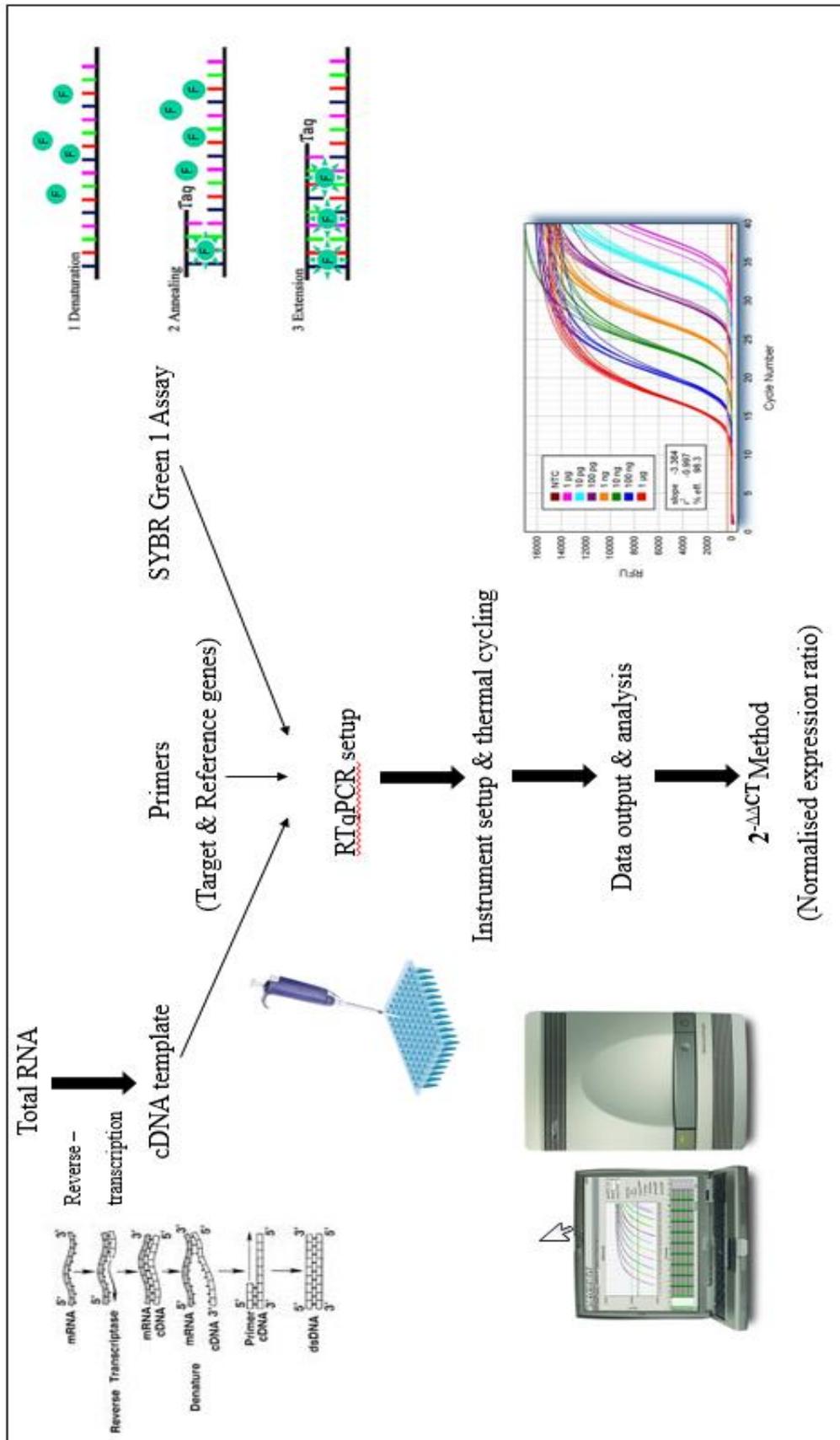


Figure 2.4 legend: Quantitative detection of TSH-R mRNA by RTqPCR

TSH-R mRNA was detected and quantified using RTqPCR. Total RNA was isolated from FFPE tissue sections and reverse-transcribed to form cDNA templates for use in quantitative amplification reactions along with specific primers and SYBR Green 1 fluorescent DNA-binding dye. When SYBR Green 1 binds double-stranded DNA, it emits a fluorescence that increases up to a 1000-fold. Therefore, as PCR products amplify during the reaction, the SYBR Green fluorescent signal also increases.

fluorescent DNA-binding dye. When free in solution, SYBR Green 1 emits little fluorescence. However, when SYBR Green 1 binds to double-stranded DNA, its fluorescence dramatically increases up to a 1000-fold. The overall fluorescent signal obtained from RTqPCR is therefore proportional to the amount of double-stranded DNA that is present in the reaction. Hence, as PCR product is amplified in the reaction, the SYBR Green 1 fluorescent signal will also increase (Bio Rad, 2006).

The Comparative C_T method was used to quantify the results obtained by RTqPCR. This quantitative approach requires comparing the C_T (threshold cycle) values of the test samples with a calibrator (control). “The threshold cycle (C_T) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold” (Livak and Schmittgen, 2001). A calibrator sample is known as a reference or control sample that represents the normal physiological state of the study and therefore can be an untreated or baseline sample. When comparing multiple samples, a single sample is selected as the calibrator, and the expression of the target gene in all test samples is expressed as either, an increase or decrease relative to the calibrator sample (Bio Rad, 2006). In the present study, RNA isolated from normal brain limbic tissue from each of amygdala, cingulate gyrus and frontal cortex, represented the calibrator samples for these experiments.

Further to the requirements for an ideal RTqPCR assay, is the normalisation of samples with suitable reference genes for “correct data interpretation, since its accuracy is significantly affected by sample quality, reagents and operator technique” (Bustin et al., 2009). Therefore the C_T values of both the calibrator and samples of interest were normalised to an endogenous *housekeeping* gene. In the present study, GAPDH was used as a reference gene for the normalisation of gene expression in human brain limbic tissue, since studies have reported GAPDH to be a suitable and stable endogenous reference gene in RTqPCR analysis of human brain samples (Grunblatt et al., 2004, Coulson et al., 2008, Kreth et al., 2010).

The data output for the Comparative C_T method is determined by the normalised expression ratio, $2^{-\Delta\Delta C_T}$, and reported as a fold-change or fold-difference of the target gene expression levels between the experimental study groups (Livak and Schmittgen, 2001). Therefore, in the present study, we determined the expression fold-change of our target gene (TSH-R) in bipolar brain limbic tissue, normalised to the endogenous reference gene, GAPDH, and relative to normal brain limbic tissue (calibrator).

2.5.1 Extraction and quantification of RNA from FFPE tissue specimens for gene expression analyses

2.5.1.1 Silanation of glass slides

Glass slides used to capture tissue sections were cleaned and cleared of RNases and other contaminants by immersion in chloroform for 30 minutes at RT, followed by absolute ethanol for 30 minutes at RT. They were then silanated with 2% 3-aminopropyltriethoxysilane (Sigma) solution for 5 minutes, quickly dipped twice in acetone and DEPC-treated H₂O, and then dried overnight at 50°C. The following morning, the silanated slides were stored in dust-free boxes at RT.

2.5.1.2 Sectioning of wax embedded tissue

Formalin-fixed, paraffin-embedded tissue blocks were frozen at -20°C overnight. All surfaces of the microtome including: bench top, water-bath, blades holder, handles etc, were cleaned with RNAse Zap (Sigma) before tissue was sectioned. A water-bath was filled with DEPC-treated H₂O and maintained at 55°C.

Briefly, 10 µm tissue sections of wax-embedded control thyroid and brain tissue were prepared using a rotary microtome system from Thermo Scientific (Shandon Finesse® 325). Sliced tissue sections were

immersed into the pre-warmed water-bath to allow easy adherence onto silanised glass slides and then left to dry overnight in an oven at 50°C. The following morning, all slides were stored in dust-free boxes at RT.

2.5.1.3 RNA Isolation from FFPE tissue sections

Total RNA was isolated from each tissue section using the ReliaPrep FFPE Total RNA MiniPrep System (Promega, USA) according to the manufacturer's instructions. This system requires deparaffinisation of FFPE tissue sections using mineral oil as opposed to xylene deparaffinisation or deparaffinisation with alternate organic solvents. By eliminating the washing steps performed during xylene deparaffinisation, this method ensures that even small RNA fragments are retained. Prior to deparaffinisation with mineral oil, with the aid of a sterile blade, the tissue section was carefully scraped off the slide and suspended into a sterile RNase/DNase-free microcentrifuge tube. A total of 5 tissue sections of 10 µm each were processed per reaction.

2.5.1.4 Determining total RNA concentration using spectrophotometry

The concentration of purified total RNA was determined using the Nanodrop spectrophotometer (ThermoScientific). Nuclease-free H₂O was used as a blank, since it served as the elution buffer for purified total RNA.

2.5.2 Reverse-transcriptase quantitative polymerase chain reaction (RTqPCR)

2.5.2.1 Reverse-transcriptase (RT) reaction - synthesis of complementary DNA (cDNA)

Following RNA isolation, the total RNA samples (undiluted) were subjected to reverse-transcription using the SuperScript IV First-Strand cDNA Synthesis System (ThermoFisher, SA) according to the

manufacturer's instructions. A 7 µl volume of undiluted total RNA along with random hexamer primers (provided with kit) were used for the cDNA synthesis reaction.

Negative controls were prepared by substituting RNA template with DEPC-treated H₂O. Resulting cDNA concentration was measured spectrophotometrically as performed in section 2.5.1.4. The cDNA concentrations occurred in the range 2458.7 - 3319.2 ng/µl and accompanying A₂₆₀/A₂₈₀ ratios occurred in the range 1.7 - 2.0. Recovered cDNA were stored at -20°C until further use.

2.5.2.2 Quantitative PCR (qPCR) - cDNA amplification

Quantitative PCR (qPCR) assay

Quantitative PCR (qPCR) was performed on the Applied Biosystems 7500 Real-time PCR Instrument (Applied Biosystems, Life Technologies) in 96-well microtitre reaction plates (Applied Biosystems, Life Technologies). The fluorescent DNA-binding dye, SYBR Green 1, was used to monitor the amplification of the target sequence. The endogenous *housekeeping gene*, GAPDH was used as a reference gene for the normalisation of gene expression in human brain limbic tissue. Primer sequences and PCR product sizes for the target gene (TSH-R) and the reference gene (GAPDH) are presented in Table 2.3.

Each reaction was performed in a final volume of 20 µl containing 6 µl nuclease-free H₂O, 10 µl of 2x PowerUp SYBR Green Master mix (ThermoFisher, SA), 1 µl (0.5 µM final) forward primer, 1 µl (0.5 µM final) reverse primer and 2 µl (undiluted) cDNA. Non-template controls were also run in parallel under the same conditions for both target and reference genes to verify the purity of obtained measurements. Amplification was performed at 95°C for 10 minutes for enzyme activation, followed by 40 cycles of 95°C for 15 seconds (denaturation), 60°C (GAPDH and TSH-R) for 30 seconds

(annealing) and 72°C for 30 seconds (extension). All reactions were performed in duplicate and the mean was used for further calculations. To assess the specificity of the amplification of the target gene (TSH-R), a *melt curve analysis* was included at the completion of each amplification reaction. This comprised a single cycle that was performed at 95°C for 15 seconds, 60°C for 1 minute, 95°C for 30 seconds and 60°C for 15 seconds [see addendum A.3 for list of reagents (A.3.1) and detailed procedure (A.3.2)].

2.5.2.3 Comparative C_T method (2^{-ΔΔCT})

The Comparative C_T method is also known as the double delta C_T method and can be represented as the normalised ratio, 2^{-ΔΔCT},

where:

$$\Delta\Delta C_T = \Delta C_{T \text{ sample}} - \Delta C_{T \text{ calibrator}}$$

where:

$$\Delta C_{T \text{ sample}} = C_{T \text{ target}} - C_{T \text{ reference}}$$

(i.e. ΔC_{T sample} represents the C_T value for the sample normalised to the *housekeeping* gene).

Similarly,

$$\Delta C_{T \text{ calibrator}} = C_{T \text{ target}} - C_{T \text{ reference}}$$

(i.e. ΔC_{T calibrator} represents the C_T value for the calibrator normalised to the *housekeeping* gene).

Therefore, the amount of target (TSH-R), normalised to an endogenous reference (GAPDH) and relative to a calibrator (normal brain), is given by the expression ratio:

$$2^{-\Delta\Delta C_T}$$

The threshold cycle (C_T) was generated by the 7500 ABI real time PCR system software automatically. The raw fluorescent data, including C_T means, ΔC_T mean, ΔΔC_T and 2^{-ΔΔCT}, was exported to an MS Excel spreadsheet that had been generated by the authors. Normal brain limbic tissue for amygdala, cingulate gyrus and frontal cortex were chosen as the calibrator samples for these experiments. The Comparative C_T method (2^{-ΔΔCT}) was used to calculate fold-changes in gene expression differences

between the two experimental groups (normal and bipolar brain limbic tissue). The data is presented as the fold-change in TSH-R gene expression normalised to the endogenous reference gene (GAPDH) and relative to the normal brain limbic areas, i.e. amygdala, cingulate gyrus and frontal cortex.

2.5.2.4 Statistical Methods

All statistical analyses were performed using STATA Software, v 13.1 (StatCorp, USA). Statistical analyses were performed for the fold-change of expression of our target gene, TSH-R in the bipolar limbic group, relative to the normal control group. However, the data did not appear to follow a normal distribution curve; therefore, non-parametric assessments were considered for this category. This included obtaining median values which were transformed using the natural log (Ln) with *inter-quartile range-error* calculations. The *Wilcoxon rank test* was the choice of test to determine intra-group statistical comparisons between the normal and bipolar limbic groups. Significance was tested at $p < 0.05$.

Further, bipolar inter-group statistical comparisons for the fold-change of TSH-R expression across the three bipolar limbic areas examined (amygdala, cingulate gyrus and frontal cortex) were established using the *Kruskal-Wallis rank test* followed by *Dunn's Pairwise comparison with Sidák adjustment* and significance was tested at $p < 0.05$.

ADDENDUM (A)

Buffers, Reagents and Methods

A.1 IMMUNO-HISTOCHEMICAL LOCALISATION OF TSH-R AND TG BY IMMUNO-PRECIPIATION

A.1.1 Reagents:

- (a) Xylene (Merck, SA)
- (b) Absolute ethanol (Merck, SA)
- (c) Methanol (Merck, SA)
- (d) 0.1 M Sodium-Citrate, pH 6.0 (w/v) – dissolve 29.4 g Tri-sodium citrate (Sigma, SA) in 800 ml dH₂O, adjust to pH 6 with HCl and make up to final volume of 1 L.
- (e) 20% hydrogen peroxide (H₂O₂)/80% methanol (MeOH), (v/v) - mix 80 ml MeOH (Merck, SA) and 20 ml H₂O₂ (Merck, SA) just before use.
- (f) TBS (1 L) - mix 500 ml 1M Tris-HCl and 300 ml 5M NaCl,
 - 1 M Tris-HCl, pH 8.2 - dissolve 121.14 g Trizma base (Sigma, SA) in 800 ml dH₂O, adjust to pH 8.2 with HCl, make up to final volume of 1 L and store at RT
 - 5 M NaCl - dissolve 292 g NaCl in 1 L dH₂O and store at RT.
- (g) Milk blocker - dissolve 1 ml milk blocker (Roche, SA) in 9 ml 3% BSA/TBS
 - 3% BSA/TBS - dissolve 3 g BSA in 100 ml TBS and store at 4°C.
- (h) Primary (1°) Ab - polyclonal goat anti-human TSH-R IgG (Santa Cruz Biotechnology) and polyclonal rabbit anti-human TG IgG (DakoCytomation)
- (i) Anti-goat IgG Biotin link (LSAB K0690. Dako, UK)
- (j) Secondary (2°) Ab - Streptavidin-peroxidase (LSAB)
- (k) Chromogen liquid DAB (K3465, Dako, UK) - dissolve 1 drop DAB chromogen in 1 ml DAB buffer just before use.
- (l) Counter-stain - Mayers haematoxylin (Sigma, SA)

A.1.2 Procedure:

Step	Solution	Temp	Time
1.	Deparaffinisation in xylene	RT	2x 10 minutes
2.	Rehydrate in 100% ethanol	RT	2x 5 minutes
3.	Quench endogenous peroxidase with 100% methanol	RT	1x 20 minutes
4.	Rehydrate in 90% ethanol	RT	1x 5 minutes
5.	Rehydrate in 70% ethanol	RT	1x 5 minutes
6.	Rehydrate in 50% ethanol	RT	1x 5 minutes
7.	Rehydrate in dH ₂ O	RT	1x 5 minutes
8.	Antigen retrieval – boil in 0.1 M sodium-citrate, pH 6.0 in microwave.	80°C	3 minutes high
9.	Allow to cool under laminar flow	RT	20 minutes
10.	Allow to equilibrate in dH ₂ O	RT	1x 5 minutes
11.	Block endogenous peroxidase in 20% H ₂ O ₂ / 80% MeOH	RT	4x 15 minutes
12.	Wash in TBS	RT	2x 5 minutes
13.	Block non-specific binding sites 3% BSA/TBS blocking buffer	RT	2x 30 minutes
14.	Incubate with 1° Ab - goat anti-human TSH-R IgG diluted 1: 200 with blocking buffer - rabbit anti-human TG IgG diluted 1: 1500 with blocking buffer	4°C	18 hours
15.	Allow slides to reach RT	RT	20 minutes
16.	Wash in TBS	RT	2x 5 minutes

17.	Incubate with anti-goat/rabbit biotin link	RT	1x 20 minutes
18.	Wash in TBS	RT	1x 5 minutes
19.	Incubate with strepavidin-peroxidase	RT	1x 20 minutes
20.	Wash in TBS	RT	1x 5 minutes
21.	Add DAB chromogen	RT	2-5 minutes
22.	Stop reaction by immersing slides in TBS	RT	5 minutes
23.	Counter-stain with Mayers' haematoxylin	RT	1 minute
24.	Rinse slides under streaming tap H ₂ O	RT	5 minutes
25.	Dehydrate in dH ₂ O	RT	1x 5minutes
26.	Dehydrate in 50% ethanol	RT	1x 5 minutes
27.	Dehydrate in 70% ethanol	RT	1x 5 minutes
28.	Dehydrate in 90% ethanol	RT	1x 5 minutes
29.	Dehydrate in 100% ethanol	RT	2x 5 minutes
30.	Dry slides in the incubator	50°C	10 minutes
31.	Immerse slides in xylene	RT	2x 5 minutes
32.	Mount with DPX permanent mountant and allow slides to dry in fumehood	RT	45 minutes
33.	View slides under light microscope and capture images		
34.	Store slides away at 4°C	4°C	

A.2 DETECTION OF TSH-R mRNA BY *in-situ* RT-PCR

A.2.1 Pre-treatment of tissue sections for *in-situ* RT-PCR

A.2.1.1 Reagents and consumables:

1. Xylene (Merck, SA)
2. Absolute Ethanol (Merck, SA) - dilute to 90%, 70%, 50% with DEPC-treated H₂O
3. 0.1% (v/v) DEPC-treated H₂O - add 1 ml DEPC (Sigma) to 1 L dH₂O, leave to stand O/N at RT and autoclave the following day.
4. 10 x PBS - dissolve 5 PBS tablets (Sigma, pH 7.4) in 1 L DEPC-treated H₂O.
5. 1 x PBS - dilute 100 ml of 10 x PBS, pH 7.4 in 900 ml of DEPC-treated H₂O.
6. 4% (w/v) paraformaldehyde - dissolve 20 g of paraformaldehyde (Merck) in 400 ml of 1 x PBS by slowly heating the solution to 70°C on a heating plate. Once the solution is clear, top up the volume to 500 ml with 1 x PBS and allow to cool. Prepared fresh on day of use.
7. 200 mM HCl - dilute 5 ml of 10.4 M (37%) HCl with 255 ml DEPC-treated H₂O.
8. 1 M Tris, pH 7.5 - dissolve 121 g Trizma base (Sigma) in 800 ml of DEPC-treated H₂O, adjust pH to 7.5 with HCl and make up to final volume of 1 L
9. 5 M NaCl (w/v) dissolve 292.2 g of NaCl (Merck) in 800 ml of DEPC-treated H₂O. Once dissolved, make up to final volume of 1 L.
10. TBS - dilute 50 ml of 1 M Tris-HCl, 7.5 and 30 ml of 5 M NaCl in 1 L DEPC-treated H₂O
11. 65 µl Gene Frame incubation chamber and coverslips (Inqaba Biotec)
12. Proteinase K - dissolve 1 mg of proteinase K *Tritirachiu album* (Sigma) in 1 ml DEPC-treated H₂O to prepare a stock solution of 1 mg/ml concentration which was aliquoted and stored at -20°C. This stock solution was used to prepare a dilution series (refer to A.2.1.2 below).
13. Chloroform (Merck)
14. RNase Zap (Sigma)

A.2.1.2 Optimal proteinase K concentrations at 37°C for 30 minutes

Proteinase K concentration (µg/ml)	Starting concentration of proteinase K concentration	Volume (µl)	Volume of 0.1% (v/v) DEPC-H ₂ O (µl)	Dilution
50 (A)	1 mg/ml	50	950	1: 20
45	50 µg/ml (A)	900	100	9: 10
40	50 µg/ml (A)	800	200	8: 10
35	50 µg/ml (A)	700	300	7: 10
30	50 µg/ml (A)	600	400	6: 10
25	50 µg/ml (A)	500	500	1: 2
20	50 µg/ml (A)	400	600	2: 5
15	50 µg/ml (A)	300	700	3: 10
10	50 µg/ml (A)	200	800	1: 5
5	50 µg/ml (A)	100	900	1: 10
0	0	0	1000	0

Control tissue	Proteinase K concentration (µg/ml)
Control salivary gland	50
Control kidney	20
Control brain	10
Control thyroid	10

A.2.1.3 Procedure:

Step	Solution	Temp	Time
1.	Deparaffinisation in xylene	RT	2 x 10 minutes each
2.	Rehydrate in 100% Ethanol	RT	2 x 5 minutes each
3.	Rehydrate in 90% Ethanol	RT	1 x 5 minutes
4.	Rehydrate in 70% Ethanol	RT	1 x 5 minutes
5.	Rehydrate in 50% Ethanol	RT	1 x 5 minutes
6.	Rehydrate in DEPC-treated H ₂ O	RT	1 x 5 minutes
7.	Fixation in freshly prepared 4% paraformaldehyde	RT	1 hour
8.	Rinse in TBS	RT	2 x 2 minutes each
9.	Digest in 200 mM HCl	RT	10 minutes
10.	Rinse in 2 changes of fresh TBS	RT	2 x 2 minutes each
11.	Protease digestion with proteinase K, in order to permeabilise cell membrane	37°C	30 minutes
12.	Rinse in 3 changes of fresh, cold TBS to inactivate enzyme	4°C	3 x 10 minutes
13.	Dehydrate in DEPC-H ₂ O	RT	1 x 5 minutes
14.	Dehydrate in 50% Ethanol	RT	1 x 5 minutes
15.	Dehydrate in 70% Ethanol	RT	1 x 5 minutes
16.	Dehydrate in 90% Ethanol	RT	1 x 5 minutes
17.	Dehydrate in 100% Ethanol	RT	1 x 5 minutes
18.	Dry in Chloroform	RT	1 minute
19.	Dry in fumehood	RT	10 minutes
20.	Store in RNase-free/dust-free slide box	RT	

A.2.2 Reverse-Transcriptase and Polymerase Chain Reaction - RTase reaction

A.2.2.1 Reagents:

1. First-strand cDNA kit (Separation Scientific, SA, Pty Ltd)
 - Bulk first-strand cDNA mix [cloned, FPLCpure murine reverse transcriptase, RNAGuard (porcine), RNase/DNase-free BSA, dATP, dCTP, dGTP, and dTTP in aqueous buffer]
 - DTT solution (200 mM)
 - DEPC-treated H₂O
2. Reverse primer - GAPDH/TSH-R (IDT)
3. Parafilm sheets

A.2.2.2 *in-situ* RT reaction mix:

Reagents	Positive reaction (µl)	Negative control (µl)
DEPC-H ₂ O	48	50
Bulk mix	8	8
DTT (200mM)	2	2
Reverse primer (10 µM)	2	-
TOTAL	60	60

Incubation:

- 37°C for 2 hours

A.2.3 *in-situ* PCR Reaction

A.2.3.1 Reagents:

1. PCR-grade H₂O
2. Kapa Hifi reaction kit (Kapa Biosystems, Inqaba Biotech)
 - 5x Kapa Hifi Fidelity Buffer

- Kapa Hifi (Hotstart) DNA polymerase (1U/ μ l)
- 3. Forward and Reverse Primers - GAPDH/TSH-R (IDT)
- 4. 10x PCR DIG labelling mix (Roche)
- 5. 65 μ l Gene Frame incubation chamber and coverslips (Inqaba Biotec)

A.2.3.2 PCR mix:

Reagents	Positive reaction (μ l)	Negative control (μ l)
PCR-grade H ₂ O	30	34
5x Kapa Hifi Fidelity Buffer	10	10
10x DIG labelling mix	5	5
Forward primer (10 μ M)	2	-
Reverse primer (10 μ M)	2	-
Kapa Hifi (Hotstart) DNA polymerase (1U/ μ l)	1	1
TOTAL	50	50

Cycling conditions: GAPDH and TSH-R

- Initial denaturation: 98°C for 3 minutes \longrightarrow 1 cycle
 - Denaturation: 95°C for 30 seconds
 - Annealing: 60°C for 30 seconds
 - Extension: 72°C for 30 seconds
 - Final extension: 72°C for 5 minutes \longrightarrow 1 cycle
- } 30 cycles

A.2.4 Post-hybridisation washes and immuno-detection of bound DIG-labelled amplicants

A.2.4.1 Reagents:

1. 20x Sodium citrate (SSC, w/v) - dissolve 175.3 g NaCl and 88.2 g sodium citrate in 800 ml DEPC-treated H₂O, adjust the pH to 7.0 and make up to a final volume of 1 L. Filter and autoclave.

Prepare SSC solutions (2 x, 1 x, 0.5x, 0.1x) as follows:

- 2x SSC: dilute 100 ml 20 x SSC with 900 ml DEPC-treated H₂O
 - 1x SSC: dilute 50 ml 20 x SSC with 950 ml DEPC-treated H₂O
 - 0.5x SSC: dilute 25 ml 20 x SSC with 975 ml DEPC-treated H₂O
 - 0.1x SSC: dilute 10 ml 20 x SSC with 990 ml DEPC-treated H₂O
2. 1x TBS (refer to A.2.1.1)
 3. DIG Wash and Block Buffer Set (Roche). Contains washing, blocking and detection buffers (each at 10x concentration) for the immunological detection of DIG-labelled amplicants
 - 1x Blocking Solution: dilute 1 ml of 10x Maleic acid buffer with 9 ml of dH₂O to prepare a 1x Maleic acid buffer solution. Then dilute 1 ml of 10x Blocking solution with 9 ml of the freshly prepared 1x Maleic acid buffer solution. Always prepared fresh.
 - 1x Detection Buffer: dilute 1 ml of the 10 x Detection buffer with 9 ml of dH₂O. Store at 4°C and allow it to reach RT before each use.
 4. Polyclonal Sheep Anti-DIG-Alkaline Phosphatase-Conjugate (F_{ab}) [150 U (200 µl), Roche]
 - Alkaline Phosphatase Solution: the anti-DIG-AP (Fab) was diluted 1:500 with 1x Blocking solution just before use
 5. Chromogen, NBT/BCIP Stock Solution (Roche)
 - Detection Solution: The NBT/BCIP stock solution was diluted 1:50 with 1x Detection buffer just before use. The solution contains NBT (18.75 mg/ml) and BCIP (9.4 mg/ml) in 67% DMSO.
 6. Mayer's Haematoxylin (Sigma)
 7. DPX permanent mountant (Merck)

A.2.4.2 Procedure:

Step	Solution	Temp	Time
1.	Wash tissue slides in 2 x SSC	37°C	30 minutes
2.	Wash tissue slides in 1 x SSC	55°C	20 minutes
3.	Wash tissue slides in 0.5 x SSC	55°C	20 minutes
4.	Wash tissue slides in 0.1 x SSC	55°C	20 minutes
5.	Rinse slides in TBS	RT	2 x 2 minutes
6.	Block non-specific binding sites with 1x blocking buffer application per tissue section	RT	15 minutes
7.	Polyclonal sheep anti-DIG-IgG alkaline phosphatase conjugate was diluted 1:500 in blocking solution and added to each tissue section	4°C	18 hours
8.	Rinse slides in TBS	RT	2 x 2 minutes
9.	Chromogen - NBT/BCIP diluted 1:50 in 1x detection buffer was added to each tissue section and left to develop in the dark	RT	≤ 15 minutes
10.	Upon colour change, the slides were immersed in dH ₂ O to stop the reaction	RT	5 minutes
11.	Counter-stain with Mayer's haematoxylin	RT	2 minutes
12.	Slides were rinsed under running tap water	RT	5 minutes
13.	Dehydrate tissue sections in dH ₂ O	RT	5 minutes
14.	Dehydrate in 50% Ethanol	RT	5 minutes
15.	Dehydrate in 70% Ethanol	RT	5 minutes
16.	Dehydrate in 90% Ethanol	RT	5 minutes

17.	Dehydrate in 100% Ethanol	RT	2 x 5 minutes each
18.	Dry slides in incubator	50°C	10 min
19.	Immerse slides in Xylene	RT	2 x 5 minutes each
20.	Mount with DPX permanent mountant and allow to dry in fumehood	RT	45 minutes
21.	View slides under light microscope and capture images		
22.	Store slides away at 4°C	4°C	

A.3 DETECTION OF TSH-R mRNA BY QUANTITATIVE PCR (qPCR)

A.3.1 Reagents and consumables:

1. 96-well microtitre plates (Applied Biosystems)
2. PowerUp 2x SYBR Green master mix (Applied Biosystems)
3. PCR-grade H₂O
4. Forward primers (GAPDH/TSH-R)
5. Reverse primers (GAPDH/TSH-R)
6. Undiluted cDNA (limbic brain)
7. Undiluted calibrator sample (representative normal limbic group)

A.3.2 qPCR mix

Reagents	Volume (µl)
PCR-grade H ₂ O	6
SYBR Green master mix (2x)	10
Forward primer (GAPDH/TSH-R, 10µM)	1
Reverse primer (GAPDH/TSH-R, 10µM)	1
cDNA (undiluted) or calibrator (undiluted)	2
TOTAL	20

Cycling conditions: GAPDH and TSH-R

- Initial denaturation: 95°C for 10 minutes → 1 cycle
 - Denaturation: 95°C for 15 seconds
 - Annealing: 60°C for 30 seconds
 - Extension: 72°C for 30 seconds
- } 40 cycles

Melt Curve Analysis: Cycling conditions:

- Step 1: 95°C for 15 seconds → 1 cycle
- Step 2: 60°C for 1 minute → 1 cycle
- Step 3: 95°C for 30 seconds → 1 cycle
- Step 4: 60°C for 15 seconds → 1 cycle

CHAPTER 3

Results

3.1 LOCALISATION OF TSH-R AND TG BY IMMUNO-HISTOCHEMISTRY

3.1.1 Control tissues

Immuno-labelling of normal human thyroid tissue demonstrated TSH-R in the thyroid follicular cells that constitute the simple cuboidal, follicular epithelia (Figure 3.1A). The colloid, colloidal spaces and colloidal vesicles were devoid of TSH-R labelling. Further, TSH-R could not be detected within thyroid vasculature.

Thyroid protein (TG) expression in brain tissue is described in detail under section (3.1.3). However, controls for TG immuno-staining on normal thyroid tissue is described here. Immuno-reactive TG was evident within the cytoplasm of thyroid follicular cells (Figure 3.1C). In addition, intense TG labelling was visualised within the colloidal space and colloidal vesicles, and within the smooth muscle cells of the tunica media and tunica adventitia of thyroidal vasculature.

Method controls for thyroid tissue demonstrated the absence of staining when TSH-R (Figure 3.1B) and TG (Figure 3.1D) antibodies were substituted with dilution buffer.

3.1.2 Thyroid-stimulating hormone receptor (TSH-R)

(a) Observations

Immuno-reactive TSH-R was observed in all six limbic regions in all five brains examined for normal brain, as well as all three matched limbic regions (amygdala, cingulate gyrus and frontal cortex) investigated in all five brains of bipolar subjects. Positive immuno-labelling was demonstrated in the large motor neurons of the frontal cortex (Figure 3.2A and B), hippocampus (Figure 3.2C), cingulate gyrus (Figure 3.2D), amygdala (Figure 3.2E and F), hypothalamus and thalamus of normal brain.

Figure 3.1: Immuno-localisation of TSH-R and TG in normal human control thyroid tissue

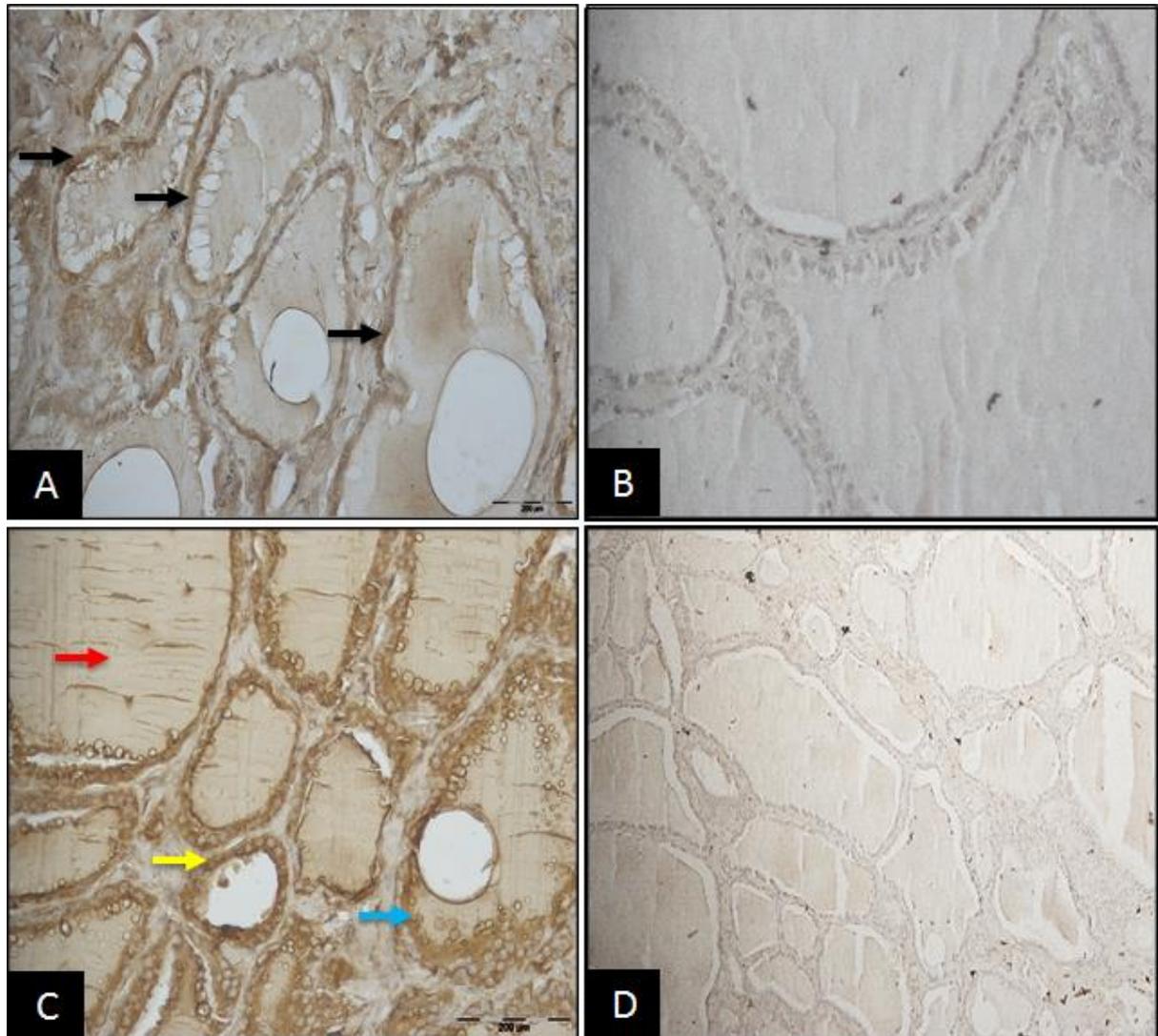


Figure 3.1 legend: Immuno-localisation of TSH-R and TG in normal human control thyroid tissue

Immuno-labelling of normal control thyroid tissue demonstrated TSH-R in the thyroid follicular cells (black arrows), (A, x400). Antibody method control for TSH-R in thyroid tissue was devoid of stain (B, x400). Immuno-reactive TG was demonstrated within the cytoplasm of thyroid follicular cells (yellow arrow), colloid (red arrow) and colloidal vesicles (blue arrow) of normal thyroid tissue (C, x 400). Antibody method control for TG in thyroid tissue demonstrated the absence of stain within thyroidal structures (D, x400). Positive labelling for TSH-R and TG in thyroid tissues were identified as brown colour. Scale bars = 200 μ m.

Figure 3.2: Immuno-localisation of TSH-R in normal human adult brain limbic areas

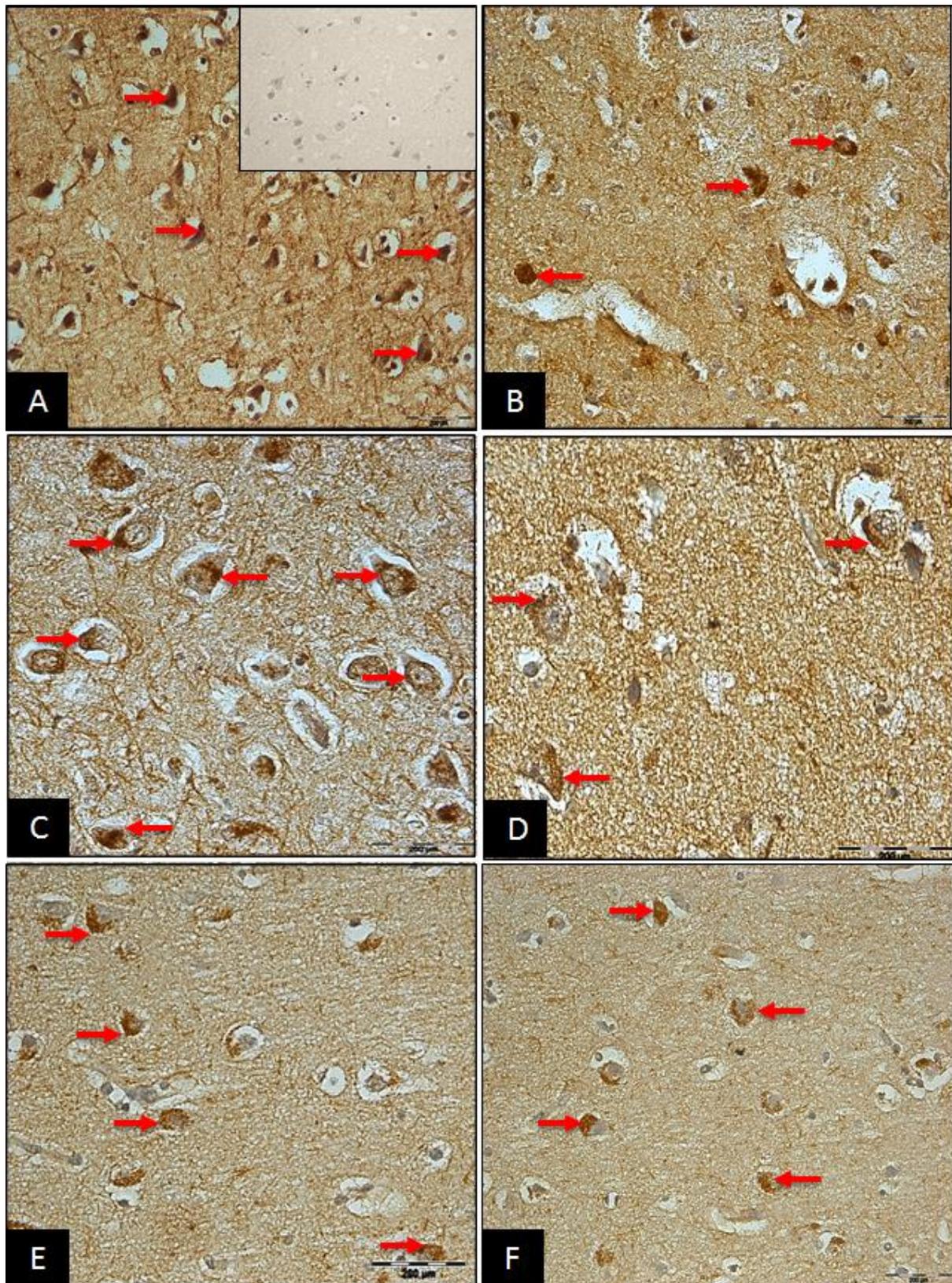


Figure 3.2 legend: Immuno-localisation of TSH-R in normal human adult brain limbic areas

In brain limbic areas, immuno-reactive TSH-R was evident in neuronal cell bodies (red arrows) in the frontal cortex (A and B, x400), hippocampus (C, x400), cingulate gyrus (D, x400) and amygdala (E and F, x400). Antibody method controls for brain tissue showed absence of stain in neurons (A, inset, x400). Positive labelling for TSH-R in brain tissues were identified as brown colour. Scale bars = 200 μ m.

Similarly, in bipolar brain limbic areas, immuno-reactive TSH-R was demonstrated within large motor neuronal cell bodies in the amygdala (Figure 3.3B), cingulate gyrus (Figure 3.3C and D) and frontal cortex (Figure 3.3E and F). Further, TSH-R was not detected in either cerebral vasculature or neuronal support cells that include the oligodendrocytes, astrocytes, neuroglia and satellite cells, in both normal and bipolar limbic areas.

(b) Image Analysis

The labelling intensities of TSH-R across all normal brain limbic regions were analysed and quantified by image analysis, and appeared to be confined to within the range $4.62 - 540.42 \times 10^2$ pixels. μm^{-2} (*arithmetic mean intensity* values). Specifically, regions such as the frontal cortex (540.42×10^2 pixels. μm^{-2}) and cingulate gyrus (395.47×10^2 pixels. μm^{-2}) displayed more intense staining, whilst the hypothalamus (11.24×10^2 pixels. μm^{-2}) and thalamus (4.62×10^2 pixels. μm^{-2}) stained to a lesser degree by a few hundred-fold (Table 3.1). Figure 3.4 displays the *Log mean (Ln) intensity* for TSH-R immuno-labelling in all normal brain limbic regions examined in the range 1.5 - 5.8. These results demonstrate an approximate 3-fold difference each for cingulate gyrus (5.80), frontal cortex (5.80), hippocampus (5.50) and amygdala (5.10) when compared to both, hypothalamus (1.90) or thalamus (1.50) (Table 3.1, Figure 3.4). Statistical comparisons demonstrated that TSH-R in both the hypothalamus and thalamus differed significantly when compared to each of the other four normal limbic areas ($p < 0.0001$) but not when compared to each other (Table 3.1, Figure 3.4). Further, no appreciable significance was noted among the remaining four normal limbic regions when compared amongst each other.

Figure 3.3: Immuno-localisation of TSH-R in bipolar human adult brain limbic areas and normal human control thyroid tissue

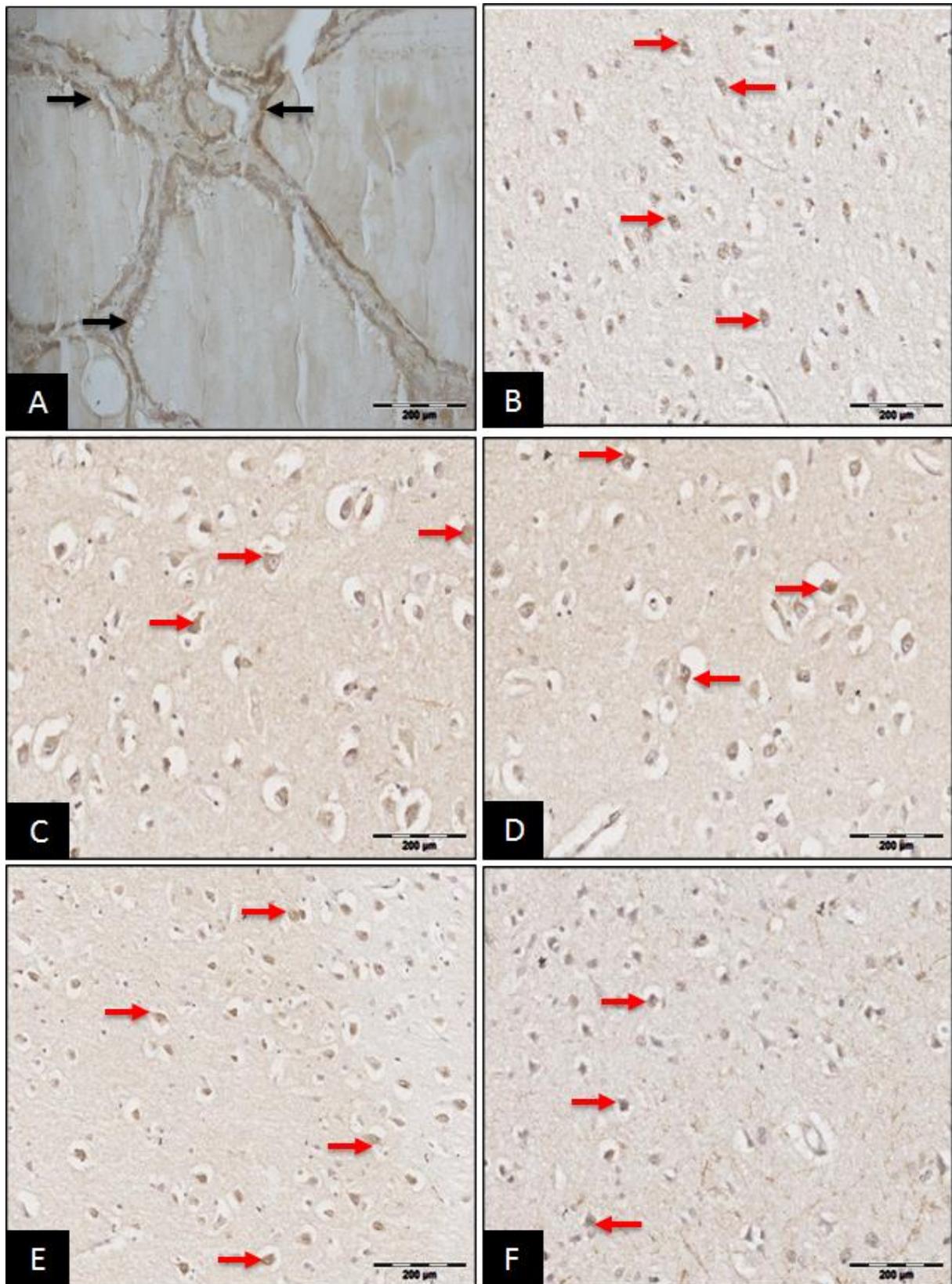


Figure 3.3 legend: Immuno-localisation of TSH-R in bipolar human brain limbic areas and normal human control thyroid tissue

Immuno-labelling of normal control thyroid tissue demonstrated TSH-R within the thyroid follicular cells especially on the luminal edge of the thyroid follicular cells bordering the colloidal spaces (black arrows) (A, x400). In bipolar brain limbic areas, immuno-reactive TSH-R was evident within neuronal cell bodies (red arrows) in the amygdala (B, x 400), cingulate gyrus (C and D, x400) and the frontal cortex (E and F, x400). Positive labelling for TSH-R in thyroid and brain sections were identified as brown colour. Scale bars = 200µm.

Table 3.1: Quantification of TSH-R neuronal labelling intensity in normal brain limbic regions

TSH-R in normal brain limbic neurons				
Limbic Regions	Arithmetic Mean Labelling Intensity		Log (Ln) Intensity	
	Mean (pixels.µm ²)	±SEM	Log Mean (Ln)	±Log SEM
Amygdala	243.99	50.23	5.10*†	0.29
Cingulate gyrus	395.47	62.71	5.80*†	0.17
Frontal cortex	540.42	85.38	5.80*†	0.25
Hippocampus	353.97	41.06	5.50*†	0.23
Hypothalamus	11.24	3.05	1.90	0.28
Thalamus	4.62	0.30	1.50	0.06

* vs hypothalamus, $p < 0.0001$

† vs thalamus, $p < 0.0001$

n = 6 limbic regions

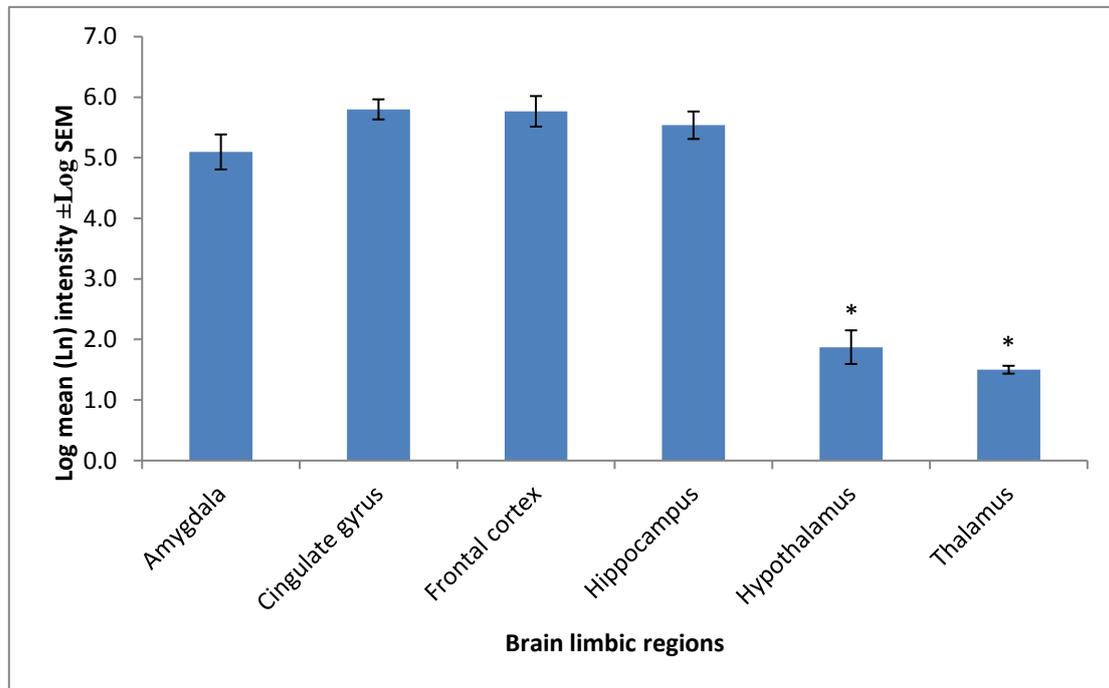
n = 5 normal human brains

n = 5 fields of view for each specimen

Image analysis: AnalySIS 5 (Soft Imaging Systems, Germany)

Statistical comparisons: STATA Software, v13.1 (StataCorp, USA)

Figure 3.4: Log mean (*Ln*) intensity of immuno-labelling for TSH-R in cortical neurons of normal human brain limbic regions



* vs amygdala, cingulate gyrus, frontal cortex, hippocampus, $p < 0.05$

Figure 3.4 legend: *Log mean (Ln) intensity of immuno-labelling for TSH-R in cortical neurons of normal human brain limbic regions*

Log mean (Ln) intensity values obtained for TSH-R in neuronal staining occurred in the range 1.5 - 5.8. These results indicate an approximate 3-fold difference when the cingulate gyrus (5.80), frontal cortex (5.80), hippocampus (5.50) and amygdala are each compared to the hypothalamus (1.90) or thalamus (1.50). Inter-group statistical comparisons showed that TSH-R in the hypothalamus and thalamus differed significantly when compared to each of the other limbic areas but not against each other. Further, there was no difference noted among the other four limbic regions when compared to each other.

Quantification of TSH-R immuno-labelling across all matched bipolar brain limbic regions provided a narrow *arithmetic mean intensity* range of 14.15 - 19.15 x10² pixels.µm⁻² (Table 3.2). Specifically, the cingulate gyrus (19.15 x10² pixels.µm⁻²) displayed the greatest intensity within this category, whilst the amygdala (14.15 x10² pixels.µm⁻²) was reported to have the least intensity for TSH-R. Table 3.2 also displays the *Log mean (Ln) intensity* for TSH-R immuno-labelling in all bipolar brain limbic regions in the range 1.97 - 2.30. Due to the narrow range reported in both *arithmetic mean* values and *Log mean (Ln) intensity* values, there is no notable fold difference amongst these regions. In addition, statistical comparisons among all bipolar regions indicates no appreciable difference within this category (Table 3.2, Figure 3.5). Hence, there appeared to be a fairly consistent level of TSH-R in these diseased limbic regions.

(c) Statistical comparisons between normal and bipolar limbic regions

Statistical comparisons obtained for TSH-R labelled neurons in normal versus bipolar brain limbic regions indicates a significant difference in all matched regions examined, i.e. amygdala ($p < 0.0001$), cingulate gyrus ($p < 0.0001$) and frontal cortex ($p < 0.0001$), (Figure 3.5). All demonstrate an appreciable decrease in TSH-R levels in the bipolar brain regions, respectively.

3.1.3 Thyroglobulin (TG)

3.1.3.1 TG in cerebral vasculature

(a) Observations

Positive immuno-labelling for TG was detected within the cerebral vasculature of all normal limbic regions. Specifically, immuno-reactive TG was localised to the smooth muscle cells of the tunica media and tunica adventitia of cerebral blood vessels in the amygdala (Figure 3.6A), frontal cortex (Figure 3.6B), thalamus (Figure 3.6C), hypothalamus (Figure 3.6D), cingulate gyrus (Figure 3.6E) and hippocampus (Figure 3.6F).

Table 3.2: Quantification of TSH-R neuronal labelling intensity for bipolar brain limbic regions

TSH-R in bipolar brain limbic neurons				
Limbic Regions (Bipolar)	Arithmetic Mean Labelling Intensity		Log (Ln) Intensity	
	Mean (pixels.µm ²)	±SEM	Log Mean (Ln)	±Log SEM
Amygdala	14.15	2.53	1.97	0.16
Cingulate gyrus	19.15	3.65	2.30	0.17
Frontal cortex	15.01	2.42	2.09	0.16

n = 5 human bipolar brains

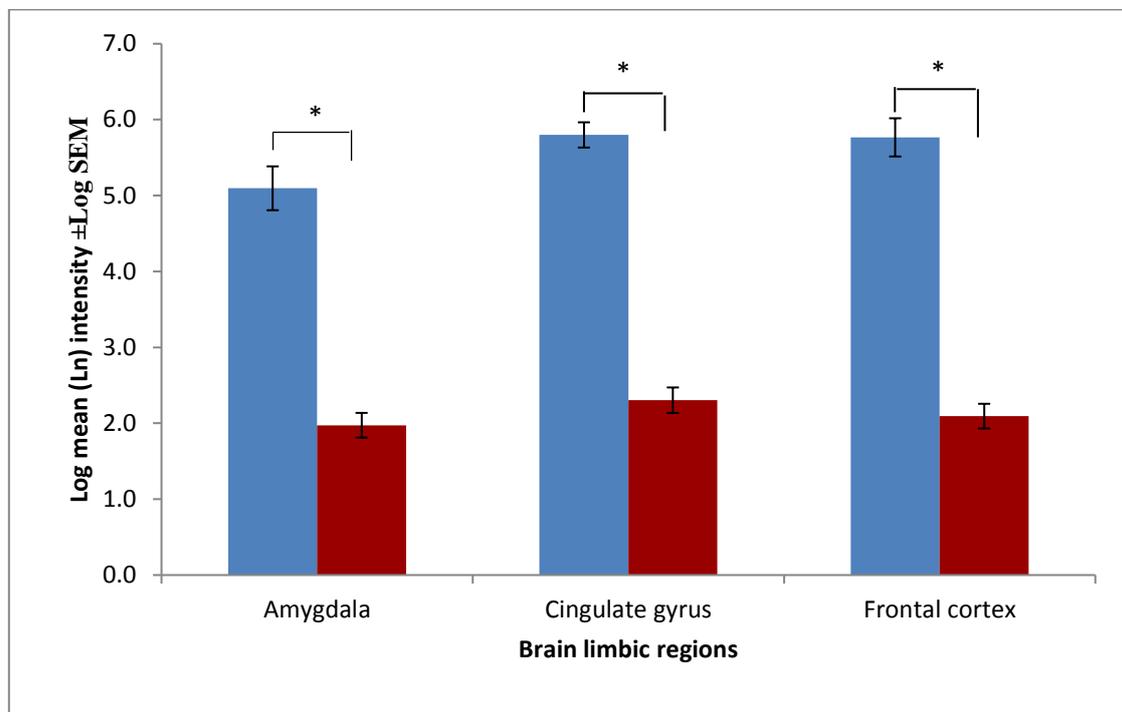
n = 3 limbic regions

n = 10 fields of view for each specimen

Image analysis: AnalySIS 5 (Soft Imaging Systems, Germany)

Statistical comparisons: STATA Software, v13.1 (StataCorp, USA)

Figure 3.5: *Log mean (Ln) intensity of immuno-labelling for TSH-R in cortical neurons of matched normal versus bipolar brain limbic regions*



■ Normal
■ Bipolar

* $p < 0.0001$

Figure 3.5 legend: *Log mean (Ln) intensity of immuno-labelling for TSH-R in cortical neurons of matched normal versus bipolar brain limbic regions*

Log mean (Ln) intensity values were obtained for TSH-R neuronal staining in normal and bipolar brain limbic areas. Intra-group statistical comparisons demonstrates significant differences between matched normal and bipolar limbic areas examined, amygdala ($p < 0.0001$), cingulate gyrus ($p < 0.0001$) and frontal cortex ($p < 0.0001$). Further, there was no inter-group difference noted among the bipolar groups when compared to each other.

Figure 3.6: Immuno-localisation of TG in normal human adult brain limbic areas

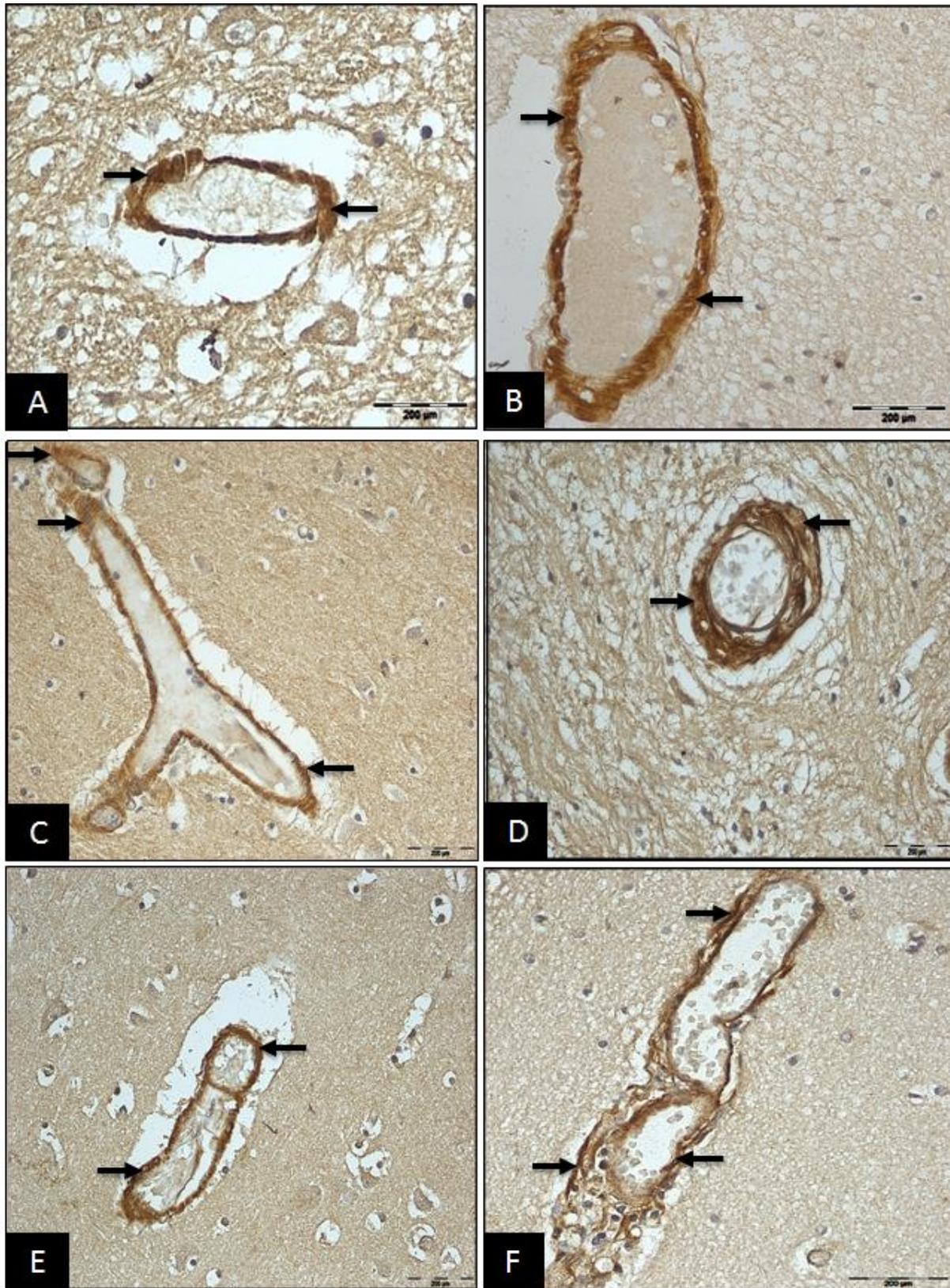


Figure 3.6 legend: Immuno-localisation of TG in normal human adult brain limbic areas

Immuno-reactive TG was highly concentrated within smooth muscle of the tunica media and tunica adventitia of cerebral vasculature in the amygdala (A, x400), frontal cortex (B, x400), thalamus (C, x400), hypothalamus (D, x400), cingulate gyrus (E, x400) and hippocampus (F, x400). Positive labelling for TG in brain tissues were identified as brown colour and are indicated by arrows. Scale bars = 200 μ m.

In matched bipolar limbic regions, immuno-reactive TG was identified within cerebral vasculature in all areas examined. However, the degree of staining distribution compared to that in normal limbic vasculature, was observed to be less. Similar to normal brain, TG immuno-labelling was localised specifically to smooth muscle cells of the tunica media and tunica adventitia of cerebral vasculature in the frontal cortex (Figure 3.7B), amygdala (Figure 3.7C and D), and cingulate gyrus (Figure 3.7E and F).

(b) Image Analysis

Quantification of TG immuno-labelling within limbic vasculature of the normal group, demonstrated a wide *arithmetic mean intensity* range of 423.84 - 2994.00 $\times 10^2$ pixels. μm^{-2} (Table 3.3). Specifically, the cingulate gyrus (2994.00 $\times 10^2$ pixels. μm^{-2}), thalamus (1474.34 $\times 10^2$ pixels. μm^{-2}) and hippocampus (1407.14 $\times 10^2$ pixels. μm^{-2}) displayed the greatest intensities within this category, whilst the hypothalamus (571.04 $\times 10^2$ pixels. μm^{-2}) and amygdala (423.84 $\times 10^2$ pixels. μm^{-2}) stained with lesser intensities, by a few thousand-fold. Table 3.3 also displays the *Log mean (Ln) intensity* values for TG in limbic vasculature which occurs in the range of 5.14 - 6.40 (Table 3.3). Inter-group statistical comparisons demonstrated that when compared to the amygdala, there was a significant difference in the cingulate gyrus ($p = 0.02$), frontal cortex ($p = 0.03$) and thalamus ($p = 0.02$). None of the other inter-regional comparisons showed statistical differences (Figure 3.8).

Quantification of TG immuno-labelling within limbic vasculature of the bipolar regions, presented an *arithmetic mean intensity* range of 75.48 - 98.96 $\times 10^2$ pixels. μm^{-2} (Table 3.4). The cingulate gyrus (98.96 $\times 10^2$ pixels. μm^{-2}) indicated the greatest intensity within this category, whilst the frontal cortex (75.48 $\times 10^2$ pixels. μm^{-2}) stained the least. *Log mean (Ln) intensity* values were also obtained in the range of 2.99 - 3.96 (Table 3.4). Inter-group statistical comparisons among all bipolar limbic regions demonstrated that when compared to the frontal cortex, there were significant differences in the cingulate gyrus ($p < 0.005$) and amygdala ($p < 0.002$), (Table 3.4, Figure 3.9)

Figure 3.7: Immuno-localisation of TG in bipolar human brain limbic areas and normal human control thyroid tissue

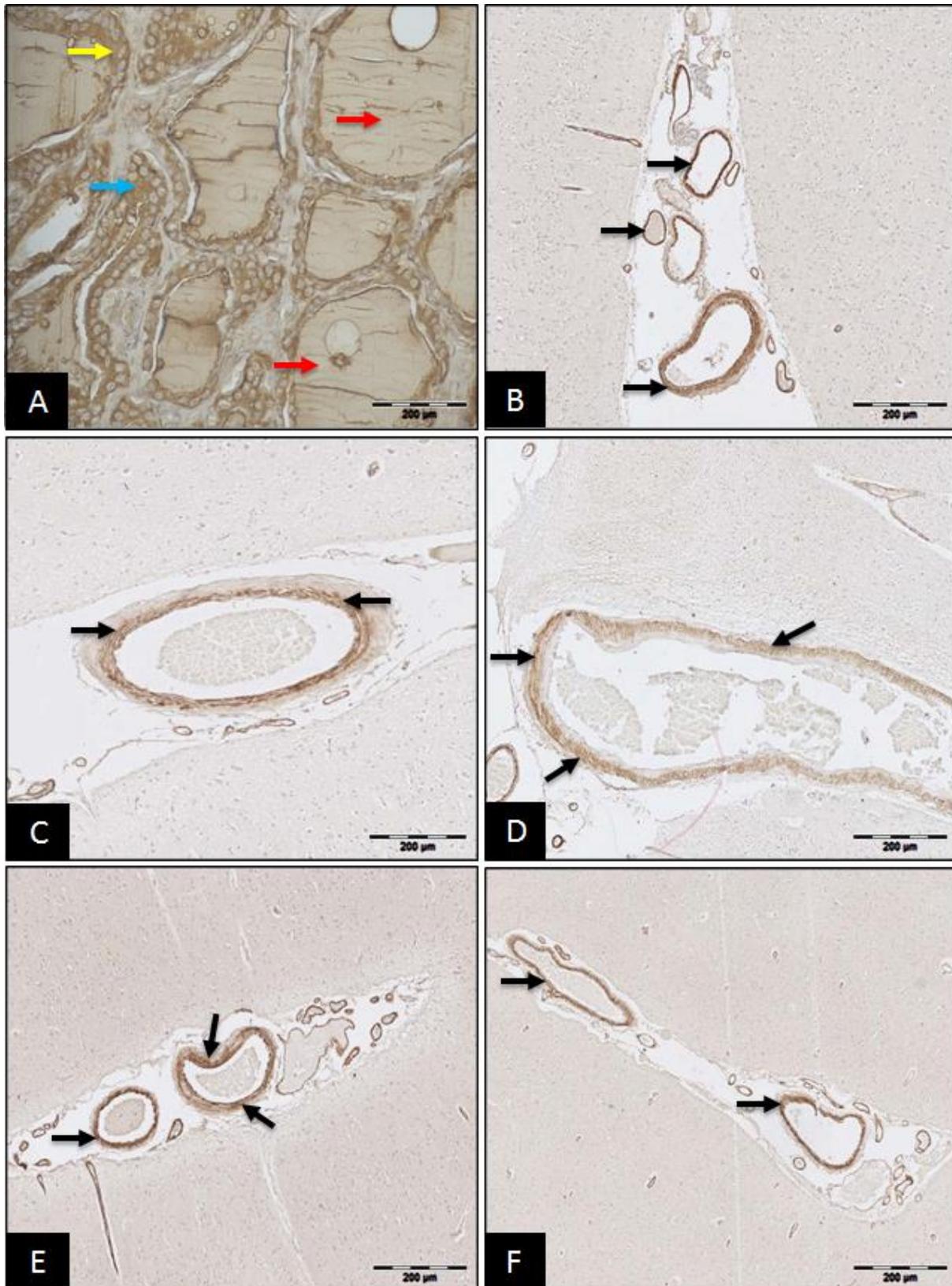


Figure 3.7 legend: Immuno-localisation of TG in bipolar human brain limbic areas and normal human control thyroid tissue

Immuno-labelling of normal control thyroid tissue demonstrated TG within the cytoplasm of thyroid follicular cells (yellow arrow), colloid (red arrow) and colloidal vesicles (blue arrow) (A, x400). Immuno-reactive TG was highly concentrated within the smooth muscle of the tunica media/adventitia of cerebral vasculature in the frontal cortex (B, x200), amygdala (C and D, x200) and cingulate gyrus (E and F, x200) of bipolar brain. Positive labelling for TG in thyroid and brain tissues were identified as brown colour. Scale bars = 200µm.

Table 3.3: Quantification of TG cerebral vasculature labelling intensity in normal brain limbic regions

TG in normal brain limbic cerebral vasculature				
Limbic Regions	Arithmetic Mean Labelling Intensity		Log (Ln) Intensity	
	Mean (pixels.µm ²)	±SEM	Log Mean (Ln)	±Log SEM
Amygdala	423.84	132.30	5.14	0.37
Cingulate gyrus	2994.00	2315.11	6.40*	0.30
Frontal cortex	884.70	229.70	6.28*	0.19
Hippocampus	1407.14	922.26	5.97	0.26
Hypothalamus	571.04	94.44	6.09	0.15
Thalamus	1474.34	500.46	6.35*	0.28

* vs amygdala, $p < 0.05$

n = 6 limbic regions

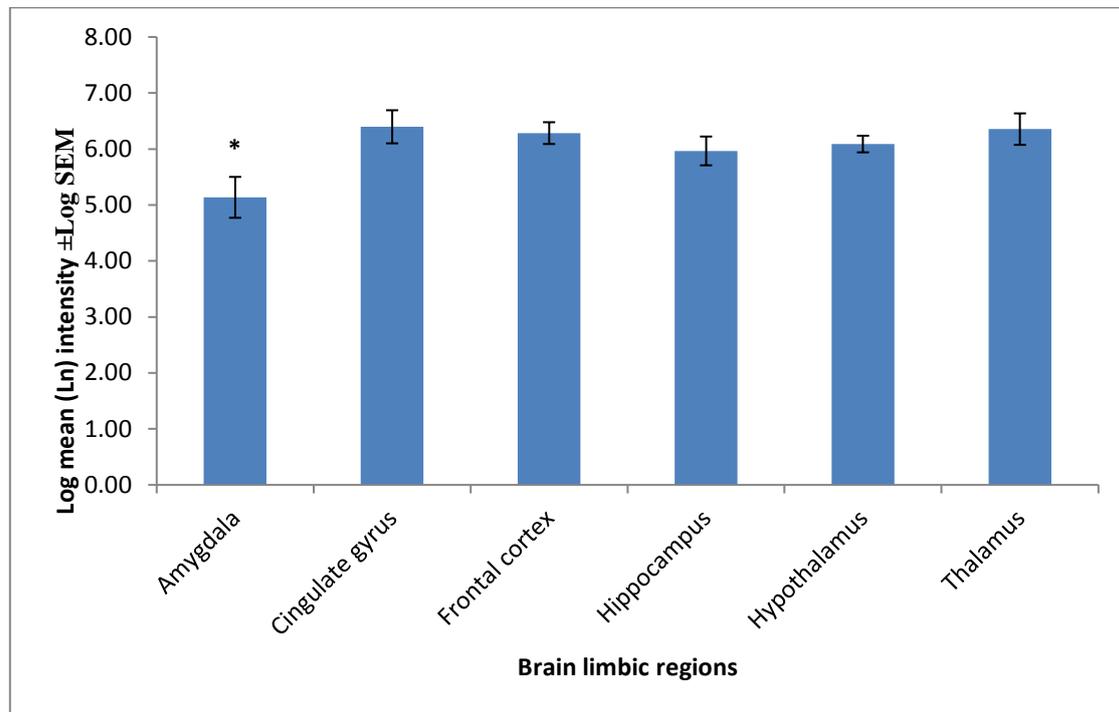
n = 5 normal human brains

n = 5 fields of view for each specimen

Image analysis: AnalySIS 5 (Soft Imaging Systems, Germany)

Statistical comparisons: STATA Software, v13.1 (StataCorp, USA)

Figure 3.8: Log mean (Ln) intensity of immuno-labelling for TG in cerebral vasculature of normal human brain limbic regions



* versus cingulate gyrus, frontal cortex, thalamus, $p < 0.05$

Figure 3.8 legend: *Log mean (Ln) intensity of immuno-labelling for TG in cerebral vasculature of normal human brain limbic regions*

Log mean (Ln) intensity values obtained for TG in normal limbic cerebral vasculature occurred in the range 5.14 - 6.40. Statistical comparisons indicates that the cingulate gyrus, frontal cortex and thalamus differed significantly from the amygdala. No statistical differences occurred among other inter-regional comparisons.

Table 3.4: Quantification of TG cerebral vasculature labelling intensity in bipolar brain limbic regions

TG in bipolar brain limbic cerebral vasculature				
Limbic Regions (Bipolar)	Arithmetic Mean Labelling Intensity		Log (Ln) Intensity	
	Mean (pixels.µm²)	±SEM	Log Mean (Ln)	±Log SEM
Amygdala	89.82	14.36	3.96*	0.15
Cingulate gyrus	98.96	17.14	3.88*	0.18
Frontal cortex	75.48	18.34	2.99	0.25

* vs frontal cortex, $p < 0.05$

n = 5 human bipolar brains

n = 3 limbic regions

n = 10 fields of view for each specimen

Image analysis: AnalySIS 5 (Soft Imaging Systems, Germany)

Statistical comparisons: STATA Software, v13.1 (StataCorp, USA)

(c) Statistical comparisons between normal and bipolar limbic regions

Intra-group statistical comparisons determined for TG in cerebral vasculature in matched normal versus bipolar limbic regions demonstrated significant differences in all areas, amygdala ($p = 0.0007$), cingulate gyrus ($p < 0.0001$) and frontal cortex ($p < 0.0001$), (Figure 3.9). Thus, there appeared to be appreciable decreases in the TG levels found in bipolar brain regions

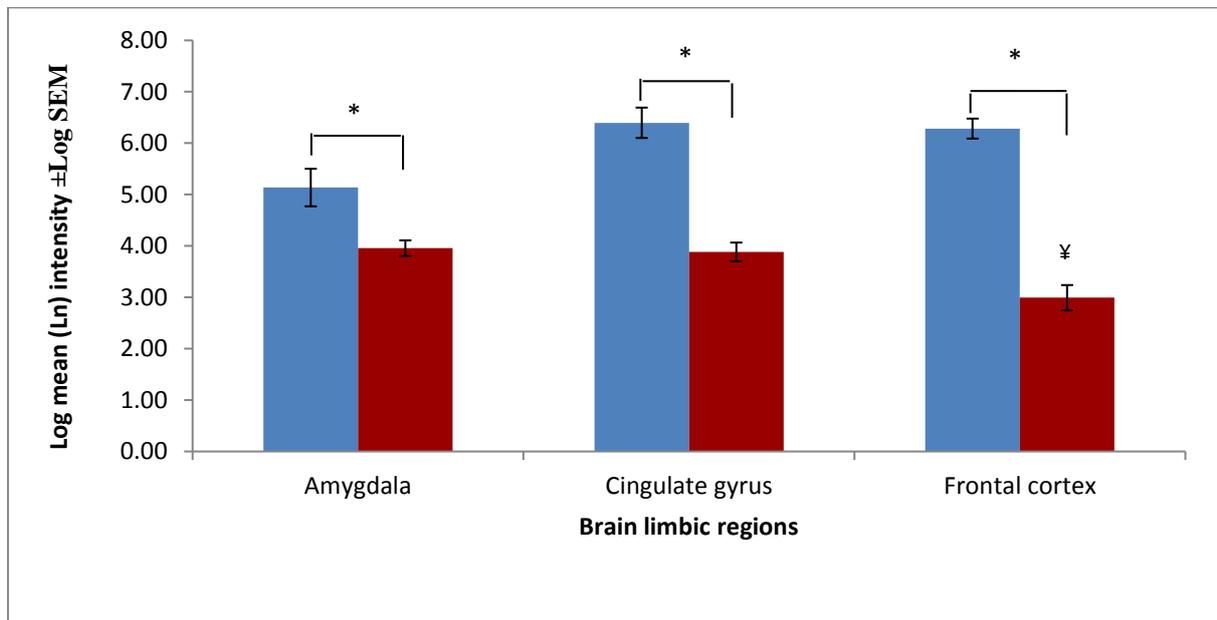
3.1.3.2 TG in cerebral neuronal cells

(a) Observations

In normal limbic brain, immuno-reactive TG was demonstrated in most neuronal cell bodies and axons of the cingulate gyrus (Figure 3.10A-D), whilst similar cellular structures in the frontal cortex exhibited less staining distribution (Figure 3.10E and F). There was no evidence of positive TG labelling within neuronal cell bodies and supporting cerebral cellular structures in the other four limbic regions.

In matched bipolar brain limbic regions, immuno-reactive TG was evident within neuronal cell bodies of the amygdala (Figure 3.11A and B), cingulate gyrus (Figure 3.11C and D) and the frontal cortex (Figure 3.11E and F). In contrast to normal brain, bipolar brain neuronal axons, specifically within the cingulate gyrus, were devoid of stain. Further, there was no evidence of TG labelling within other supporting cerebral cellular structures in all matched bipolar limbic regions.

Figure 3.9: Log mean (Ln) intensity of immuno-labelling for TG in cerebral vasculature of matched normal versus bipolar brain limbic regions



■ Normal
■ Bipolar

* $p < 0.0001$

‡ vs amygdala, cingulate gyrus, $p < 0.05$

Figure 3.9 legend: *Log mean (Ln) intensity* of immuno-labelling for TG in cerebral vasculature of matched normal versus bipolar brain limbic regions

Log mean (Ln) intensity values were obtained for TG in normal and bipolar brain limbic cerebral vasculature. Intra-group statistical comparisons demonstrates significant differences between respective normal and bipolar limbic areas examined, amygdala ($p = 0.0007$), cingulate gyrus ($p < 0.0001$) and frontal cortex ($p < 0.0001$). Within the bipolar groups only, inter-group significant increases were demonstrated in the cingulate gyrus ($p < 0.005$) and amygdala ($p < 0.002$), when compared to the frontal cortex.

Figure 3.10: Immuno-localisation of TG in normal human brain limbic neurons

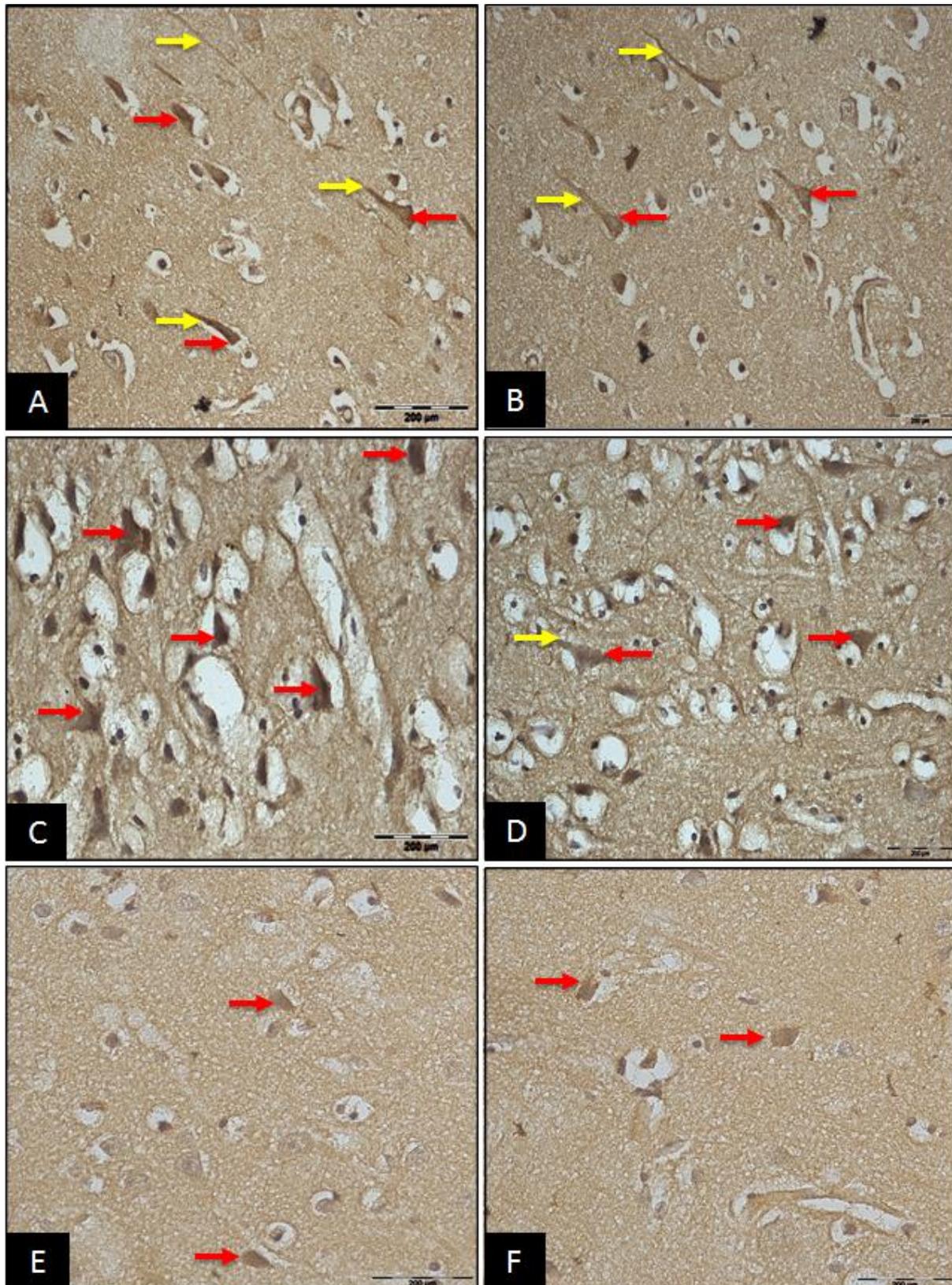


Figure 3.10 legend: Immuno-localisation of TG in normal human brain limbic neurons

Immuno-reactive TG was highly concentrated in neuronal cell bodies (red arrows) and axons (yellow arrows) of normal cingulate gyrus (A, x400), (B, x400), (C, x400) and (D, x400). TG was also evident within neuronal cell bodies of normal frontal cortex (E, x400) and (F, x400). Positive labelling for TG in brain tissues were identified as brown colour. Scale bars = 200µm.

Figure 3.11: Immuno-localisation of TG in bipolar human brain limbic neurons

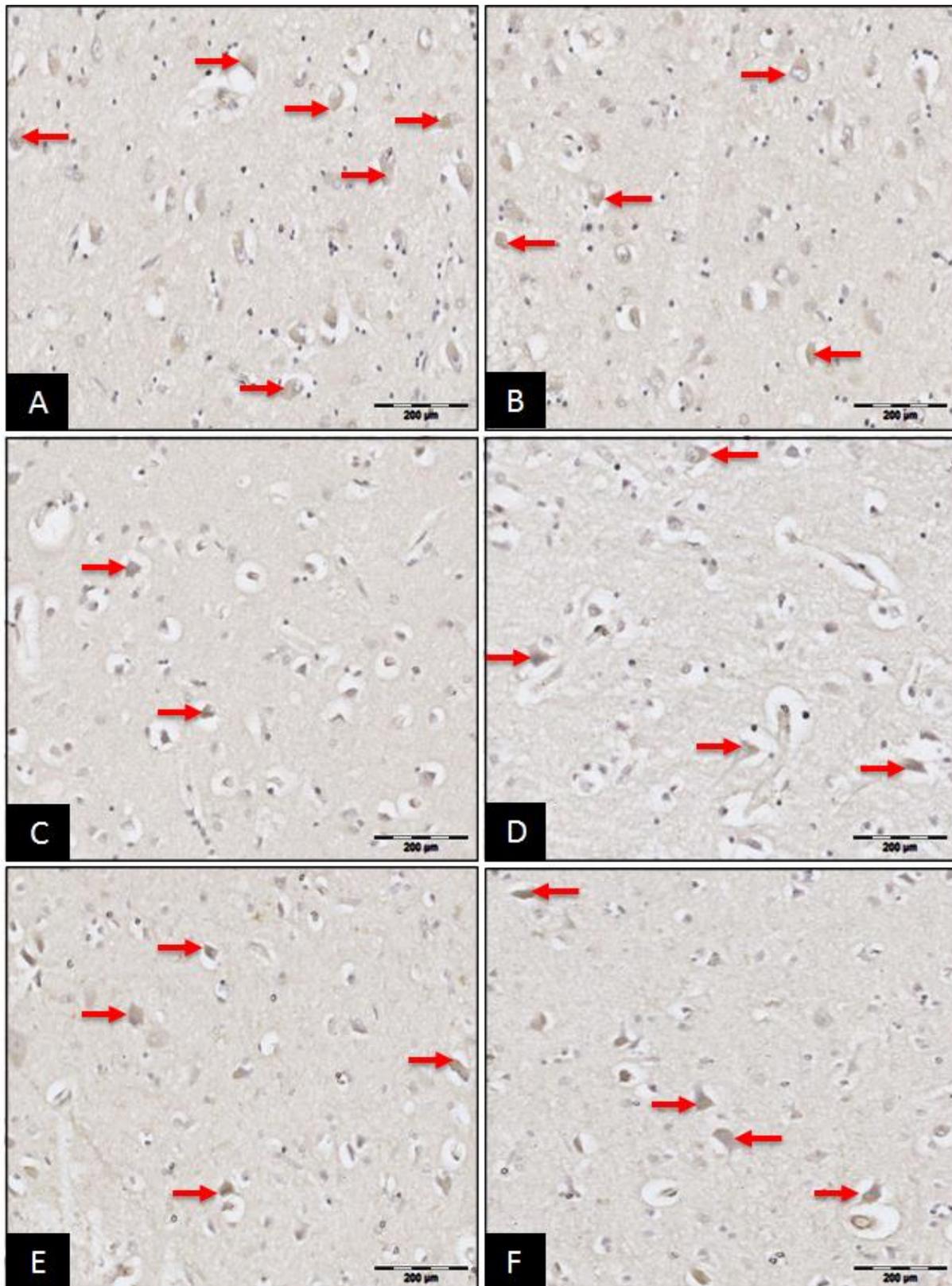


Figure 3.11 legend: Immuno-localisation of TG in bipolar human brain limbic neurons

Immuno-reactive TG was evident within neuronal cell bodies (red arrows) of the amygdala (A and B, x400), cingulate gyrus (C and D, x400) and frontal cortex (E and F, x400) of bipolar brain. Neuronal axons and other cerebral structures were devoid of stain. Positive labelling for TG in brain sections were identified as brown colour. Scale bars = 200 μ m.

(b) Image Analysis

Quantification of TG-neuronal staining in the normal group, presented *arithmetic mean intensity* values for the cingulate gyrus (118.20×10^2 pixels. μm^{-2}), whilst the frontal cortex (91.90×10^2 pixels. μm^{-2}) stained to a lesser degree, by approximately 30% (Table 3.5). *Log median (Ln) intensity* values obtained for this category had a range of -3.23 - 3.05 (Table 3.5). Specifically, the cingulate gyrus obtained a *Log mean (Ln)* value of 1.25, whilst the frontal cortex was reported to be -3.00. Inter-group statistical comparisons, tested at $p < 0.003$, demonstrated that the normal hypothalamus was significantly higher than all other normal limbic regions except the cingulate gyrus ($p = 0.07$).

Quantification of the staining intensities in the matched bipolar group, presented *arithmetic mean* values for amygdala (2.33×10^2 pixels. μm^{-2}), cingulate gyrus (5.53×10^2 pixels. μm^{-2}) and frontal cortex (4.73×10^2 pixels. μm^{-2}), (Table 3.6). Further, *Log median (Ln) intensity* values obtained for this category had a range of 0.43 - 0.89 (Table 3.6). Specifically, the following *Log median (Ln)* values were obtained: Amygdala (0.43), cingulate gyrus (0.79) and frontal cortex (0.89). Bipolar inter-group statistical comparisons tested at $p < 0.05$ demonstrated that the amygdala was significantly less than the frontal cortex ($p = 0.0183$) but not the cingulate gyrus ($p = 0.0876$).

(c) Statistical comparison between normal and bipolar limbic regions

Intra-group statistical comparisons of neuronal-labelled TG in matched normal versus bipolar brain limbic regions, indicates a significant difference in the amygdala groups ($p < 0.0001$) and in the frontal cortex groups ($p < 0.001$). In contrast, there was no appreciable difference in TG immuno-localisation observed between normal and bipolar cingulate gyrus.

Table 3.5: Quantification of TG neuronal labelling intensity in normal brain limbic regions

TG in normal brain limbic neurons					
Limbic Regions	Arithmetic Mean Labelling Intensity		Log (Ln) Intensity		
	Mean (pixels.µm ²)	±SEM	Log Median (Ln)	<i>p</i> 25	<i>p</i> 75
Amygdala	0.10	0.02	-2.52 [¥]	-3.72	-1.85
Cingulate gyrus	118.20	56.41	1.25	-1.81	5.02
Frontal cortex	91.90	46.90	-3.00 [¥]	-3.18	0.43
Hippocampus	0.20	0.10	-3.23 [¥]	-3.50	-2.67
Hypothalamus	145.10	52.62	3.05	1.84	4.71
Thalamus	9.10	3.77	-1.72 [¥]	-2.83	0.43

¥ vs hypothalamus, $p < 0.003$

n = 6 limbic regions

n = 5 normal human brains

n = 5 fields of view for each specimen

Inter-quartile range errors: *p*25 and *p*75

Image analysis: AnalySIS 5 (Soft Imaging Systems, Germany)

Statistical comparisons: STATA Software, v13.1 (StataCorp, USA)

Table 3.6: Quantification of TG neuronal labelling intensity for bipolar brain limbic regions

TG in bipolar brain limbic neurons					
Limbic Regions (Bipolar)	Arithmetic Mean Labelling Intensity		Log (Ln) Intensity		
	Mean (pixels.µm ²)	±SEM	Log Median (Ln)	p25	p75
Amygdala	2.33	0.24	0.43*	0.21	1.04
Cingulate gyrus	5.53	1.17	0.79	0.23	1.54
Frontal cortex	4.73	0.68	0.89	0.25	2.04

* vs frontal cortex, $p < 0.05$

n = 5 human bipolar brains

n = 3 limbic regions

n = 10 fields of view for each specimen

Inter-quartile range errors: $p25$ and $p75$

Image analysis: AnalySIS 5 (Soft Imaging Systems, Germany)

Statistical comparisons: STATA Software, v13.1 (StataCorp, USA)

3.2 CELLULAR DISTRIBUTION OF TSH-R mRNA BY *in-situ* RT-PCR

3.2.1 GAPDH and TSH-R mRNA expression in various human control tissues

(a) Observations

For the optimisation of the *in-situ* RT-PCR experiments, we used the mRNA expression of the ubiquitous control (*housekeeping*) gene, GAPDH, in various normal human tissues. Tissue controls for these experiments included kidney, thyroid and brain (cingulate gyrus). The GAPDH mRNA was positively localised in all normal human tissue examined (Figure 3.12). In normal human kidney tissue, labelled GAPDH mRNA was observed within the renal tubular epithelial cells of the proximal and distal convoluted tubules (Figure 3.12A). In thyroid tissue, GAPDH mRNA was evident within the thyroid follicular cells (Figure 3.12C). Normal human brain (cingulate gyrus) tissue demonstrated GAPDH mRNA within the cytoplasm of neuronal cell bodies (Figure 3.12E). Methods controls that were included for each type of human tissue, by the omission of primers from the PCR reactions, showed no localisation of GAPDH mRNA (Figure 3.12B, D and F).

For our target gene, TSH-R, we used normal human control thyroid tissue to demonstrate TSH-R mRNA labelling in thyroid follicular cells (Figure 3.13A). There was no evidence of TSH-R mRNA in other thyroidal structures such as the colloid and colloidal vesicles. Method control for thyroid tissue showed absence of TSH-R mRNA in all thyroidal structures when PCR primers were omitted from the reactions (Figure 3.13A, inset).

3.2.2 TSH-R gene expression in normal and bipolar brain limbic regions

(a) Observations

In normal cingulate gyrus, positive labelling for TSH-R mRNA expression was observed in neuronal cell bodies as well as some axons and dendrites (Figure 3.13E and F). Similarly, normal frontal cortex

Figure 3.12: The distribution of GAPDH mRNA in various normal human tissue by *in-situ* RT-PCR

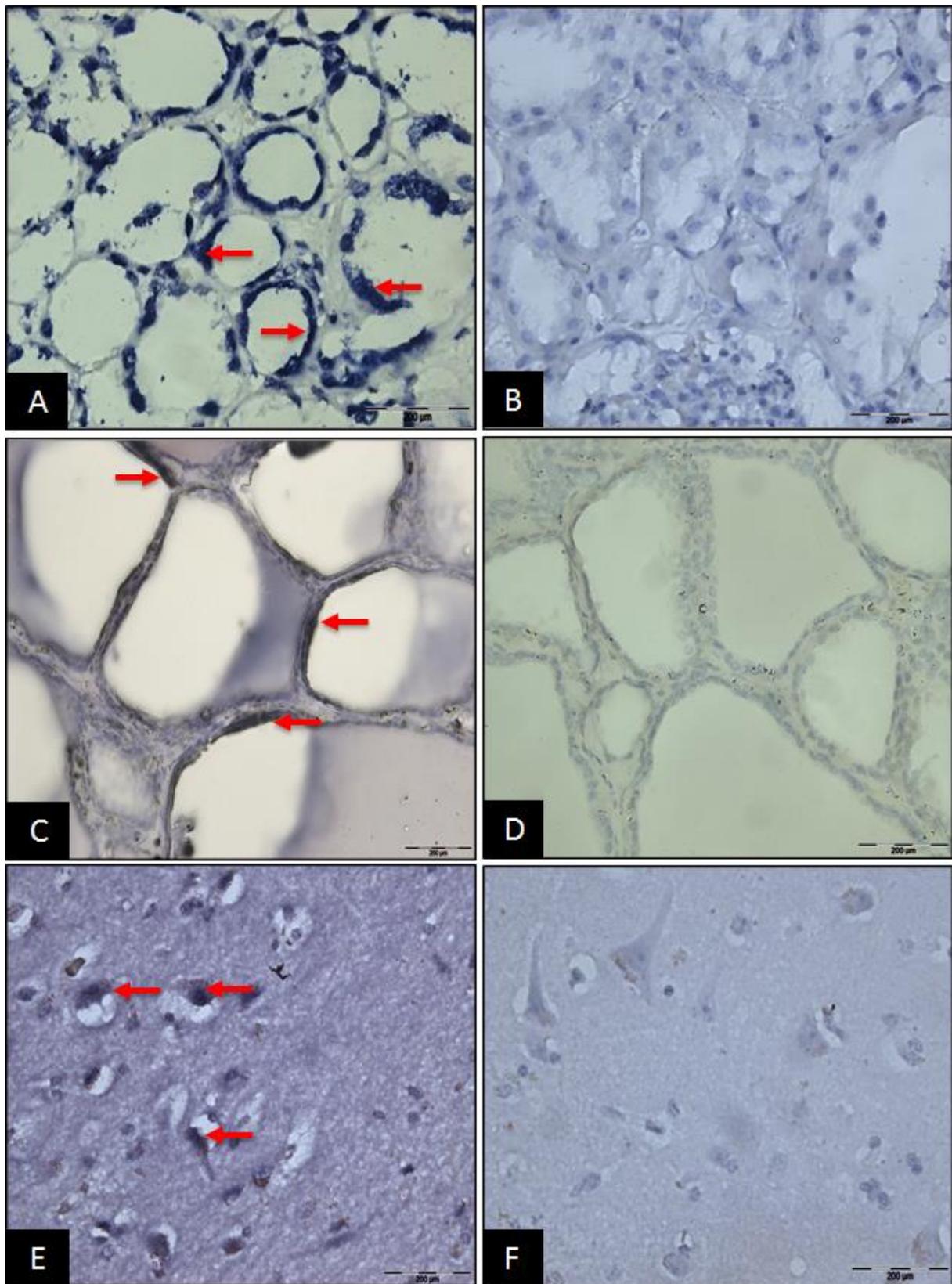


Figure 3.12 legend: The distribution of GAPDH mRNA in various normal human tissue by *in-situ* RT-PCR

Examination of normal human kidney tissue, demonstrated GAPDH mRNA within renal tubular epithelial cells of the proximal and distal convoluted tubules (A, x400). Human thyroid tissue showed GAPDH mRNA within thyroid follicular cells (C, x400). Human brain (cingulate gyrus) tissue demonstrated GAPDH mRNA within the cytoplasm of neuronal cell bodies (E, x400). Method controls for normal kidney (B), thyroid (D) and cingulate gyrus (F) were devoid of GAPDH mRNA labelling. Arrows in A, C, E indicate immuno-localised GAPDH mRNA, due to the blue-black NBT/BCIP precipitate. Scale bars = 200µm.

Note: due to the colloid in the normal thyroid tissue sample in C, there were multiple focal planes. The focal plane selected in C was optimal for the colloidal epithelial cells, in which the control (housekeeping) gene expression for GAPDH was localised.

Figure 3.13: The distribution of TSH-R mRNA in normal human brain limbic areas and normal human control thyroid tissue by *in-situ* RT-PCR

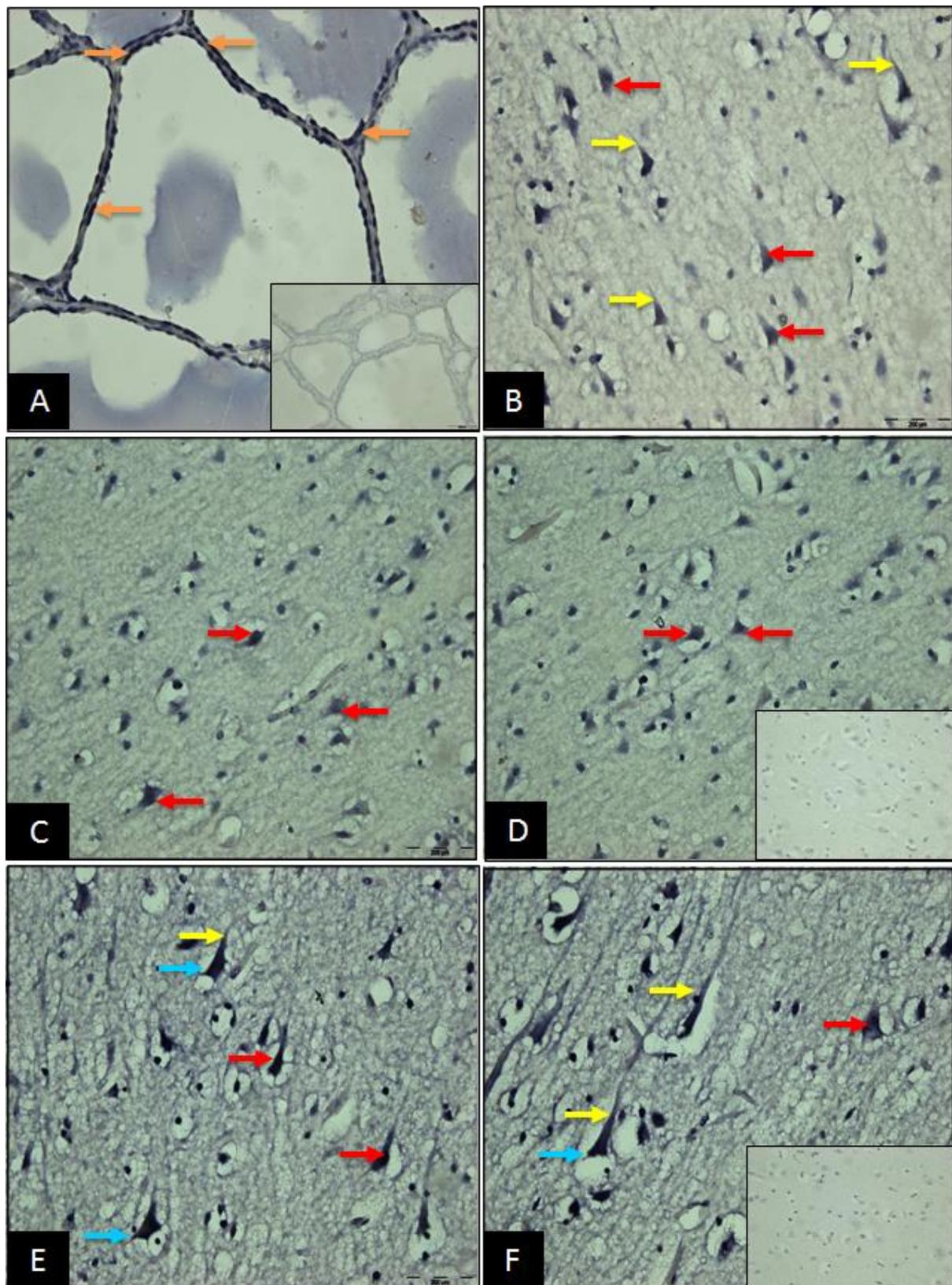


Figure 3.13 legend: The distribution of TSH-R mRNA in normal human brain limbic areas and normal human control thyroid tissue by *in-situ* RT-PCR

Examination of normal human control thyroid tissue demonstrated TSH-R mRNA labelling in thyroid follicular cells (A, x400). Method control for thyroid tissue showed absence of TSH-R mRNA when primers were omitted from the PCR reactions (A, inset, x 400). In normal limbic areas, intense labelling for TSH-R mRNA was detected in neuronal cell bodies (red arrows) in the frontal cortex (B, x400), amygdala (C and D, x400) and the cingulate gyrus (E and F, x400). The frontal cortex (B, x400) and cingulate gyrus (E and F, x400) also demonstrated TSH-R in neuronal axons (yellow arrows). Further, TSH-R mRNA was also evident in neuronal dendrites (light blue arrows) of the cingulate gyrus (E and F, x400). Method controls for brain tissue showed absence of TSH-R mRNA label in limbic structures (D and F, insets, x400). Positive labelling for TSH-R mRNA in thyroid and brain tissues were identified as blue-black colour indicating the precipitation of the chromogen NBT/BCIP. Scale bars = 200µm.

Note: due to the colloid in the normal thyroid tissue sample in A, there were multiple focal planes. The focal plane selected in A was optimal for the colloidal epithelial cells, in which the target gene expression for TSH-R was localised.

demonstrated TSH-R mRNA in neuronal cell bodies and axons; however, there was no labelling evident within dendrites (Figure 3.13B). In normal amygdala, TSH-R mRNA labelling was confined to neuronal cell bodies only (Figure 3.13C and D). Further, TSH-R mRNA was not identified in cerebral vasculature nor in neuronal support cells including oligodendrocytes, astrocytes, neuroglia and satellite cells, in any of the normal limbic areas. Method controls for normal brain tissue showed the absence of label for TSH-R mRNA expression in limbic structures, when primers were omitted from the PCR reactions (Figure 3.13D and F, insets).

In bipolar brain limbic areas, TSH-R mRNA expression was confined exclusively to neuronal cell bodies in the amygdala (Figure 3.14A and B), frontal cortex (Figure 3.14C and D) and cingulate gyrus (Figure 3.14E and F). There was no evidence of TSH-R mRNA in cerebral vasculature nor in neuronal support cells including oligodendrocytes, astrocytes, neuroglia and satellite cells, in both normal and bipolar limbic areas. Method controls for brain tissue demonstrated an absence of immuno-localised TSH-R mRNA label in limbic structures (Figure 3.14B, D and F, insets, x400).

(b) Semi-quantitative analysis

The distribution of the TSH-R mRNA in normal and bipolar experimental groups, by *in-situ* RT-PCR, was semi-quantitatively graded on a scale of 0 to 3 according to (i) visual intensity of labelling, as well as (ii) the extent of cellular distribution. Determinations were performed by two independent observers and the mean scores were collated (Table 3.7).

Semi-quantitative analysis of the normal limbic groups indicated intense visual labelling intensity as a maximum score of (3) in limbic structures of the frontal cortex, amygdala and cingulate gyrus (Table 3.7). The extent of TSH-R mRNA distribution within these regions ranged from 25-75% in the

Figure 3.14: The distribution of TSH-R mRNA in bipolar human brain limbic areas by *in-situ* RT-PCR

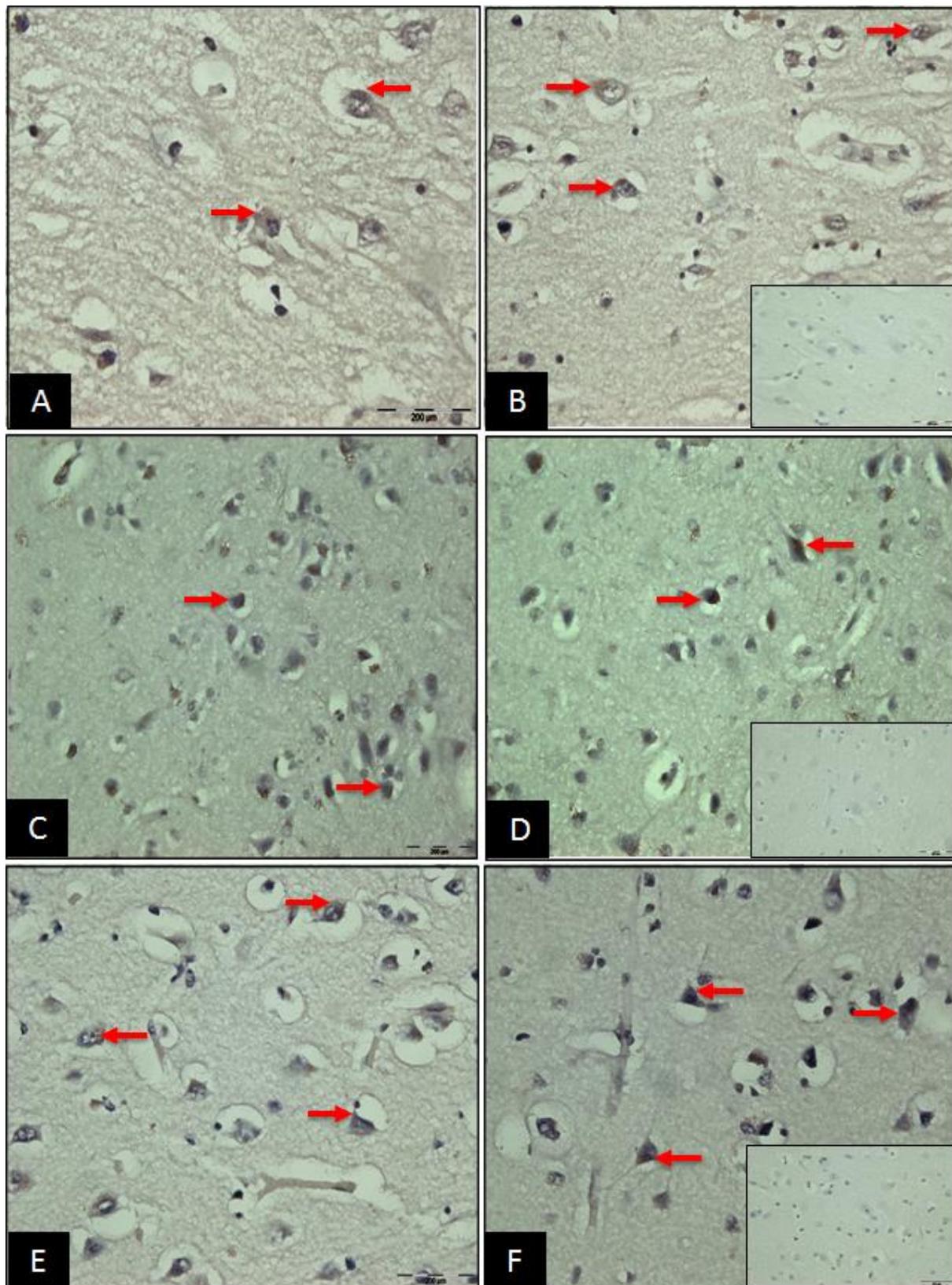


Figure 3.14 legend: The distribution of TSH-R mRNA in bipolar human brain limbic areas by *in-situ* RT-PCR

Examination of bipolar limbic areas demonstrated a sparse distribution of labelling for TSH-R mRNA in neuronal cell bodies (red arrows) of the amygdala (A and B, x400). Moderate distribution of TSH-R mRNA labelling was detected in neuronal cell bodies (red arrows) of the frontal cortex (C and D, x400) and cingulate gyrus (E and F, x400). Method controls for brain tissue showed absence of TSH-R mRNA label in limbic structures (B, D and F, insets, x400). Positive labelling for TSH-R mRNA in thyroid and brain tissues were identified as blue-black colour. Scale bars = 200µm.

Table 3.7: Semi-quantitative comparison of *in-situ* RT-PCR cellular labelling intensity and extent of regional distribution of TSH-R mRNA in normal and bipolar brain limbic regions

Experimental group	Limbic region	Cellular distribution	Visual intensity of labelling	Extent of cellular labelling
Normal	Amygdala	Neurons	3	3
	Cingulate gyrus	Neurons, axons & dendrites	3	2
	Frontal cortex	Neurons & axons	3	2
Bipolar	Amygdala	Neurons	1	2
	Cingulate gyrus	Neurons	2	3
	Frontal cortex	Neurons	2	2

Determination by 2 independent observers

Semi-quantitative grading scale of TSH-R mRNA distribution in normal and bipolar limbic areas:

- (i) Visual intensity of labelling (0) zero labelling
 - (1) low labelling
 - (2) moderate labelling
 - (3) intense labelling

- (ii) Extent of cellular labelling (0) no cells stained
 - (1) < 25%
 - (2) 25-75%
 - (3) > 75%

cingulate gyrus and frontal cortex and > 75% in the amygdala (Table 3.7). In contrast, the visual labelling intensity of TSH-R mRNA distribution in the bipolar experimental groups indicated low labelling with a score of (1) in the amygdala, whilst moderate labelling (2) was observed in the frontal cortex and cingulate gyrus (Table 3.7). The extent of cellular distribution in the amygdala and frontal cortex were observed to be within 25-75%, whilst the cingulate gyrus demonstrated labelling in > 75% of cells (Table 3.7). *In-situ* RT-PCR semi-quantitative comparisons between normal and bipolar groups therefore indicated an under-expression of the TSH-R mRNA in all bipolar limbic areas examined when compared to their matched normal limbic groups.

In other normal limbic areas (non-matched for bipolar disorder) that were examined for the distribution of TSH-R mRNA by *in-situ* RT-PCR. TSH-R mRNA was detected only in hypothalamic neurons, albeit at low labelling intensity (1), (see addendum B1 for this non-matched normal limbic data). The extent of TSH-R distribution in the hypothalamus occurred in < 25% of cells. There was no evidence of TSH-R mRNA labelling in the other areas examined (hippocampus and thalamus).

3.3 REAL-TIME QUANTITATIVE PCR OF TSH-R GENE EXPRESSION

3.3.1 TSH-R gene expression in normal versus bipolar group

Statistical analyses for the fold-change in gene expression of our target gene, TSH-R, in the bipolar group, relative to the normal control limbic group was determined. Table 3.8 presents data where the target (TSH-R) and reference (GAPDH) genes were amplified in separate wells of a 96-well microtitre plate. Figure 3.15 displays the fold-change of TSH-R gene expression in the normal and bipolar groups. The method formulated by Livak and Schmittgen (2001) was used in qPCR calculations to determine relative change in expression of the target gene (Livak and Schmittgen, 2001). The normal (control) group was considered to be the reference against which the bipolar regions were measured. The double delta C_T ($\Delta\Delta C_T$) values obtained from qPCR for each normal control was considered to be zero (0). Therefore, calculating $2^{-\Delta\Delta C_T}$ for the controls (i.e. 2^0) gave a value of one (1) for each control (normal)

Table 3.8: RTqPCR data calculations to determine TSH-R gene regulation in bipolar human limbic regions

Limbic Regions	Sample Number	TSH-R C_T Mean	GAPDH C_T Mean	Δ C_T Mean	ΔΔC_T	Fold difference 2^{-ΔΔC_T}
Amygdala (Normal)	Calibrator	36.57124	32.92617	3.645073	0	1
Cingulate (Normal)	Calibrator	9.059217	33.12026	-24.061	0	1
Frontal (Normal)	Calibrator	28.20495	32.17434	-3.96939	0	1
Amygdala (Bipolar)	1	36.86412	29.19463	7.669487	4.024414	0.061451
	2	21.92845	28.8778	-6.94935	-10.5944	1546.11
	3	35.57757	27.33126	8.246309	4.601236	0.041199
	4	36.84208	30.66835	6.173722	2.528649	0.173301
	5	38.01571	27.30877	10.70695	7.061874	0.007485
Cingulate (Bipolar)	1	26.54842	30.00072	-3.4523	20.60874	6.25E-07
	2	37.13113	28.3683	8.762827	32.82387	1.32E-10
	3	17.77519	26.7646	-8.98942	15.07163	2.9E-05
	4	36.02329	29.49221	6.531087	30.59213	6.18E-10
	5	-	-	-	-	-
Frontal (Bipolar)	1	21.73064	29.60411	-7.87347	-3.90408	14.97081
	2	36.86812	28.60583	8.262291	12.23168	0.000208
	3	28.72767	25.72587	3.001803	6.97119	0.00797
	4	35.43441	30.46318	4.971224	8.940611	0.002035
	5	35.41467	27.38278	8.031889	12.00128	0.000244

TSH-R: target gene

GAPDH: reference gene

Calibrator: normal brain limbic samples were selected as reference samples for these experiments

- : below the detection threshold limits of the real-time PCR system

C_T: threshold cycle for target amplification

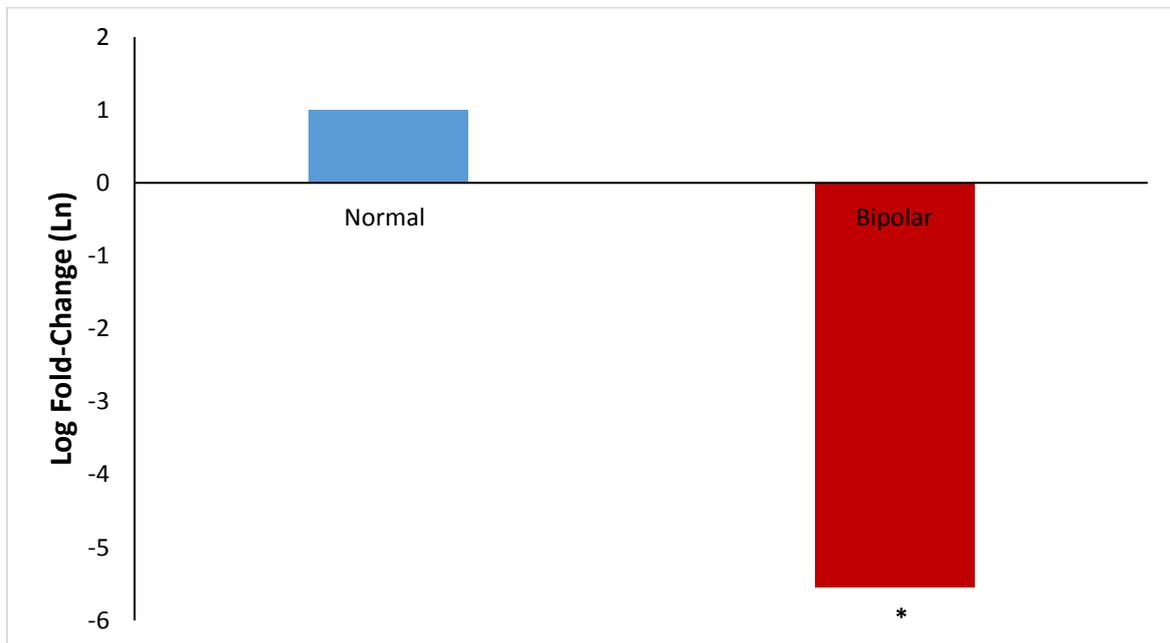
Δ C_T = C_T(Target) - C_T(Reference) i.e. C_T(TSH-R) - C_T(GAPDH)

Δ C_T Mean = Mean of (C_T(TSH-R) - C_T(GAPDH))

ΔΔC_T = Δ C_Tsample - Δ C_Tcalibrator

2^{-ΔΔC_T}: Expression fold-change of the target (TSH-R), normalised to the endogenous reference (GAPDH), and relative to the calibrator

Figure 3.15: *Log median (Ln)* fold-change of TSH-R gene expression in normal versus bipolar limbic groups



* normal vs bipolar, $p = 0.05$

Figure 3.15 legend: *Log median (Ln)* fold-change of TSH-R gene expression in normal versus bipolar limbic groups

The *Log median (Ln)* expression fold-change difference of TSH-R mRNA in normal and bipolar limbic groups were determined. Statistical comparisons demonstrates the down-regulation of TSH-R gene expression in the bipolar group when compared to the normal control group.

limbic region. Thus, the resulting $\Delta\Delta C_T$ values obtained from qPCR for each of the bipolar limbic regions were calculated as a fold-change relative to the control (equal to one).

Therefore, when compared to the normal control group, the expression levels of TSH-R in the bipolar limbic region appeared to be down-regulated (Figure 3.15). Further, statistical comparisons by *Wilcoxon rank test* between normal and bipolar categories, confirmed a significant down-regulation ($p = 0.05$).

3.3.2 Inter-regional and intra-regional differences of TSH-R gene expression in bipolar limbic areas

For gene expression in specific bipolar regions, the fold-change in gene expressions had to be compared to the expression in normal tissue (calibrated to 1 as the external control). Thus, in contrast to immuno-histochemistry (section 3.1.2) where inter-group variations could be determined within normal and bipolar tissue separately, here the inter-group comparison had to be calibrated for each bipolar limbic region against a single combined normal control. Table 3.9 provides *Log median (Ln)* quantification values for TSH-R expression fold-change differences among all matched bipolar limbic areas examined viz. amygdala, cingulate gyrus and frontal cortex. *Log median (Ln)* values obtained for this category had a range of -2.78951 to -17.7449. Bipolar inter-group statistical comparisons demonstrated a significant difference in the cingulate gyrus group ($p = 0.0037$) when compared to the amygdala (Figure 3.16). In contrast, none of the other comparisons showed appreciable statistical differences. Thus, these results demonstrate that TSH-R expression is significantly reduced in the cingulate gyrus when compared to the other two limbic groups. In addition, we obtained intra-group comparisons in all individual normal and bipolar limbic groups (Figure 3.16). Statistical analyses demonstrated a down-regulated TSH-R in all bipolar limbic groups when compared to their normal control groups.

Table 3.9: *Log median (Ln)* quantification of TSH-R gene expression fold-change differences in bipolar brain limbic areas

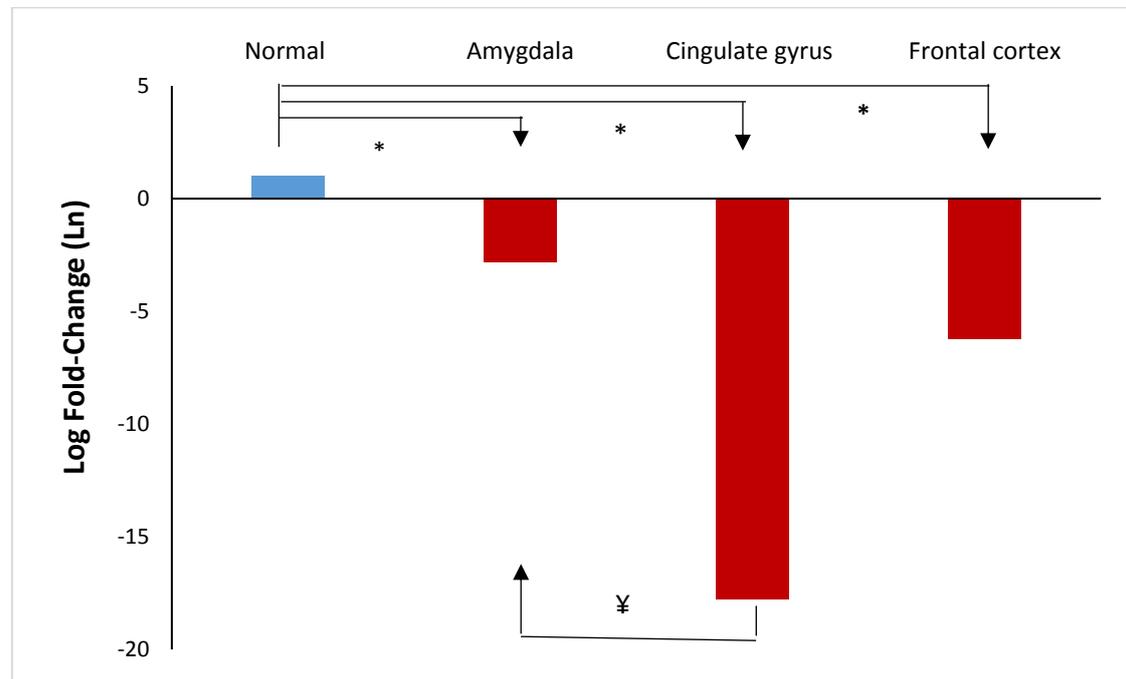
TSH-R mRNA Expression			
Limbic Areas (Bipolar)	Log Fold-change (Ln)		
	Log median (Ln)	<i>p</i> 25	<i>p</i> 75
Amygdala	-2.78951*	-3.18933	-1.75273
Cingulate gyrus	-17.7449	-21.9783	-12.3659
Frontal cortex	-6.19716	-8.31865	-4.83206

* vs cingulate gyrus, $p = 0.0037$

Inter-quartile range errors: *p*25 and *p*75

Fold-change vs normal calibrator sample (=1)

Figure 3.16: *Log median (Ln)* TSH-R gene expression fold-change differences in bipolar brain limbic areas



* vs amygdala, cingulate gyrus and frontal cortex, $p < 0.05$

¥ vs amygdala, $p = 0.0037$

Normal: calibrator samples for amygdala, cingulate gyrus and frontal cortex comparison

No error bars for individual data since each region is a comparison to the calibrator

Figure 3.16 legend: *Log median (Ln)* TSH-R gene expression fold-change differences in bipolar brain limbic areas

The *Log median (Ln)* expression fold-change differences of TSH-R mRNA among all bipolar limbic regions were determined. Bipolar inter-group statistical comparisons demonstrated a significant difference in the cingulate gyrus group when compared to the amygdala. These results illustrate that the largest down-regulation of TSH-R gene expression in bipolar limbic regions was observed in the cingulate gyrus.

Table 3.10: Overview of the results obtained for TG and TSH-R gene expression and protein localisation in matched normal and bipolar brain limbic regions

	Immuno-histochemistry				<i>in-situ</i> RT-PCR			RT-qPCR
Limbic region	TG protein		TSH-R protein		TSH-R mRNA			TSH-R expression fold-change
	Cellular location	Up-/down-regulation	Cellular location	Up-/down-regulation	Cellular location	Semi-quantitative grading	Up-/down-regulation	Up-/down-regulation
Amygdala (normal)	Vasculature *	-	Neurons (cell bodies)*	-	Neurons (cell bodies)	intense labelling > 75%	-	-
Amygdala (bipolar)	Vasculature Neurons	↓	Neurons (cell bodies)	↓	Neurons (cell bodies)	low labelling 25-75%	↓	↓*

	Immuno-histochemistry				<i>in-situ</i> RT-PCR			RT-qPCR
Limbic region	TG protein		TSH-R protein		TSH-R mRNA			TSH-R expression fold-change
	Cellular location	Up-/down-regulation	Cellular location	Up-/down-regulation	Cellular location	Semi-quantitative grading	Up-/down-regulation	Up-/down-regulation
Cingulate (normal)	Vasculature * Neurons (cell bodies, axons)*	-	Neurons *(cell bodies)	-	Neurons (cell bodies, axons, dendrites)	intense labelling 25-75%	-	-
Cingulate (bipolar)	Vasculature Neurons (cell bodies)	↓	Neurons (cell bodies)	↓	Neurons (cell bodies)	moderate labelling > 75%	↓	↓*

	Immuno-histochemistry				<i>in-situ</i> RT-PCR			RT-qPCR
Limbic region	TG protein		TSH-R protein		TSH-R mRNA			TSH-R expression fold-change
	Cellular location	Up-/down-regulation	Cellular location	Up-/down-regulation	Cellular location	Semi-quantitative grading	Up-/down-regulation	Up-/down-regulation
Frontal (normal)	Vasculature * Neurons * (cell bodies, axons)	-	Neurons * (cell bodies)	-	Neurons (cell bodies, axons)	intense labelling 25-75%	-	-
Frontal (bipolar)	Vasculature Neurons (cell bodies)	↓	Neurons (cell bodies)	↓	Neurons (cell bodies)	moderate labelling 25-75%	↓	↓*

↑ up-regulation of target gene/protein

↓ down-regulation of target gene/protein

* $p < 0.05$

ADDENDUM (B)

B.1 Semi-quantitative comparison of *in-situ* RT-PCR labelling intensity and extent of regional distribution of TSH-R mRNA in normal limbic regions that were non-matched for bipolar disorder

Experimental group	Limbic region	Cellular distribution	Visual intensity of labelling	Extent of cellular labelling
Normal	Hippocampus	Neurons	0	0
	Hypothalamus	Neurons	1	1
	Thalamus	Neurons	0	0

Determination by 2 independent observers

Semi-quantitative grading scale of TSH-R mRNA distribution in normal limbic areas (non-matched for bipolar disorder):

- (i) Visual intensity of labelling (0) zero labelling
 - (1) low labelling
 - (2) moderate labelling
 - (3) intense labelling

- (ii) Extent of cellular labelling (0) no cells stained
 - (1) < 25%
 - (2) 25-75%
 - (3) > 75%

The results shown in Table B1 of Addendum (B) are for the normal limbic regions that could not be matched with commercial bipolar tissue. However, we have included these results should there be any future project that is able to do a comparative assessment with bipolar tissue

CHAPTER 4

Discussion

Following extensive review of the literature, we report for the first time the expression and cellular distribution of thyroid-specific proteins specifically in the limbic regions of normal and bipolar human adult brains. The aim of our study was to determine whether our previous pilot findings of TG and TSH-R on other areas of normal brain could be reflected in normal human adult limbic brain at the protein level as well as a molecular level. Further, we aim to determine whether increased or decreased thyroid-specific protein expression in limbic brain is specific for bipolar disorder.

Interestingly, our comparisons obtained from protein chemistry methods indicates a significant reduction of both TSH-R and TG in limbic regions of bipolar subjects when compared to our observations in normal brain. In addition, molecular methods including *in-situ* RT-PCR and RTqPCR were also employed to confirm the down-regulation of TSH-R gene expression in the bipolar state. Immuno-reactive TSH-R mRNA and protein was detectable in the large neurons in all matched normal and bipolar brain limbic regions examined. Our findings of TSH-R in human brain tissue is consistent with the work of Prummel and colleagues (2000), who immuno-detected TSH-R proteins in folliculo-stellate cells of human anterior pituitary tissue (Prummel et al., 2000). Those authors postulated that the expression of TSH-R in the anterior pituitary gland may provide a short-loop regulatory function within the HPT axis, by modulating TSH secretion at the pituitary level. In the present study, we also demonstrate TSH-R in normal human hypothalamus, albeit at a lower concentration. Although, the scope of our investigation did not extrapolate to TSH-R localisation within the HPT axis, our observations of TSH-R in hypothalamus is consistent with the findings of Bockmann (1997), Crisanti (2001) and Bolborea (2015), (Bockmann et al., 1997, Crisanti et al., 2001, Bolborea et al., 2015). The Crisanti group identified TSH-R in hypothalamic neurons of rat brain, whilst the Bolborea group reported TSH-R gene expression in tanycytes of rat hypothalamus. The Bockmann group confirmed the expression of TSH-R in sheep hypothalamus. More specifically, we identified TSH-R in human neurons and these observations correlate with Crisanti's reports of TSH-R mRNA in a rodent neuronal cell model (Crisanti et al., 2001). Interestingly, neurons are a primary target of thyroid hormones, specifically T₃, and the effects of thyroid hormones are exerted mainly by

regulation of gene expression following T₃ binding to thyroid hormone nuclear receptors expressed in rodent brain neuronal cells as well as human cultured neurons (Freitas et al., 2010). In addition, the Crisanti group (2001) also identified TSH-R in human primary cultured astrocytes (Crisanti et al., 2001). In contrast, our observations do not include TSH-R in astrocytes or any other neuronal support cells of human limbic tissue. However, our observations did demonstrate an absence of TSH-R in astrocytes which are consistent with the findings of Carlson (1994) and Munoz (1991) (Munoz et al., 1991, Carlson et al., 1994). The Carlson group (1994) demonstrated the immuno-fluorescent localisation of thyroid hormone receptors in neurons (and oligodendrocytes) in rat brain, but not in astrocytes. In addition, the Munoz group (1991) suggested that astrocytes may not be a target of thyroid hormones since the alteration of astrocyte-specific genes, due to changes in thyroidal status, were not observed. Moreover, Carlson and colleagues (1994) suggested that any effect the thyroid hormones may have on astrocyte structure and function may be indirectly mediated by thyroid-hormone interaction with receptors expressed on non-astrocytic cells, or the result of non-nuclear mechanisms (Carlson et al., 1994).

We detected TG-like proteins in neuronal cell bodies and axons of the cingulate gyrus and some neurons of the frontal cortex in normal brain. Similarly, the bipolar cingulate gyrus and frontal cortex groups also demonstrated TG-like proteins in neurons. However, in contrast to our observations for normal amygdala, the bipolar amygdala group showed evident neuronal labelling for TG-like proteins. Interestingly, structural and functional neuro-imaging studies associates bipolar disorder with increased amygdala volume (Phillips et al., 2003, Strakowski et al., 2005) and elevated activity in the amygdala, respectively (Kruger et al., 2003, Chang et al., 2004, Lawrence et al., 2004). Our findings of TG-like proteins exclusive to bipolar amygdala neurons, is suggestive of an increase in amygdala content when compared to normal amygdala. Further, it is reasonable to speculate that the presence of TG-like proteins specifically in amygdala neurons of bipolar brain may alter neuronal transmission and functioning, thereby contributing to a hyperactive amygdala in bipolar subjects. Interestingly,

Miklowitz and Johnson (2006) suggest that elevated amygdala activity may contribute to emotional sensitivity, and these are clinical features of bipolar disorder (Miklowitz and Johnson, 2006).

Other novel findings in the present study includes TG-like proteins within the cerebral vasculature of all limbic areas in both, normal and bipolar groups. Specifically, immuno-reactive TG was localised to the smooth muscle cells of the tunica media and tunica adventitia of cerebral vasculature; however, quantification of TG-staining intensity in cerebral vasculature indicated a significant reduction in the bipolar groups compared with the normal groups. Our observations of TG-like proteins in smooth muscle of cerebral vasculature is supported by the work of Ojamaa and colleagues (1996) where those authors described the effects of thyroid hormones on vascular smooth muscle cells of rat aorta (Ojamaa et al., 1996). Specifically, they reported on the vasodilatory effects of T₃ when it acts directly on vascular smooth muscle cells *in vitro* to cause rapid relaxation of coronary arteries. Similarly, Mizuma and colleagues (2001) demonstrated the local T₃ production by DII activation in human vascular smooth muscle cells (Mizuma et al., 2001). Further, the significant role of mammalian thyroid hormones in the development, homeostasis and regeneration of skeletal muscle has been well-documented (Ramsay 1966, Nwoye et al., 1982, Yu et al., 2000, Clement et al., 2002, Udayakumar et al., 2005, Brent, 2012). In addition, muscular alterations that occur secondary to thyroid dysfunction (including hypo-thyroidism and hyper-thyroidism) has been described previously, further illustrating the important role of thyroid hormones in mammalian muscle physiology (Ramsay, 1966, Nwoye et al., 1982, Yu et al., 2000, Clement et al., 2002, Udayakumar et al., 2005, Brent, 2012).

Our findings of TG-like proteins in cerebral vasculature of human brain together with our postulate that circulating anti-thyroid antibodies may bind limbic targets during AITD, suggests the migratory potential of these proteins across the seemingly-impervious BBB. In the recent past, there has been much controversy generated around the findings of non-CNS proteins and enzymes in brain areas, thus, appearing to breach the seemingly-impervious BBB. Our study has also faced some criticism with

regards to the presence of thyroid-associated proteins in the human limbic system. Thus, we provide in the following paragraphs a detailed description of numerous studies that demonstrate translocation of non-CNS agents via the BBB. Multiple modes of egress beyond the BBB have been described, whereby the integrity of the BBB may be altered during neuro-pathological diseases including Alzheimer's disease, HIV-associated dementia, multiple sclerosis, and bipolar disorder (Lou et al., 1997, Minagar et al., 2002, Patel and Frey, 2015). Alteration of the protective BBB increases its permeability to leukocytes and other circulating compounds. The consequential influx of leukocytes triggers signal transduction mechanisms that facilitate the loss of tight junction proteins that constitute the highly specialised cerebral endothelium of the BBB (Bolton et al., 1998). Normally, the BBB restricts the entry of endogenous IgG from plasma, as well as exogenously-administered monoclonal antibodies (Rojko and Price-Schiavi, 2008). However, one study by Terasaki and Ohtsuki (2005), reported that the BBB can be breached by endogenous IgG and some larger hydrophilic molecules by the brain-to-blood transport mechanism (Terasaki and Ohtsuki, 2005). Their proposed mechanism involves several transporters that are located within the brain capillary endothelial cells of the BBB whose function is to reduce accumulating levels of hydrophilic endogenous molecules generated within the brain. More recently, the translocation of large macromolecules such as amyloid- β protein into mice CNS has also been described (Bu et al., 2017), whereby it induces Alzheimer's disease-related pathologies.

Further evidence of BBB breach occurs via transferrin receptor molecules that are expressed in high concentration by brain capillary endothelial cells of the BBB, and which can be targeted by specific monoclonal antibodies, as described in a rat model (Pardridge et al., 1991). It is thought that these anti-transferrin antibodies could function as potential drug transport vectors across the BBB and into the CNS. The process of cellular uptake of antibodies into the brain is known as receptor-mediated transcytosis. Moreover, St-Amour and co-workers (2013) reported the migration of up to 0.1% of peripherally-administered IgG into mice brain parenchyma (St-Amour et al., 2013). Further reports indicate that antibodies traverse the BBB by penetrating the CSF and then progressing through the

ventricular circulation to reside in brain parenchymal regions (Berer et al., 2011, Saunders et al., 2012). Lastly, whilst the BBB is known to protect brain parenchymal regions, it is unable to protect the meningeal tissue, since the meninges lack a barrier (Bard et al., 2000). Thus, there appears to be an accumulation of evidence that illustrate the probability of antibodies such as those in AITD crossing the BBB and affecting various cerebral regions.

There are reports that suggest an association between BBB disruption and bipolar disorder (Patel and Frey, 2015), and these interactions bear relevance to the present study. Similar to the major neuro-psychiatric disorders, bipolar disorder also involves inflammatory processes. Several reports also suggest an association between bipolar disorder and increased inflammation as well as oxidative stress (Patel and Frey, 2015). Increased neuro-inflammation is believed to facilitate cognitive decline as well as gray and white-matter abnormalities in bipolar patients (Patel and Frey, 2015). Studies have demonstrated increased levels of pro-inflammatory cytokines (such as IL-1, IL-6 and TNF α) in plasma (Stertz et al., 2013, Haarman et al., 2014) and an abnormal expression of pro-inflammatory genes in circulating monocytes (Padmos et al., 2008). In addition, psychotropic agents have been shown to have modulatory effects on the immune system (Drzyzga et al., 2006, Haarman et al., 2014). Further, “activation of the immune system is linked with neuro-inflammation through activation of microglia which is a central player in neuro-inflammatory pathways” (Patel and Frey, 2015). It has also been demonstrated that neuro-inflammation is linked with increased microglial activity within the hippocampal brain regions of bipolar patients (Stertz et al., 2013). In addition, decreased expression of growth-associated proteins as well as oxidative damage to RNA have roles in neuro-inflammation and interestingly, these have been observed in the hippocampal regions of bipolar patients (Kato et al., 2003, Tian et al., 2007, Che et al., 2010). The oxidative stress reported in bipolar disorder is thought to be due to mitochondrial alterations (Scola et al., 2013) as well as the increased levels of circulating markers of oxidative damage to lipids, RNA and DNA in bipolar patients (Andreazza et al., 2008, Brown et al., 2014).

Interestingly in bipolar disorder research, there is growing evidence that available treatment such as mood-stabilising and anti-depressant agents may reverse or prevent the effects of inflammation and oxidative stress (Patel and Frey, 2015). Lithium has been shown to protect against the effects of oxidative stress and reactive oxygen species (ROS)-induced damage, thus preserving the BBB integrity (Gonzalez-Mariscal et al., 2008, Maurer et al., 2009, Cummins, 2012, Lochhead et al., 2012). Further, chronic lithium treatment down-regulates the arachidonic acid-prostaglandins pathway (Murphy et al., 1973, Karmazyn et al., 1978) which has implications in neuro-inflammation (Chang et al., 1996, Rao et al., 2008). In addition, several other studies have demonstrated how lithium treatment significantly increased the levels of anti-inflammatory cytokines (Sztejn et al., 1987, Kucharz et al., 1993, Rapaport et al., 1999, Ballanger et al., 2008, Tay et al., 2012, Agrawal et al., 2013), whilst attenuating levels of pro-inflammatory cytokines (Boufidou et al., 2004, Beurel and Jope, 2009, Wang et al., 2009, Zhang et al., 2009, Tan et al., 2010, Green and Nolan, 2012, Rowse et al., 2012, Tay et al., 2012, Agrawal et al., 2013, Chen et al., 2013, Himmerich et al., 2013, Wang et al., 2013).

There does appear to be a strong link between oligodendrocyte dysfunction with accompanying myelin damage in bipolar disorder. Studies have also demonstrated alterations in oligodendrocyte structure and function in bipolar disorder (Patel and Frey, 2015). “Oligodendrocytes facilitates the formation and stability of neural circuits by insulating axons with myelin sheath” (Patel and Frey, 2015). The down-regulation of oligodendrocyte-specific markers were reported in the brain of bipolar subjects (Konradi et al., 2012). In addition, Uranova and colleagues (2001) demonstrated possible apoptosis and necrosis of oligodendrocyte cells in prefrontal cortical areas of bipolar patients (Uranova et al., 2001). Previously these authors had reported decreased levels in oligodendrocyte density within layer VI of bipolar patients thus further implicating the disruption of oligodendrocytes in the pathophysiology of bipolar disorder (Orlovskaya et al., 2000). In addition, several other studies have demonstrated myelin abnormalities in bipolar subjects (Aston et al., 2005, Sokolov, 2007, Heng et al., 2010, Herring and Konradi, 2011, Konradi et al., 2012).

In summary, increased peripheral inflammation, oxidative stress as well as white matter and oligodendrocyte alterations have been implicated in the pathogenesis of bipolar disorder (Patel and Frey, 2015). Further, treatment for bipolar disorder such as lithium has been shown to modulate the effects of inflammatory and oxidative stress pathways (Patel and Frey, 2015). Recent neuro-imaging and post-mortem studies have reported increased neuro-inflammation during bipolar disorder through elevated microglial activation (Patel and Frey, 2015). The microglia along with other neuronal support cells, i.e. oligodendrocytes and astrocytes lie in close proximity to the BBB. Taking this into consideration along with expanding reports suggesting BBB disruption in various other neuro-psychiatric conditions, Patel and Frey (2015) have proposed a novel model that depicts BBB disruption with consequent permeability increase in bipolar disorder (Figure 4.1), (Patel and Frey, 2015).

It appears that BBB disturbance can occur during various neuro-pathological conditions including bipolar disorder, and this supports our present postulate that circulating auto-antibodies (as well as TG-like proteins) gain ingress into the CNS to bind brain limbic targets. The Patel and Frey (2015) model suggests that loss of BBB integrity (transient or persistent) may lead to an influx of pro-inflammatory molecules such as cytokines and ROS from the peripheral blood into the brain. This influx will trigger the activation of localised microglial cells which together with ROS would promote neuro-inflammatory processes, by inducing damage to nearby oligodendrocytes and the resulting myelin sheath, thereby compromising myelination and the integrity of the neural circuit.

A study by Rybakowski and colleagues (2013), provides support for the proposed model in bipolar disorder, where those authors report increased levels of matrix metalloproteinase-9 (MMP9), which promotes increased BBB permeability during bipolar depression, by aiding proteolysis of the basal lamina, tight junctions and extracellular matrix that constitute the protective BBB

Figure 4.1: Novel model depicting BBB disruption in bipolar disorder. Adapted from Patel and Frey (2015)

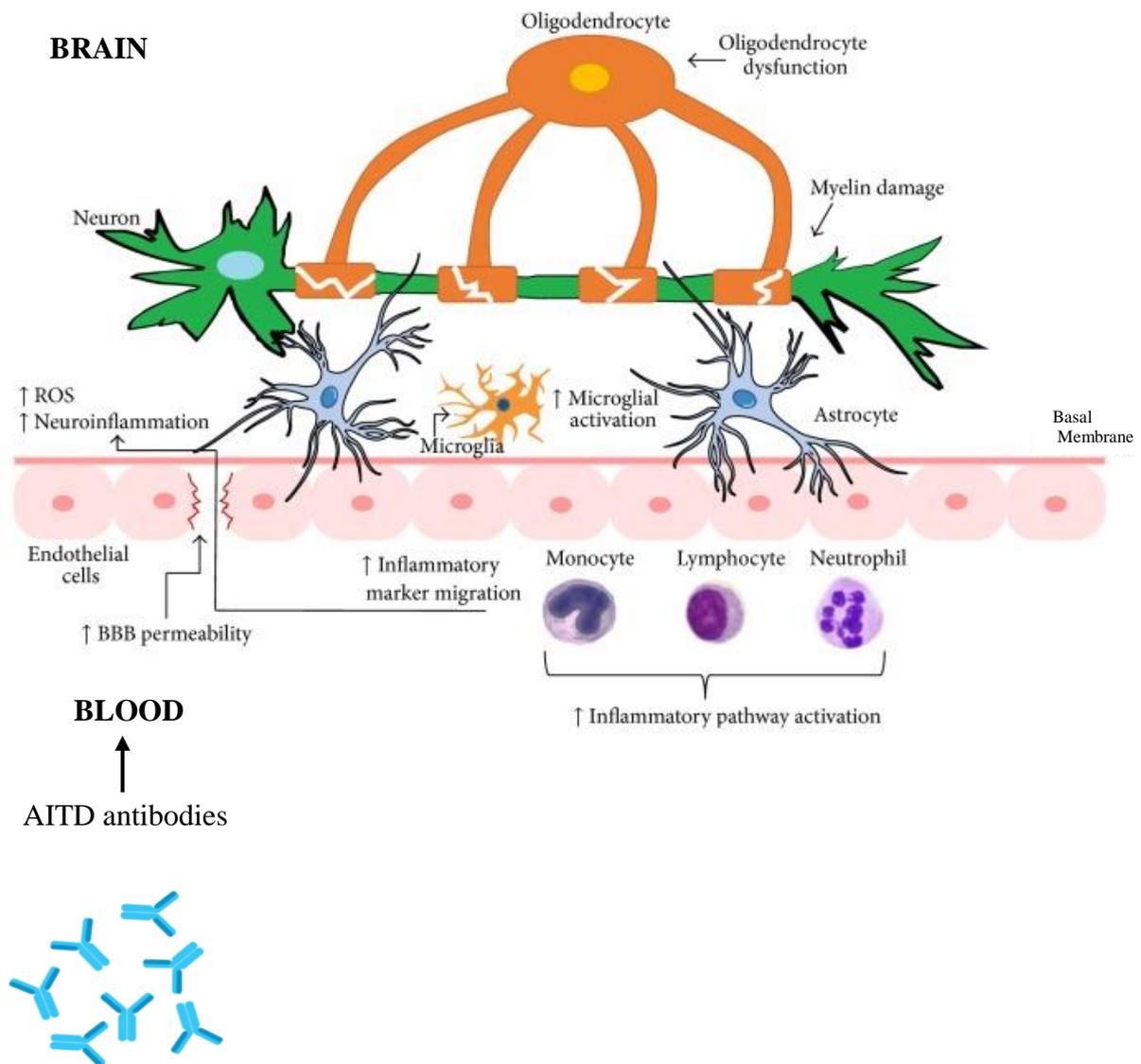


Figure 4.1 legend: Novel model depicting BBB disruption in bipolar disorder

The loss of BBB integrity in bipolar disorder, may increase the permeability of BBB to circulating inflammatory molecules such as cytokines and ROS, into the brain. This influx will activate nearby microglial cells thus facilitating neuro-inflammatory processes by inducing localised oligodendrocyte damage and thereby compromising the process of myelination.

(Rybakowski et al., 2013). Further, Goldstein and colleagues (2009) report increased levels of pro-inflammatory cytokines during the manic and depressive episodes, thereby suggesting increased BBB permeability during these major episodes in bipolar patients (Goldstein et al., 2009).

It is well-documented that thyroid hormones (T_3 and T_4) mediate their effects in the CNS by binding to thyroid hormone receptors that are widely distributed within predominant cell types of the mammalian CNS including neurons, astrocytes and oligodendrocytes (Mellstrom et al., 1991, Strait et al., 1991, Bradley et al., 1992, Carlson et al., 1994, Carlson et al., 1996, Strait et al., 1997, Carre et al., 1998, Ahmed et al., 2008). There are two major CNS isoforms of thyroid hormone receptors, viz. $TR\alpha 1$ and $TR\beta 1$ (Chan and Privalsky, 2009, Shroeder and Privalsky, 2014). Whilst $TR\alpha 1$ is expressed during early embryonic development and thereafter widely distributed in the adult brain, in contrast, $TR\beta 1$ occurs in the latter stages of embryonic development and demonstrates restricted-tissue expression in the adult brain (Calvo et al., 1990, Murata, 1998, Forrest and Vennstrom, 2000, Zhang and Lazar, 2000, Wondisford, 2003, Cheng, 2005, Chatonnet et al., 2013). Functionally, thyroid hormones have critical roles in mammalian foetal and post-natal brain development and maturation whereby they affect neuronal processing and integration, myelination, proliferation of glial cells, and the synthesis of important enzymes essential for neurotransmission (Porterfield and Hendrich, 1993, Bernal and Nunez, 1995, Morreale de Escobar et al., 2004, Wallis et al., 2010). In the developing brain, thyroid hormones are also involved in the regulation of energy metabolism (Bernal and Nunez et al., 1995). Severe thyroid hormone deficiency during the early developmental stages is associated with structural and functional abnormalities that results in various neurological and behavioural impairments including cretinism (Bernal and Nunez et al., 1995). In the adult mammalian brain, thyroid hormones have been reported to regulate energy metabolism (Lindvall-Axelsson et al., 1985, Atterwill and Collins, 1987, St Germain and Galton, 1997) and influence gene expression (Ceccatelli et al., 1992, Giordano et al., 1992, Iniguez et al., 1992). Further, in adults, altered thyroidal status appears to correlate with various neurological and behavioural conditions which can then be corrected with proper circulating thyroid hormone adjustment (Krishnan, 2005, Whybrow and Bauer, 2005a, Whybrow and Bauer, 2005b,

Bernal, 2007). It has also been reported that circulating thyroid hormone levels decrease as a function of age in humans (Chakraborti et al., 1999, Hertoghe, 2005) and rodents (Cao et al., 2012). Interestingly, neurogenesis is also known to decrease with age (Enwere et al., 2004, Gould et al., 1999, Kuhn et al., 1996) and this has been attributed to the vital role of thyroid hormones in adult neurogenesis (Lemkine et al., 2005). Therefore, decreasing age-related thyroid hormone levels with a consequent reduction in neurogenesis may be associated with adverse cognitive effects reported in adults (Flamant et al., 2015). Thus, the importance of thyroid hormones, T₃ and T₄, in mammalian CNS during various life stages is well-established. It is important to bear in mind that our commercial bipolar samples were obtained from older individuals than the normal controls. This may influence the functional capacity of circulating thyroid levels in bipolar individuals. In the current project, however, we chose to consider age-related physiological endocrine function separate from the influence on neuropathology such as in bipolar-related AITD since the focus of the project was the co-incidence of thyroid-related factors in this disease rather than the regulation associated with changes in endocrine levels. In the present study, we demonstrate thyroid-related proteins, TSH-R and TG in limbic structures of normal human adult brain (Naicker and Naidoo, 2017). Limbic-derived TSH-R/TG may provide an alternate neuro-physiological purpose, specifically related to mood control. In addition, we provide evidence demonstrating down-regulated TSH-R and TG protein expression in matched bipolar limbic regions. Thus, we may indeed speculate that a reduced expression of TSH-R and TG in limbic structures (e.g. neurons) will result in reduced thyroid function in the adult brain, which may then predispose or exhibit some involvement in the pathophysiology of mood dysregulation. In the following paragraphs we provide a detailed description of previous studies that suggest potential neuro-functional roles of TSH-R and how these correlate with our present findings.

In the thyroid gland, the TSH-R is located on the basolateral surface of thyroid follicular cells and is regulated by TSH that is secreted by the thyrotropes of the anterior pituitary gland, thereby functioning as the main pituitary regulator of thyroid hormone synthesis (Landek and Caturegli, 2009). TSH binds to, and agonises TSH-R causing an increase in intracellular cAMP followed by the stimulation of

secondary messenger systems for the synthesis of thyroid hormones, T₃ and T₄. Our findings of TSH-R expression in limbic structures suggests that TSH does not only serve as a hormonal messenger for the thyroid gland, but may also have a neuro-functional role in human limbic brain. We speculate that TSH may act directly on limbic components by first binding TSH-R (we were the first to report TSH-R in limbic neurons, Naicker and Naidoo, 2017). Similarly, as described in thyroid physiology, the interaction between TSH and TSH-R may stimulate intra-cellular signalling (Landek and Caturegli, 2009); however, the precise role of TSH in human limbic brain remains unknown. We can further speculate that the presence of functional TSH-R in limbic brain could provide an additional short-loop feedback mechanism on the neuro-endocrine control of the thyroid gland; hence, the regulation of thyroid hormone synthesis. Our postulate does not appear to be so far-fetched if one considers other seemingly-improbable theories. One such is the potential role of brain-derived TSH-R which was proposed by Hosaka and co-workers (1992) where those authors related the increase in GLUT1 glucose transporter mRNA, along with an increase in GLUT1 protein and 2-deoxyglucose transport activity, to the interaction between TSH and TSH-R on cAMP dependent pathways (Hosaka et al., 1992). Further, Morgello and co-workers (1995) performed electron-microscopy studies to localise GLUT1 to the CNS endothelium and in astrocytes that constitute the BBB of gray matter in humans and monkeys (Morgello et al., 1995). The site of location of GLUT1 suggests its involvement in glucose and glucose metabolite transport to astrocytic and neuronal areas. Thus, there appears to be evidence that supports the work of the Hosaka group (1992) that proposes a direct role of TSH in the regulation and facilitation of glucose uptake into brain cells, where glucose is an important source of energy (Hosaka et al., 1992). Our present study results demonstrate a significant reduction of TSH-R in limbic neurons of bipolar brain. We can, therefore, speculate that a reduced expression of TSH-R in limbic neurons will likely result in a decreased ligand-receptor interaction and this may have a direct effect on the uptake of glucose for utilisation by neuronal cells, thereby contributing to altered neuronal functioning within the mood-regulatory centres of the human brain. Altered neuronal functioning in mood control regions may be associated with disturbed mood in these patients. Interestingly, this correlates with previous reports describing neuro-inflammation due to microglial alterations in depressed and suicidal bipolar subjects, where microglial alterations may lead to reduced glucose uptake

and metabolism, thus directly effecting normal neuronal functioning in these patients (Ongur et al., 1998, Cotter et al., 2002, Steiner et al., 2008, Holmes et al., 2017).

An alternative clinically-related school of thought is that the presence of TSH-R in limbic brain may provide an extra-thyroidal target for auto-immunity. The evidence is particularly convincing when one considers the paradigms resulting from the following studies: Patients with AITD display immuno-reactivity directed against thyroid factors such as TSH-R, TG and TPO, and can present as either hyper-thyroidism or hypo-thyroidism (Davies et al., 2002, Ai et al., 2003, Parvathaneni et al., 2012). During hyper-thyroidism (Grave's disease), stimulatory-type anti-thyroid antibodies bind to TSH-R causing an over-activity of the thyroid gland resulting in the classical goiter presentation (Davies et al., 2002, Szkudlinski et al., 2002, Ai et al., 2003). In contrast, the inhibitory-type anti-thyroid antibodies bind to TSH-R in hypo-thyroidism (Hashimoto's disease), causing cell-mediated destruction of thyroid follicular cells thus resulting in thyroid follicle atrophy. As a result, this leads to reduced TSH-R, TG, TPO, with a compensatory rise in TSH, a classical hallmark for clinical hypo-thyroidism (Ai et al., 2003, Jameson and Weetman, 2005). Similarly, we can speculate that during AITD, especially in Hashimoto's disease (the most common form of hypo-thyroidism), inhibitory-type auto-antibodies may recognise and agonise TSH-R expressed in limbic neurons. This interaction may well lead to cell-mediated destruction or inactivation of limbic neurons with a consequential reduction in the levels of expressed TSH-R. Interestingly, in the present study, we report the reduced expression of TSH-R in limbic neurons of bipolar brains. It is therefore possible that a loss of limbic neurons or diminished neuronal activity in limbic regions (due to thyroid auto-immunity such as in Hashimoto's disease) may indeed contribute towards the development of symptoms of mood dysregulation as observed in bipolar disorder (Figure 4.2). Alternatively, the consequential loss of TSH-R (i.e. loss of localised thyroid function), due to limbic neuronal damage, will result in decreased ligand-receptor interaction. As discussed in the previous paragraph, this decreased ligand-receptor interaction may lead to reduced glucose uptake into limbic brain cells, thus depriving limbic regions of an energy source and creating an imbalance affecting mood regulation.

Figure 4.2: Overview of how the study postulate relates to the results

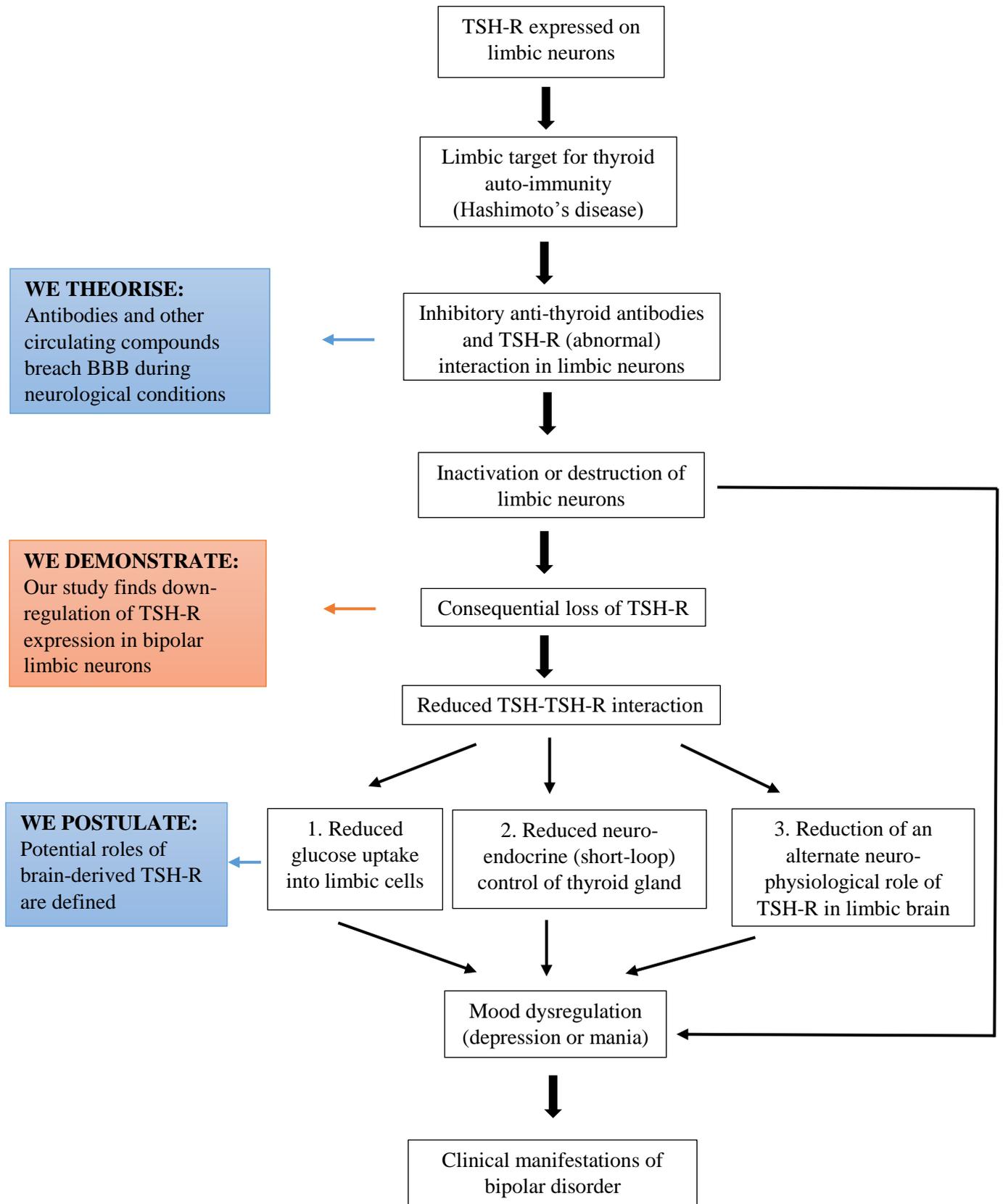


Figure 4.2 legend: Overview of how the study postulate relates to the results

The expression of thyroid-specific proteins, associated with Hashimoto's disease, in limbic neurons of human bipolar brain provides an extra-thyroidal target for auto-immunity. The abnormal association between inhibitory-type auto-antibodies and thyroid-specific receptors may inactivate or destroy limbic neurons with consequential reduction of limbic-derived TSH-R. Both, loss of normal neuronal functioning and decreased TSH-TSH-R interaction in limbic regions, may predispose to mood dysregulation and bipolar disorder.

Interestingly, intra-group comparisons obtained for normal and bipolar limbic brain in the present study, demonstrate a significant down-regulated expression of thyroid proteins (TSH-R and TG) in all bipolar limbic areas. This reduction of thyroid factors in bipolar brain suggests that these thyroid-specific proteins may exhibit a much greater role in neuro-physiology in addition to its primary role in the regulation of the synthesis of thyroid hormones (T₄ and T₃) in the thyroid gland. Since the limbic brain is responsible for mood and behaviour control, we postulate that a significant reduction of thyroid protein concentration in human limbic brain may be associated with developing symptoms of mood dysregulation (i.e. depression and/or mania), the classical manifestations of bipolar disorder. Our present findings therefore implicate a significant, yet unknown role of the thyroid system and thyroid auto-immunity in the development of bipolar disorder.

There are some other theories that also support an association between bipolar disorder and anti-thyroid antibodies: Firstly, the anti-thyroid antibodies may present initially as a marker for sub-clinical hypo-thyroidism and may predispose patients to specific types of bipolar disorder (Haggerty et al., 1997). Cowdry (1983) and Bauer (1990a) have reported increased prevalences of sub-clinical and overt hypo-thyroidism in patients with the rapid-cycling type of bipolar disorder (Cowdry et al., 1983, Bauer et al., 1990a). However, the mechanism by which these patients develop bipolar disorder is unclear. Secondly, an association may exist “between the predisposition to bipolar disorder and the predisposition to thyroid auto-immunity” (Haggerty et al., 1997). This arises from evidence that bipolar disorder has been previously associated with other auto-immune diseases including multiple sclerosis (Schiffer et al., 1988, Haggerty et al., 1997). Thirdly, a linked predisposition together with the effects of a thyroid hormone deficit could determine the clinical course of the bipolar disorder. Thus, bipolar disorder may predispose to auto-immunity, and those patients that develop a thyroid auto-immune disorder will most likely display a gradual decline in thyroid function. This low thyroid capacity could influence whether the bipolar patient develops predominantly depressed or mixed symptoms (Haggerty et al., 1997).

Bipolar disorder is not considered to be a disease caused by neuro-degeneration. Unlike conventional neuro-degenerative disorders which are characterised by both neuronal loss as well as glial cell pathology, the clinical manifestations of bipolar disorder are mainly attributed to glial cell abnormalities rather than neuronal pathology (Muneer et al., 2016). However, several reports from neuro-imaging studies provide evidence of both structural (Rajkowska et al., 2001, Lopez-Larsen et al., 2002, Phillips et al., 2003, DelBello et al., 2004, Lochhead et al., 2004, Lyoo et al., 2004, Strakowski et al., 2005, Abe et al., 2015) and functional (Kruger et al., 2003, Chang et al., 2004, Lawrence et al., 2004, Lennox et al., 2004, Mayberg et al., 2004, Townsend et al., 2013) abnormalities in key limbic regions of bipolar subjects (discussed in greater detail in subsequent paragraphs), (Figure 4.3). In parallel with neuro-imaging reports, biochemical studies have reported neuronal alterations in limbic regions of bipolar subjects by investigating important neuronal metabolic marker measurements that indicate neuronal loss or dysfunction during bipolar disorder (Moore et al., 2000, Winsberg et al., 2000, Cecil et al., 2002). Altogether, these various findings (discussed in greater detail below) suggest degeneration within key limbic regions and this may be associated with clinical symptoms of bipolar disorder. Interestingly, our findings of a down-regulated expression of TSH-R and TG in major limbic regions of human bipolar brain correlates with neuro-imaging reports that associates bipolar disorder with both structural and functional alterations to these specific brain regions that are important in regulating emotion (Figure 4.3). Moreover, we have also localised this reduction of thyroid proteins in neurons. Further, we suggest the possibility of a yet unknown neuro-physiological role of thyroid-specific proteins in limbic regions of normal human brain (Naicker and Naidoo, 2017). Thus, it is reasonable to assume that reduction of thyroid-specific proteins in limbic neurons affects normal neuronal functioning in limbic brain which may predispose to mood disturbance (Figure 4.3).

An important finding in several functional imaging studies in bipolar disorder demonstrates elevated activity in the amygdala (Kruger et al., 2003, Chang et al., 2004, Lawrence et al., 2004). The amygdala is “a key brain structure involved in identifying the significance of emotionally relevant stimuli of both negative and positive valence” (Miklowitz and Johnson, 2006). Hence, amygdala hyperactivity appears

Figure 4.3: Evidence of degeneration of limbic structures in bipolar disorder

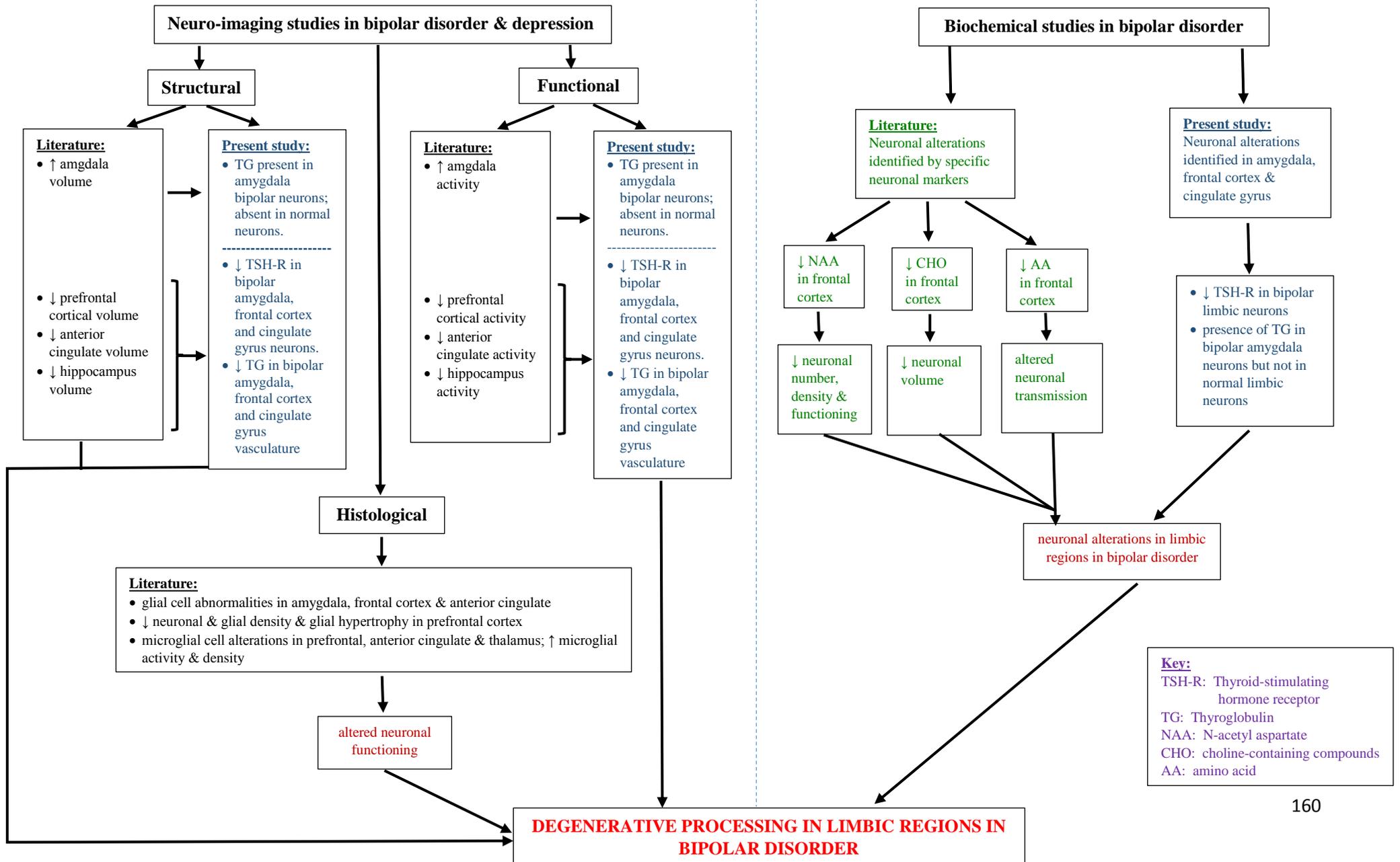


Figure 4.3 legend: Evidence of degeneration of limbic structures in bipolar disorder

Structural, functional and histological neuro-imaging studies provide evidence that suggest neuro-degeneration within major limbic regions of bipolar brain (black font). Other biochemical studies indicate neuronal alterations in bipolar disorder by specific neuronal marker determinations (green font). The present study also suggests neuronal alterations indicated by reduced levels of expressed thyroid-specific protein receptors in bipolar limbic neurons (blue font). Further, present study findings correlate with structural and functional imaging studies (blue font). Altogether, these various study findings indicate that bipolar disorder may be associated with neuro-degeneration in key limbic regions (red font).

to be associated with enhanced emotional sensitivity. Interestingly, whilst we did not observe TG-like protein in limbic neurons of normal amygdala in the current study, we did find evidence of TG-like proteins in neurons in the bipolar amygdala group. The current study did not include neuro-physiological studies; however, we may speculate that the presence of TG-like proteins in bipolar limbic neurons may alter normal neuronal functioning.

Other interesting functional observations in limbic regions of bipolar subjects include the decreased neuro-physiological activity of the hippocampus and prefrontal cortex (Kruger et al., 2003), whilst decreased activity in the anterior cingulate was reported in clinically-depressed patients (Mayberg et al., 2004). It has been suggested that the decreased activity of these cortico-limbic regions hinders “effective planning and goal pursuit in relation to emotion, thus resulting in a reduced capacity to regulate emotion” (Miklowitz and Johnson, 2006). This, then, allows us to speculate that our findings of reduced TSH-R/TG protein expression in cortico-limbic neurons of bipolar brain, may be a contributing factor for the decreased neuro-physiological activity within limbic regions in bipolar disorder. Our speculation is based on reports indicating neurons as a primary target for thyroid hormones, specifically T₃, (Freitas et al., 2010) and that the effects of thyroid hormones are exerted mainly by regulation of gene expression following binding to thyroid hormone receptors that are widely expressed in neurons, as well as astrocytes and oligodendrocytes of mammalian CNS (Mellstrom et al., 1991, Strait et al., 1991, Bradley et al., 1992, Carlson et al., 1994, Carlson et al., 1996, Strait et al., 1997, Carre et al., 1998, Ahmed et al., 2008).

Curiously, the neural activity in limbic regions appears to alter according to “episode status” in bipolar disorder (Lennox et al., 2004). In fact, during the manic phase of bipolar disorder, patients demonstrate a decreased reactivity to negative stimuli when compared with healthy or euthymic control subjects (Lennox et al., 2004). In that study, the authors identified decreased activation of the amygdala and subgenul anterior cingulate in manic subjects. Hence, it appears that during manic episodes, the level

of activity in limbic regions that is responsible for identifying negative stimuli and then regulating appropriate responses, becomes reduced (Lennox et al., 2004). A more recent neuro-imaging study by Abe and co-workers (2015) correlated decreased cortico-frontal volume with the manic phase of bipolar disorder (Abe et al., 2015). Moreover, neuro-imaging studies by Townsend and co-workers (2013) investigating the functioning of the neural network during down-regulated emotion in euthymic bipolar subjects demonstrated the hypo-activation of the ventral lateral prefrontal cortex in these subjects (Townsend et al., 2013). Those authors related their findings of reduced frontal connectivity in bipolar disorder to decreased emotion-regulation and predisposition towards acute mood-state relapse. Thus, they were able to report that “ventral lateral prefrontal cortex inhibitory inputs to the amygdalae may be abnormal in bipolar disorder due to local alterations (e.g. neuronal) and/or disrupted connections (e.g. white matter tracks) between regions” (Townsend et al., 2013). Interestingly, in the present study we have also identified neuronal alterations in these limbic regions of bipolar subjects, whereby the expression of TSH-R was significantly down-regulated in bipolar limbic neurons when compared with normal limbic brain. In addition, we found TG-like proteins in neurons of bipolar amygdala but not in neurons of normal amygdala and this may possibly relate to an enhanced emotional sensitivity.

Numerous previous studies have examined functional and structural imaging in bipolar disorder. Interestingly, many findings indicate structural brain abnormalities in bipolar disorder that positively correlate with observations in functional studies. These include a smaller-than-average volume of the prefrontal cortex, hippocampus and anterior cingulate, whilst the amygdala demonstrates an above-average volume (Phillips et al., 2003, Strakowski et al., 2005). In contrast, DelBello and co-workers (2004) reported decreased amygdala volumes in bipolar adolescents (DelBello et al., 2004). “These findings suggest that amygdala enlargement may therefore be a consequence of abnormal development of this structure during adolescence and early adulthood” (Strakowski et al., 2005). Further related studies of the bipolar prefrontal cortex has demonstrated reduced volumes of gray matter in prefrontal cortical regions (Lopez-Larsen et al., 2002, Lochhead et al., 2004, Lyoo et al., 2004, Strakowski et al., 2005). These neuro-anatomical abnormalities in the prefrontal cortex are consistent

with prefrontal histological abnormalities that have been reported in bipolar patients and which included decreased glial and neuronal density with glial hypertrophy (Rajkowska et al., 2001, Strakowski et al., 2005). Glia (astrocytes, oligodendrocytes and microglia) abnormalities have also been identified in other limbic regions, including the anterior cingulate gyrus, orbito-frontal cortex and amygdala of untreated bipolar patients (Rajkowska et al., 2001); however, upon treatment with lithium, the loss of glia appear to be reduced (Bowley et al., 2002). The diminished volumes of the cortico-limbic regions in bipolar disorder positively correlate with our findings demonstrating a reduced expression of thyroid proteins in these regions. Similarly, previous findings of an enlarged amygdala in bipolar disorder (Phillips et al., 2003, Strakowski et al., 2005), correlate well with our demonstration of TG protein in amygdala neurons of the bipolar brain and its absence in normal controls. Further, whilst previous structural imaging studies have described glial cell abnormalities in bipolar disorder, we did not detect thyroid proteins in any neuronal support cells including oligodendrocytes, astrocytes, neuroglia and satellite cells, in both normal and bipolar limbic areas.

In a major post-mortem study conducted by Steiner and co-workers (2008), the possible role of microglial cells in the pathophysiology of suicidal mood disorders, including bipolar disorder, was investigated (Steiner et al., 2008). Those authors demonstrated elevated microglial density in the prefrontal cortex, anterior cingulate gyrus and thalamus of suicidal patients. Further, the critical role of microglia in inflammation of the CNS is well-documented (Stertz et al., 2013, Watkins et al., 2014). Thus, it has been proposed that the association between suicidality and neuro-inflammation is caused by microglial cell alterations. More recently, Holmes and co-workers (2017) positively correlated neuro-inflammation (attributed to increased microglial activity) in depressed patients to suicidal tendencies (Holmes et al., 2017). Further, that group identified the highest microglial activity in the anterior cingulate cortex. “Glia affect several processes, including regulation of extracellular potassium, glucose storage and metabolism, and glutamate uptake, all of which are crucial for normal neuronal activity” (Ongur et al., 1998). Considering the important influence that glial cells have on

neurons, it is presumed that a glial cell deficit would have negative consequences on normal neuronal functioning (Cotter et al., 2002).

Previous studies have used proton magnetic resonance spectroscopy (MRS) to describe altered neurochemical and metabolic markers for neuronal volume and functioning in cortico-limbic regions of bipolar subjects, in the implication of neuro-degenerative processing in bipolar disorder (Moore et al., 2000, Winsberg et al., 2000, Cecil et al., 2002). Those studies report a decrease in N-acetyl aspartate (NAA) concentrations in bipolar frontal-gray matter; NAA is an amino acid that is highly concentrated in neurons and believed to relay information on neuronal viability (Winsberg et al., 2000, Cecil et al., 2002). Hence, low concentrations of an important neuronal marker such as NAA would likely indicate neuronal dysfunction or decreased neuronal number and density. Other important factors that were investigated for neurochemical and metabolic abnormalities in neurons included choline-containing compounds (CHO) and composite amino acid (AA), where changes in gray matter CHO concentration was associated with alterations in membrane phospholipid metabolism and, thus, associated with cellular loss (Cecil et al., 2002). That same group demonstrated reduced CHO concentration in bipolar frontal gray matter, thus, suggesting decreased neuronal volume. In addition, elevated levels of AA in bipolar frontal white matter, was related to altered neuronal transmission. The reduced concentrations of NAA and CHO in gray matter are consistent with findings from radio-isotope studies that indicate hypo-metabolism (Drevets et al., 1997) and suggests the occurrence of a neuro-degenerative process in gray matter of the frontal cortex (Cecil et al., 2002). This finding is extremely important since bipolar disorder is not classified as a typical neuro-degenerative disorder as it is mainly characterised by glial cell pathology rather than neuronal abnormalities (Muneer et al., 2016).

Currently, there exists limited literature implicating neuronal changes in the pathophysiology of mood disorders. Moreover, available evidence does not lend enough support to bipolar disorder being

considered a neuro-degenerative type disorder. However, those studies that describe altered neurochemical and metabolic neuronal marker abnormalities in cortico-limbic regions of bipolar subjects, implicate neuro-degenerative processing in the pathophysiology of bipolar disorder (Moore et al., 2000, Winsberg et al., 2000, Cecil et al., 2002). In the present study, we report the extra-thyroidal expression and localisation of thyroid-specific proteins in limbic components of the bipolar human brain, possibly providing potential targets in thyroid auto-immunity whereby the abnormal interaction of auto-antibodies and thyroid-related proteins may facilitate the inactivation and/or destruction of limbic neurons. The proposed loss of normal neuronal functioning in primary mood regulatory centres may predispose to mood dysregulation. Thus, it would appear that our hypothesis inferring that the presence of Hashimoto's disease-related AITD targets in the human limbic system having a neuro-pathological relevance to bipolar disease warrants merit.

CHAPTER 5

Conclusions

The association between thyroid auto-immunity and neuro-psychiatric disorders is well-documented (Krishnan, 2005, Whybrow and Bauer, 2005a, Whybrow and Bauer, 2005b, Hage and Azar, 2012). However, there exists limited literature linking AITD, specifically with clinical features of bipolar disorder. The present study identifies this lack of available research and explores a possible relationship between these two disease states, through the extra-thyroidal localisation of thyroid-specific proteins, TSH-R and TG, in major limbic regions of human brain. Thus, let us now summarise and conclude our discussion on the relevance of this limbic-derived thyroid protein expression in providing potential therapeutic targets in future bipolar disorder research endeavours:

- We found TG-like proteins to be present in amygdala neurons of bipolar brain, thus associating this with findings of amygdala hyperactivity and heightened emotional sensitivity in bipolar disorder (Kruger et al., 2003, Chang et al., 2004, Lawrence et al., 2004, Miklowitz and Johnson, 2006).
- Taking into account the significant roles of thyroid hormones, T₃ and T₄ in foetal and post-natal brain development as well as the maintenance of adult brain functioning including mental and psychological stability (Morreale de Escobar et al., 2004, Bernal, 2007, Wallis et al., 2010), it is reasonable to speculate that our findings of thyroid-related proteins, TSH-R and TG, in normal limbic brain components, may also exhibit an important alternate neuro-physiological role, specifically related to mood control. Related to this are the additional findings in the present study of reduced TSH-R/TG protein expression levels in limbic regions that may very likely predispose or, at least, bear some relevance in the pathophysiology of mood dysregulation and clinical manifestations of bipolar disorder.
- Further, we attribute symptoms of mood dysregulation in bipolar disorder to limbic-derived TSH-R which may provide potential targets for thyroid auto-immunity during Hashimoto's disease. Consequently, this may lead to inactivated neurons and/or neurodegeneration.
- An area that has yet to be explored, and perhaps will prove to be extremely controversial, is the fact that we have demonstrated both TSH-R and TG proteins within neurons of the same limbic

regions. In the traditional HPT axis, TSH-R and TG represent significant-role players in thyroid hormone synthesis. It is therefore tempting to speculate that stimulation of TSH-R expressed in limbic neurons may initiate a series of intra-cellular processes to synthesize and modify TG into T₃ and T₄, the classical thyroid hormones that are produced in the thyroid gland. However, there is no evidence to suggest that the presence of TG-like proteins in those same limbic neurons that also express TSH-R, is the result of downstream activation of TSH-R-ligand binding. Further, where this inference falls short is the lack of evidence indicating the cerebral location of other key-role players in thyroid hormone synthesis such as TPO and NIS.

- Bipolar disorder has not been previously classified as a neuro-degenerative type disorder since it is mainly attributed to glial cell pathologies rather than neuronal (Muneer et al., 2016). Our study suggests that neuronal alterations may be due to changes in thyroidal status in bipolar disorder. However, further research in this area needs to correlate our study findings of reduced thyroid protein levels with neuronal structural and functional determinations in bipolar limbic areas before one can ascertain whether neuronal pathology (due to altered thyroidal status) may be implicated in the pathophysiology of bipolar disorder.
- Nevertheless, our findings of reduced bipolar limbic thyroid protein expression correlates well with neuro-imaging study reports that demonstrate reduced cortico-limbic volumes and activity during bipolar disorder (Lopez-Larsen et al., 2002, Kruger et al., 2003, Lyoo et al., 2004, Lochhead et al., 2004, Mayberg et al., 2004, Strakowski et al., 2005, Townsend et al., 2013, Abe et al., 2015) and is, therefore, suggestive of degenerative processing in limbic brain. This may very well provide an alternate mechanism for a neuro-degenerative aetiology in bipolar disorder.

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APPENDIX

Expression of thyroid-stimulating hormone receptors and thyroglobulin in limbic regions in the adult human brain

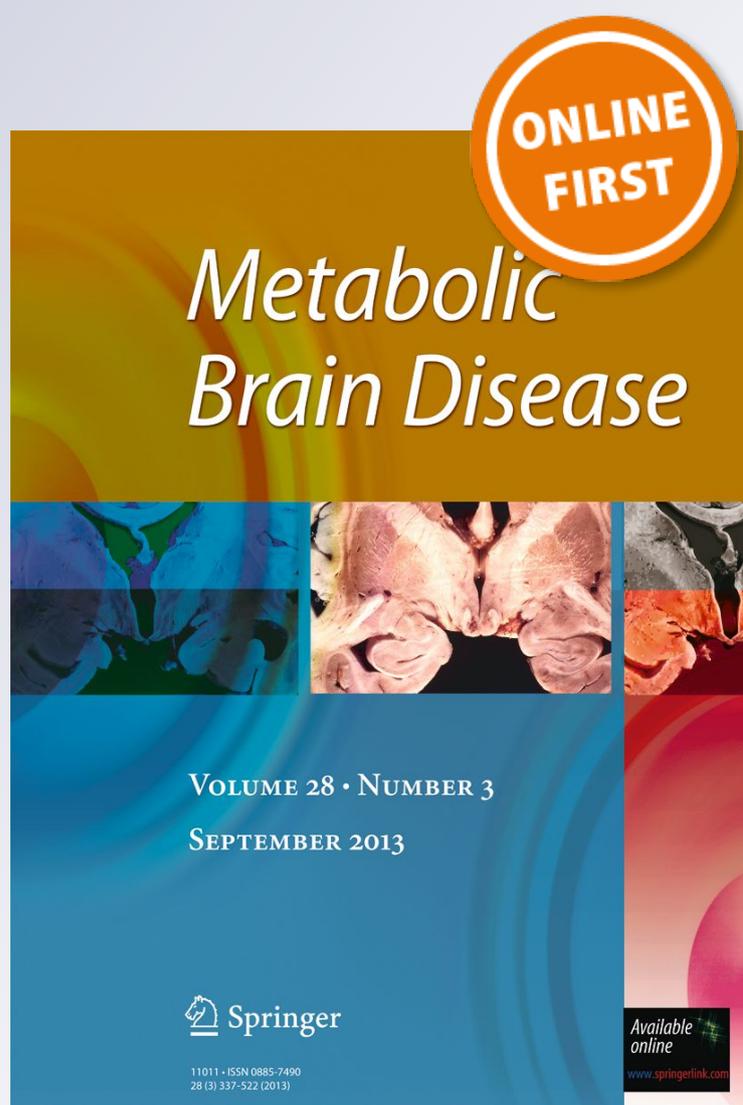
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Expression of thyroid-stimulating hormone receptors and thyroglobulin in limbic regions in the adult human brain

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Abstract Expression of the human thyroid-specific proteins, thyroid-stimulating hormone receptor (TSH-R) and thyroglobulin (TG) in non-thyroid tissue is well-documented. TSH-R has been identified in the heart, kidney, bone, pituitary, adipose tissue, skin and astrocyte cultures. TG has been identified in the skin, thymus and kidney. However, none of those previous studies had identified TSH-R or TG in specific human brain regions. Previously, a pilot study conducted by our group on normal adult human brain demonstrated TSH-R and TG in cortical neurons and cerebral vasculature, respectively, within various brain areas. In the present study, we extend this investigation of thyroid proteins specifically in limbic regions of normal human brain. Forensic human samples of amygdalae, cingulate gyri, frontal cortices, hippocampii, hypothalamii, and thalamii were obtained from five individuals who had died of causes unrelated to head injury and had no evidence of brain disease or psychological abnormality. Tissues were probed with commercial polyclonal antibodies against human TSH-R and TG which resulted in the significant demonstration of neuronal TSH-R in all limbic regions examined. Other novel results demonstrated TG in vascular smooth muscle of all limbic regions and in some neurons. Finding thyroid proteins in limbic areas of the human brain is unique, and this study demonstrates that cerebro-limbic localisation of thyroid proteins may have potential roles in neuro-psycho-pharmacology.

Keywords Thyroid-stimulating hormone receptor · Thyroglobulin · Thyroid gland · Limbic regions · Auto-immune thyroid disease · Immuno-histochemistry

Abbreviations

TSH-R	thyroid-stimulating hormone receptor
TG	thyroglobulin
AITD	auto-immune thyroid disorders
HPT	hypothalamic-pituitary-thyroid axis
BBB	blood-brain barrier
CNS	central nervous system
DAB	diaminobenzidine
ROI	region of interest
RT	room temperature

Introduction

During auto-immune thyroid disorders (AITD), patients express immuno-sensitivity towards thyroid proteins such as thyroid stimulating hormone receptor (TSH-R) and thyroglobulin (TG). The resulting auto-immune anti-thyroid antibodies bind to and stimulate TSH-R causing hyper-activity of the thyroid gland, for example, following the clinical onset of hyperthyroidism in Grave's disease (Ai et al. 2003; Davies et al. 2002; Szkudlinski et al. 2002). In contrast, during auto-immune hypothyroidism that presents in Hashimoto's disease, auto-antibodies to TG can cause cell-mediated destruction of thyroid follicular cells resulting in lymphocytic infiltration, thyroid follicular atrophy, glandular fibrosis and hypofunction (Ai et al. 2003). However, anti-thyroid antibodies are not limited to recognising thyroid-specific proteins located in thyroid tissue only. Several studies have demonstrated the expression and presence of TSH-R in mammalian extra-thyroid tissues

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including the heart, kidney, bone, lymphocytes, testis, thymus, pituitary, adipose, skin, hair follicles, astrocyte cultures as well as in fish testis (Szkudlinski et al. 2002; Bodo et al. 2009; Chabaud and Lissitzky 1977; Cianfarani et al. 2010; Crisanti et al. 2001; Drvota et al. 1995; Endo et al. 1995; Inoue et al. 1998; Kumar et al. 2000; Murakami et al. 1996; Perkonen and Weintraub 1978; Prummel et al. 2000; Sellitti 2000; Valyasevi et al. 1999). Further, TG has also been identified in mammalian extra-thyroid tissue such as skin, hair follicles, thymus and kidney (Bodo et al. 2009; Cianfarani et al. 2010; Sellitti 2000; Spitzweg et al. 1999). Immuno-reactive TSH-R was previously demonstrated in the human pituitary (Prummel et al. 2000), cultured human and rat astrocytes, and in rat brain (Crisanti et al. 2001), but not in other human cerebral cortical regions. This presented some interesting contradictions as some of the findings of those older studies differed with more recent work by our research group. In a pilot study, we were the first to demonstrate the immuno-localisation of anti-TSH-R IgG in normal adult human cortical neurons of the occipital lobe, frontal lobe, parieto-occipito-temporal lobe, cingulate gyrus, primary motor cortex and primary sensory cortex (Moodley et al. 2011). In addition, we had localised anti-TG IgG within the cerebral vasculature of the above regions. The results of that study contributes to the extensive school of thought that suggests a neuro-immunological association between auto-immune thyroid disorders and psychiatric disorders. Thus, in the present study, we postulate that thyroid-like antigens may be expressed in human limbic structures, and where anti-thyroid antibodies may then recognise and bind these regions during auto-immune thyroid disorders. Therefore, the present study aimed to immuno-localise TSH-R and TG-like proteins in limbic and limbic-associated regions; these regions represent the cerebral control centres for human emotional and behavioural responses as well as motivational drive (Guyton and Hall 2000).

Materials and methods

Samples

Ethical approval was granted by the University of KwaZulu-Natal Biomedical Research Ethics Committee (Reference EXPO42/06). Normal human brain tissues were obtained at post-mortem from 3 males (mean age: 36.7 years) and 2 females (mean age: 28.0 years) (Raidoo et al. 1996). All patients had died of sudden causes unrelated to head injuries. There were also no evidence of histopathological brain disease or clinical psychological abnormality. A single slice of cortical tissue was harvested from each of the following limbic regions: amygdala, cingulate gyrus, frontal cortex, hippocampus, hypothalamus and thalamus, from each of the 5 brains. Normal human thyroid gland tissue that was previously

collected and verified as histologically normal (Raidoo et al. 1996), was used as thyroid protein tissue controls in these experiments. The brain and thyroid tissues were formalin-fixed and paraffin-embedded in preparation for immuno-histochemistry, as per conventional protocols.

Antibodies and reagents

Commercial polyclonal rabbit anti-human TSH-R (200 µg/mL, Santa Cruz Biotechnology, California, USA) and anti-human TG (8.1 g/L, Dako, Ely, UK) IgGs were used as primary antibodies along with an avidin-biotin immuno-staining kit (LSAB, Dako, UK) and chromogen substrate, 3, 3'-diaminobenzidine (DAB, Dako, UK). The manufacturers confirmed the specificity of the antibodies for TG and TSH-R where traces of contaminating non-related antibodies had been removed by solid-phase adsorption with human plasma proteins (Santa Cruz Biotechnology, California, USA, Dako, Ely, UK).

For immuno-chemical staining, primary antibodies were diluted in 3% bovine serum albumin/tris-buffered saline (TBS/BSA). All other bio-reagents were purchased from Roche Diagnostics (Johannesburg, South Africa) and all chemicals were obtained from Merck (Durban, South Africa).

Localisation of TSH-R and TG by Immuno-histochemistry

A conventional avidin-biotin immuno-complexation technique (Wright et al. 2008), using polyclonal rabbit anti-human TSH-R and TG antibodies was used for the localisation of proteins in normal thyroid and brain tissue. Briefly, the fixed thyroid and brain tissues were sectioned (3 µm) onto silane-coated glass slides, dewaxed in xylene, and rehydrated through a graded series of ethanol solutions for 5 min each at room temperature [2 × 100%, 90%, 70% and 50% ethanol] and finally into distilled water, all at room temperature (RT). For antigen retrieval, the tissues were boiled in 0.1 M sodium citrate buffer, pH 6.0 (80 °C) for 5 min and allowed to cool to RT for approximately 20 min. The sections were then allowed to reach equilibrium in distilled water for 5 min/RT. The tissues were partially-permeabilised with methanol (20 min/RT) and any endogenous tissue peroxidases were quenched with 20% hydrogen peroxide/80% methanol (four times for 20 min each/RT). The sections were then washed twice in TBS for 5 min each/RT. A hydrophobic pen (Dako) was used to create a temporary “incubation well” around each tissue section. Tissue sections were blocked for non-specific protein binding with TBS/BSA/10% casein buffer and incubated for 30 min/RT, with one change of buffer for a further 30 min. The buffer was then carefully aspirated and replaced with a 150 µl volume of primary antibody (anti-TSH-R, 1:200; anti-TG, 1:1500). Incubation with primary antibody was performed at 4 °C for 18 h within a humidified

chamber. Following incubation, the sections were allowed to come to RT and then washed with TBS for 5 min. Tissues were then incubated with a streptavidin-biotin system (LSAB, Dako, UK), according to the manufacturer's instructions. Bound targets were then rendered visible with diaminobenzidine (DAB) chromogen application in order to facilitate visualisation under light microscopy. Following sufficient colour change, the sections were immediately immersed in TBS, counter-stained with Mayer's Haematoxylin for 5 min/RT, rinsed under tap-water for 5 min, and then dehydrated through a series of ethanols and finally into xylene. The sections were then mounted with DPX (Merck, SA) and air-dried. Normal human thyroid gland tissue served as thyroid protein method controls for these experiments and negative controls were prepared by substituting the primary antibody with dilution buffer.

Image analysis

Stained tissue results were captured as digital TIFF images using a Leica digital camera (DCF300X, Leica, Heidelberg, Germany) coupled to a Leica microscope (DMLB) and interfaced with Leica IM50 software. Regions of interest (ROI) were obtained by using the following considerations: Tissue integrity; presence of neurons; the least amount of white matter and minimal background staining. Immunolocalisation on the control and brain regions was then quantified by image analysis (AnalySIS v5, Soft Imaging Systems, Germany). Briefly, the DAB intensity range was converted to an 8-bit grey-scale with 255 phases. This grey-scale intensity of specific label in the digitised images was quantified and pre-determined, threshold range-limited, $\text{pixel} \cdot \mu\text{m}^{-2}$ values were calculated for the mean intensity of immuno-labelling. On the grey scale, we considered the upper range (160–255) as an indication of positive labelling, in order to exclude background and artifact. For each limbic region ($n = 6$) from each of 5 normal human brains ($n = 5$), images were analysed from 5 fields of view ($n = 5$) at 40 \times magnification (Wright et al. 2008). The average of mean integral intensities ($\times 10^2$ $\text{pixels} \cdot \mu\text{m}^{-2}$) was calculated for each limbic structure. For our determinations, the mean integral intensity was considered the mean value of the integral intensity of a specific ROI. The integral intensity is the sum of all the staining intensities of an ROI multiplied by the pixel area, and was obtained as an output of the analysis software.

Statistical methods

All statistical analyses were performed using STATA Software, v 13.1 (StataCorp, USA). It was found that some data analysis for TSH-R in neurons and TG in blood vessels did not statistically meet the assumption of normality and was therefore transformed using the natural log (Ln) \pm SEM for

mean (Ln) intensity. Parametric assessments were performed for both categories. This comprised an *F-test* followed by *Tukey's multiple comparisons for significance* test. Significance was therefore tested at $p < 0.05$.

Statistical analyses of TG in neurons did not appear to follow a normal distribution curve; therefore, non-parametric measures were considered for statistical comparisons. Median values were obtained for this category and then transformed using the natural log (Ln) with inter-quartile range error calculations. A *two-sample Wilcoxon rank-sum (Mann-Whitney)* test followed by *Bonferonni's multiple comparisons for significance* tests was used to correct for distribution. Thus, statistical significance was established at $p < 0.003$ for this category.

Results

Control tissues

Immuno-labelling of normal thyroid tissue demonstrated TSH-R in the cytoplasm of the follicular cells that constitute the simple cuboidal, follicular epithelia (Fig. 1A). Colloid, colloidal spaces and colloidal vesicles were devoid of TSH-R immuno-labelling. Further, no TSH-R could be detected within thyroid vasculature. Positive immuno-labelling for TG was evident within the cytoplasm of thyroid follicular cells (Fig. 1B). In addition, intense TG labelling was visualised within the colloidal space and colloidal vesicles, and within the smooth muscle cells of the *tunica media* and *tunica adventitia* of thyroid blood vessels. Method controls demonstrated the absence of staining when each respective antibody was substituted with dilution buffer (Fig. 1A and B, insets).

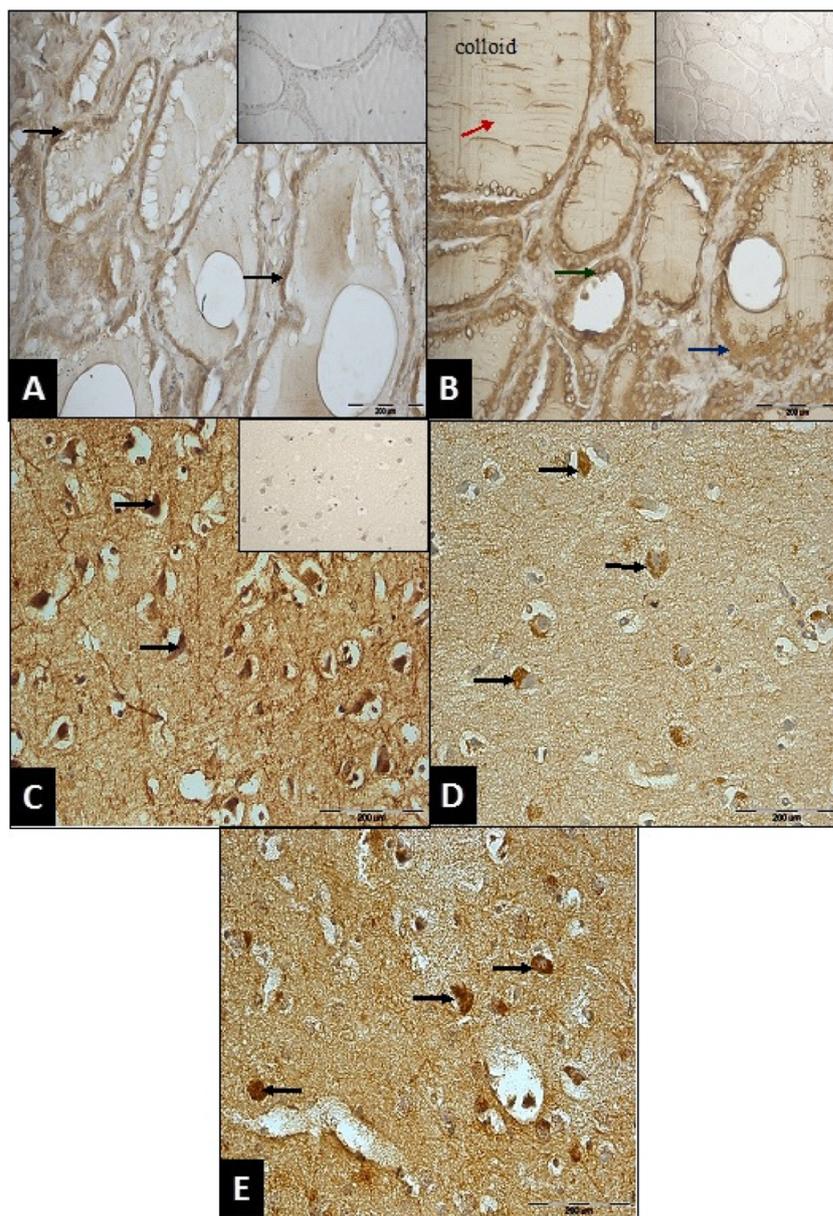
Normal human limbic tissue

Thyroid-stimulating hormone receptor (TSH-R)

Immuno-reactive TSH-R was observed in all six limbic regions in all five brains examined. Positive immuno-labelling was demonstrated in large motor neurons of the cingulate gyrus (Fig. 1C), amygdala (Fig. 1D), frontal cortex (Fig. 1E), hippocampus, hypothalamus and thalamus. TSH-R labelling was confined to the neuronal cell bodies whilst cell nuclei were negative. In contrast, there was no evidence of TSH-R immuno-labelling within the cerebral vasculature and neuronal support cells such as the oligodendrocytes, astrocytes, neuroglia and satellite cells, for any of the limbic regions examined.

The labelling intensities of TSH-R across all limbic regions were analysed and quantified by image analysis, and were confined to the range $4.62\text{--}540.42 \times 10^2$ $\text{pixels} \cdot \mu\text{m}^{-2}$

Fig. 1 Immuno-localisation of thyroid-stimulating hormone receptor (TSH-R) in normal adult, human brain limbic areas ($n = 5$) and normal control human thyroid tissue. Immuno-labelling of normal control thyroid tissue demonstrated TSH-R in the cytoplasm of thyroid follicular cells (arrows) and its absence in colloid (A, $\times 400$). Thyroglobulin was demonstrated within the cytoplasm of thyroid follicular cells (green arrow), colloid (red arrow) and colloidal vesicles (blue arrow) (B, $\times 400$). Method controls demonstrated the absence of staining when each antibody was replaced with dilution buffer (A and B, insets, $\times 400$). In brain limbic areas, immuno-reactive TSH-R was evident in neuronal cell bodies (arrows) in the cingulate gyrus (C, $\times 400$), amygdala (D, $\times 400$), frontal cortex (E, $\times 400$). Antibody method controls for brain tissue showed absence of stain in neurons and blood vessels (C, inset, $\times 400$). Positive labelling for TSH-R in thyroid and brain tissues were identified as brown colour. Scale bars = 200 μm



(arithmetic mean intensity values). Specifically, regions such as the frontal cortex ($540.42 \times 10^2 \text{ pixels} \cdot \mu\text{m}^{-2}$) and cingulate gyrus ($395.47 \times 10^2 \text{ pixels} \cdot \mu\text{m}^{-2}$) displayed more intense staining, whilst the hypothalamus ($11.24 \times 10^2 \text{ pixels} \cdot \mu\text{m}^{-2}$) and thalamus ($4.62 \times 10^2 \text{ pixels} \cdot \mu\text{m}^{-2}$) stained to a lesser degree (Table 1).

Fig. 2 displays the mean (Ln) intensity for TSH-R immuno-labelling in all normal brain limbic regions examined in the range 1.5–5.8. These results demonstrate an approximate 3 fold difference each of cingulate gyrus (5.80), frontal cortex (5.80), hippocampus (5.50) and amygdala (5.10) when compared to hypothalamus (1.90) or thalamus (1.50) (Table 1, Fig. 2).

Statistical comparisons demonstrated that TSH-R in both the hypothalamus ($p < 0.0001$) and thalamus ($p < 0.0001$) differed significantly when compared to each of other limbic

areas but not when compared to each other (Fig. 2). Further, no appreciable significance was noted among the remaining limbic regions when compared amongst each other.

Thyroglobulin (TG)

(a) TG in cerebral vasculature

Positive immuno-labelling for TG was detected within the cerebral vasculature of all six limbic regions. Specifically, immuno-reactive TG was localised to smooth muscle cells of the *tunica media* and *tunica adventitia* of cerebral blood vessels (Fig. 3B, C and D). Image analysis of TG within limbic vasculature demonstrated an arithmetic mean intensity range of $423.84\text{--}2994.00 \times 10^2 \text{ pixels} \cdot \mu\text{m}^{-2}$ and mean (Ln)

Table 1 Quantification of neuronal labelling intensity by image analysis

Limbic Regions	Arithmetic Mean Labelling Intensity				(Ln) Intensity				
	TSH-R		TG		TSH-R		TG		
	Mean (pixels.µm ²)	SEM	Mean (pixels.µm ²)	SEM	Mean (Ln)	SEM	Median (Ln)	p25	p75
	Amygdala	243.99	50.23	0.1	0.02	5.10*	0.29	-2.52 [¥]	-3.72
Cingulate gyrus	395.47	62.71	118.2	56.41	5.80*	0.17	1.25	-1.81	5.02
Frontal cortex	540.42	85.38	91.9	46.9	5.80*	0.25	-3.00 [¥]	-3.18	0.43
Hippocampus	353.97	41.06	0.2	0.1	5.50*	0.23	-3.23 [¥]	-3.5	-2.67
Hypothalamus	11.24	3.05	145.1	52.62	1.9	0.28	3.05	1.84	4.71
Thalamus	4.62	0.3	9.1	3.77	1.5	0.06	-1.72 [¥]	-2.83	0.43

*vs hypothalamus, $p < 0.0001$

vs thalamus, $p < 0.0001$

¥ vs hypothalamus, $p < 0.003$

$n = 6$ limbic regions

$n = 5$ normal human brains

$n = 5$ fields of view

Inter-quartile range errors: p25 and p75

intensity range of 5.14–6.40 (Table 2, Fig. 4). The cingulate gyrus and amygdala displayed the highest (2994.00×10^2 pixels.µm⁻²) and lowest (423.84×10^2 pixels.µm⁻²) labelling intensities, respectively. Statistical comparisons demonstrated that when compared to the amygdala, there was a significant difference in the cingulate gyrus ($p = 0.02$), frontal cortex ($p = 0.03$) and thalamus ($p = 0.02$). None of the other inter-regional comparisons showed statistical differences.

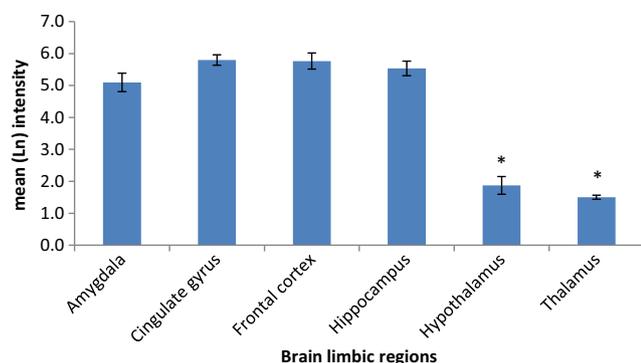


Fig. 2 Mean (Ln) intensity \pm SEM of immuno-labelling for TSH-R in cortical neurons of human brain limbic regions. * vs amygdala, cingulate gyrus, frontal cortex, hippocampus, $p < 0.05$. Mean (Ln) intensity values obtained for TSH-R in neuronal staining occurred in the range 1.5 - 5.8. These results indicate an approximate 3-fold difference when the cingulate gyrus (5.80), frontal cortex (5.80), hippocampus (5.50) and amygdala (5.10) are each compared to the hypothalamus (1.90) or thalamus (1.50). Statistical comparisons showed that TSH-R in the hypothalamus and thalamus differed significantly when compared to each of the other limbic areas but not against each other. Further, there was no difference noted among other limbic regions of higher immuno-labelling intensity values when compared to each other

(b) TG in cerebral neuronal cells

Intense immuno-reactive TG was demonstrated in most neuronal cell bodies and axons of the cingulate gyrus (Fig. 3A), whilst similar cellular structures in the frontal cortex exhibited less staining. Quantification of these staining intensities presented arithmetic mean values for cingulate gyrus (118.20×10^2 pixels.µm⁻²) and frontal cortex (91.90×10^2 pixels.µm⁻²), (Table 1). There was no evidence of positive TG labelling within neuronal cell bodies and supporting cerebral cellular structures in the other four limbic regions. Median (Ln) values obtained for this category had a range of -3.23 - 3.05 (Table 1). Statistical comparisons, tested at $p < 0.003$, demonstrated that the hypothalamus significantly differed from all other limbic regions except the cingulate gyrus ($p = 0.07$, Table 1).

When performing statistical analyses of comparison for immuno-labelling in this project, measurement of protein intensities were subject to the detection threshold limits of the avidin-biotin complexation system. Thus, the amount of TG within neurons was observed to be so minute in quantity that it could not be practically measured and was therefore considered to be negligible.

Discussion

Following extensive review of the literature, we report that this is the first study to demonstrate the localisation of thyroid-specific proteins specifically in the limbic regions of

Fig. 3 Immuno-localisation of thyroglobulin (TG) in normal adult, human brain limbic areas ($n = 5$). Immuno-reactive TG was evident in neuronal cell bodies and axons of the cingulate gyrus (A, $\times 400$). TG was highly concentrated within smooth muscle of the tunica media/adventitia of cerebral vasculature in the frontal cortex (B, $\times 400$), thalamus (C, $\times 400$) and hypothalamus (D, $\times 400$). Positive labelling for TG in brain tissues were identified as brown colour. Scale bars = 200 μm

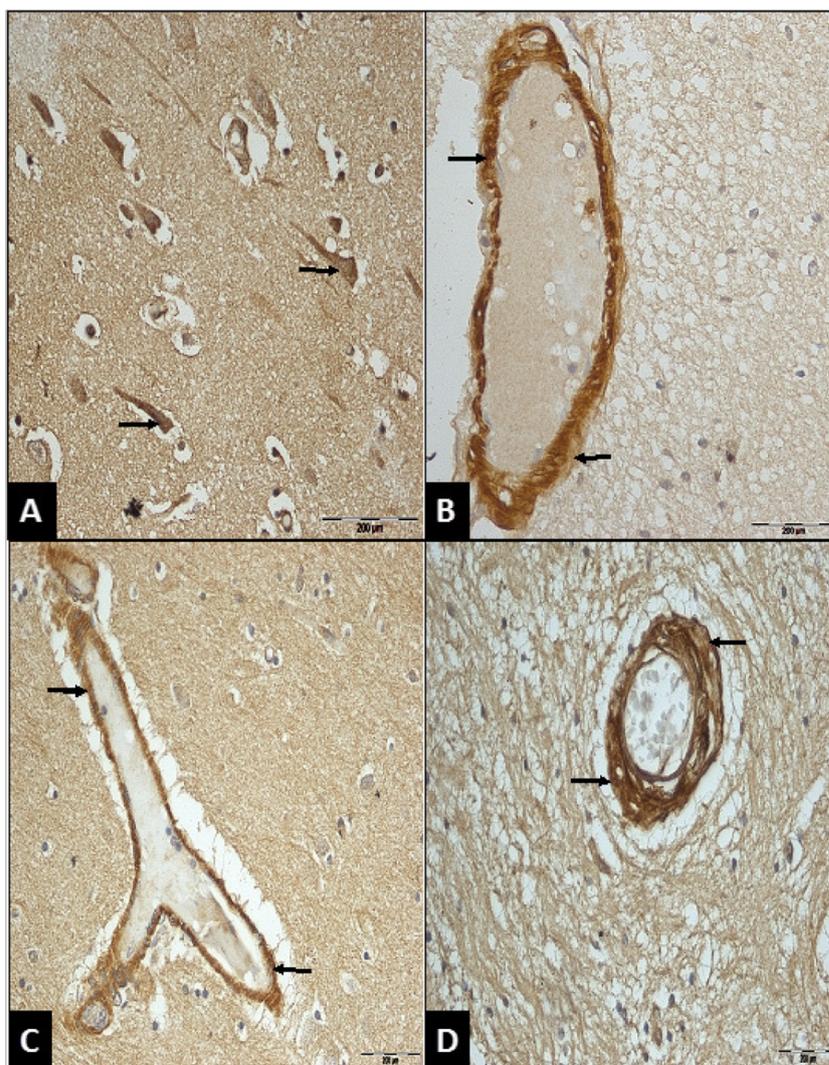


Table 2 Quantification of cerebral vasculature labelling intensity by image analysis

Limbic Blood Vessels (TG)				
Limbic Regions	Arithmetic Mean Labelling Intensity		(Ln) Intensity	
	Mean (pixels. μm^2)	SEM	Mean (Ln)	SEM
Amygdala	423.84	132.3	5.14	0.37
Cingulate gyrus	2994	2315.11	6.40*	0.3
Frontal cortex	884.7	229.7	6.28*	0.19
Hippocampus	1407.14	922.26	5.97	0.26
Hypothalamus	571.04	94.44	6.09	0.15
Thalamus	1474.34	500.46	6.35*	0.28

*vs amygdala, $p < 0.05$

$n = 6$ limbic regions

$n = 5$ normal human brains

$n = 5$ fields of view

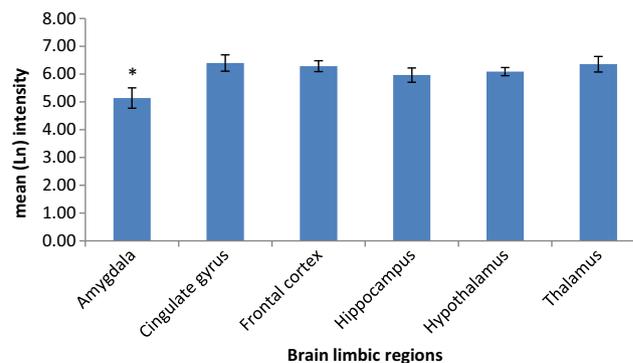


Fig. 4 Mean (Ln) intensity \pm SEM of immuno-labelling for TG in cerebral vasculature of human brain limbic regions. * versus cingulate gyrus, frontal cortex, thalamus, $p < 0.05$. Mean (Ln) intensity values obtained for TG in limbic cerebral vasculature occurred in the range 5.14 - 6.40. Statistical comparisons indicates that the cingulate gyrus, frontal cortex and thalamus differed significantly from the amygdala. No statistical differences occurred among other inter-regional comparisons

the adult human brain. The presence of TSH-R in human brain tissue is supported by Prummel et al. (2000), who immunodetected TSH-R proteins within folliculo-stellate cells in human anterior pituitary tissue (Prummel et al. 2000). Further, they postulated that the presence of the TSH-R in the anterior pituitary gland has a short-loop regulatory function within the hypothalamic-pituitary-thyroid (HPT) axis by modulating TSH secretion at the pituitary level. In the present study, we also demonstrate TSH-R in human hypothalamus, albeit at a low concentration. However, the scope of our investigation did not extrapolate to TSH-R localisation within the HPT axis. Our observations of TSH-R in hypothalamus is consistent with the findings of Crisanti et al. (2001) who demonstrated TSH-R within hypothalamic neurons of rat brain (Crisanti et al. 2001) as well as Bockmann et al. (1997) who reported expression of TSH-R in the hypothalamus of sheep (Bockmann et al. 1997). In contrast to the Prummel study (2000), we have also demonstrated for the first time, the localisation of TSH-R in non-HPT axis cerebral tissue (viz, amygdala, cingulate gyrus, frontal cortex, hippocampus and thalamus).

A pilot study previously conducted by our group was the first to demonstrate the presence of TSH-R and TG in cortical neurons and cerebral vasculature, respectively, within specific regions of normal adult human cerebral cortex (Moodley et al. 2011). In the present study, we further investigated the distribution of these thyroid proteins in major limbic regions of normal adult human cerebral cortex. Here, we demonstrate the presence of TSH-R in large neuronal cell bodies within all six limbic regions in all five normal human brains reviewed. Interestingly, we also report the localisation of TSH-R to the four primary mood centres, i.e. amygdala, cingulate gyrus, hippocampus and hypothalamus.

Our observations of TSH-R expression in human neuronal cell bodies is consistent with findings reported by the Crisanti group (2001) where those investigators localised TSH-R mRNA within a rodent neuronal cell model (Crisanti et al. 2001). It has also been reported that neurons are a primary target of thyroid hormone, T_3 , and that thyroid hormone action is exerted mostly by regulation of gene expression following the binding of T_3 to thyroid hormone nuclear receptors expressed in neuronal cells of rodent brain and human cultured cells (Freitas et al. 2010). In addition, the Crisanti group (2001) also detected TSH-R in human primary cultured astrocytes (Crisanti et al. 2001). However, we do not report findings of TSH-R in astrocytes or any other neuronal support cells of human limbic tissue.

Other novel findings in the present study include TG-like proteins in neuronal cell bodies and axons of the cingulate gyrus and some neurons of the frontal cortex. In addition, we demonstrated TG-like proteins in smooth muscle of the *tunica media* and *tunica adventitia* of cerebral vasculature in all six limbic areas examined. The presence of TG-like

proteins in the smooth muscle of cerebral vasculature also correlates with the findings of Ojamaa et al. (1996) who reported on the effects of thyroid hormones on vascular smooth muscle cells obtained from adult rat aorta (Ojamaa et al. 1996). Specifically, those authors demonstrated the rapid relaxation of vascular smooth muscle cells in culture, by the actions of T_3 (Ojamaa et al. 1996). In addition, other authors report that mammalian thyroid hormones are critical in the development, homeostasis and regeneration of skeletal muscle and that muscular alterations occur secondary to thyroid dysfunction, thus further illustrating the crucial role of thyroid hormones in mammalian muscle physiology (Yu et al. 2000; Brent 2012; Nwoye et al. 1982; Clement et al. 2002; Udayakumar et al. 2005; Ramsay 1966).

The strong neuro-immunological association between thyroid disorders and psychiatric illness is well-documented. Thus, the present study focuses only on the limbic areas which are responsible for the control of mood and emotional behaviour (Guyton and Hall 2000). This relates to our observation of TSH-R in limbic structures and it appears reasonable to then propose that auto-immune anti-thyroid antibodies may recognise and agonise limbic-associated thyroid-specific receptors. Thus, it can be speculated that such an abnormal association, should it exist, could contribute to mood dysregulation symptoms such as those often observed in bipolar disease, a brain disorder associated with unusual shifts in mood, and thus regulated by the limbic system. Indeed, this brings one to the relative argument of how thyroid auto-antibodies are able to transgress the seemingly-impervious blood-brain barrier (BBB) to bind cerebral targets in conditions such as bipolar disorder associated with Hashimoto's and Grave's disease. Curiously, there does appear to be multiple modes of egress beyond the BBB. Studies have shown that during neurological diseases such as HIV-associated dementia, multiple sclerosis, Alzheimer's disease and bipolar disorder, the integrity of the protective BBB can be altered, thus, enhancing its permeability with consequential migration of leukocytes and other circulating compounds into the brain (Lou et al. 1997; Minagar et al. 2002; Patel and Frey 2015). This influx of leukocytes triggers signal transduction cascades leading to the loss of tight-junction proteins within the highly specialised cerebral endothelium of the BBB (Bolton et al. 1998). Similarly in normal brain, the BBB is generally thought to limit entry of endogenous IgG from plasma as well as exogenously-administered monoclonal antibodies (Rojko and Price-Schiavi 2008). However, Terasaki and Ohtsuki (2005) reported that the BBB can be overcome by endogenous IgG and other larger hydrophilic molecules by undergoing brain-blood efflux (Terasaki and Ohtsuki 2005). Further evidence of how the BBB can be overcome occurs when molecules that are highly expressed by brain capillary endothelial cell membranes, such as transferrin, are targeted by specific monoclonal antibodies (Pardridge et al. 1991). In addition, St-

Amour et al. (2013) have reported that up to 0.1% of peripherally-administered IgG does reach the brain parenchyma in mice (St-Amour et al. 2013). Lastly, antibodies may also access the CNS by first migrating into the cerebrospinal fluid, followed by progression through the ventricular circulation and then finally into CNS parenchymal regions (Saunders et al. 2012; Berer et al. 2011). Thus, it does appear that BBB disturbance can occur during various neuro-pathological conditions, and supports our present postulate that circulating auto-antibodies gain ingress into the CNS and binds thyroid-like factors in limbic areas, and thus exacerbate development of symptoms of bipolar disorder.

Conclusion

The present study provides the first evidence for the distribution of thyroid-specific protein antigens in normal adult human brain limbic regions. In addition, there exists no previous evidence, until now, of any quantitative comparison of these thyroid factors in limbic regions. Whereas our previous pilot study had used semi-quantitative analysis to show TSH-R/TG localisation in various human brain regions, the present study focuses our attention specifically on limbic structures and utilised image analysis digitalisation and quantification. Given the data accumulated in the present study, the amount of thyroid protein cannot be related to function, and our observations relate only to the presence of limbic thyroid proteins. Whilst quantitative analysis and statistical comparisons are not extrapolated within the scope of the present study, it does serve to provide baseline localisation values for future comparative studies within neuro-immuno-psychiatric disorders. Thus, the cumulative results of the present study and the previous pilot project provide a general distribution of thyroid-like proteins that may be involved in neuro-physiology as well as the distinct possibility of affecting neuro-psycho-pharmacology in mood disorders.

We intend to further evolve this investigation to confirm the expression of TSH-R mRNA in limbic structures of normal human brain and bipolar cerebral tissue, by molecular determinations. There also exists the possibility of *in vitro* neuro-stimulation of limbic tissue by TSH in order to measure cyclic adenosine monophosphate (cAMP) production and ligand-binding as a function of TSH-R activation.

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Compliance with ethical standards

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Conflict of interest The authors are unaware of any conflict of interest arising from the current project, and any, and all output therefrom, including but, not limited to journal articles, conference proceedings and scientific reports.

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